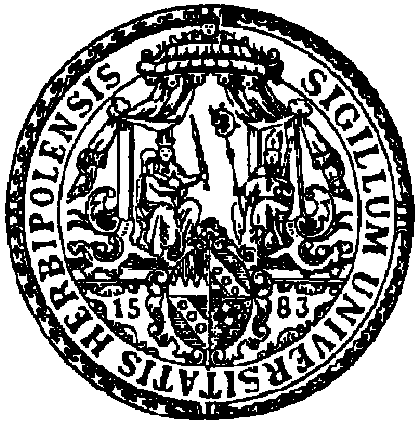

Improvement of *Salmonella* vaccine strains for cancer immune therapy based on secretion or surface display of antigens

Verbesserung von *Salmonella* Vakzinstämmen zur Krebsimmuntherapie basierend auf Sekretion oder oberflächenassoziierter Expression von Antigenen

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I hereby declare that my thesis entitled "Improvement of Salmonella vaccine strains for cancer immune therapy based on secretion or surface display of antigens"
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- Letter of GSLS Admission ☐
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I. Abstracts

I.1. Zusammenfassung

Krebsimmuntherapie ist eine viel versprechende Alternative zu den konventionellen Therapien, wie Bestrahlung, chirurgische Entfernung des Tumors oder klassische Chemotherapie. Der größte Vorteil der Krebsimmuntherapie ist Spezifität, welche durch das Zielen des Immunsystems auf so genannte Tumor-Assoziierte Antigene erreicht wird. Diese Form der Therapie sollte weniger Nebenwirkungen als Standardbehandlungen beinhalten, weiterhin könnte diese Metastasen an Orten fern des eigentlichen Tumors bekämpfen. Krebsimmuntherapie zeigte sich jedoch wenig erfolgreich in späten klinischen Studien. Zusätzlich ist die Mortalitätsrate bei Krebsleiden, trotz allen Fortschritts, immer noch sehr hoch, weshalb die Notwendigkeit der Entwicklung alternativer Immuntherapien besteht.

Ein interessanter Ansatz hierzu ist die Verwendung von Bakterien wie attenuierten Salmonellen als Träger heterologer Krebsantigene. Eine Vielzahl präklinischer Studien zeigte, dass Vakzine auf der Basis von Salmonellen CD4 und CD8 positive zelluläre Immunantworten auslösen können, welche für eine Krebsimmuntherapie entscheidend sind. Spezielle Antigenliefersysteme in Salmonellen, wie die oberflächengebundene Expression oder die Sekretion von Antigenen sind von Vorteil für die Immunogenität der Antigene.

Diese Arbeit zielte auf die Entwicklung von neuen *Salmonella* Trägerstämmen für die Immuntherapie gegen Krebs. Im ersten Projekt wurde TolC, ein multifunktionelles Protein der *E. coli* Außenmembran, als Membrananker für drei heterologe Antigene benutzt um eine oberflächenassoziierte Expression dieser Antigene zu erreichen. Die entsprechenden plasmidkodierten TolC Fusionsproteine wurden hinsichtlich ihrer Expression, Funktionalität und Plasmidstabilität in verschiedenen, rekombinanten *Salmonella* Stämmen *in vivo* und *in vitro* untersucht. Die Menge an membranständigem rekombinanten TolC war stark erhöht in *tolC*-deletierten Stämmen. Zusätzlich waren die Fusionsproteine funktionsfähig und die Plasmide wurden *in vitro* und *in vivo* äußerst stabil vererbt. Leider konnten weder spezifische $CD4^+/CD8^+$ T-Zellantworten gegen das Modelantigen Ovalbumin noch $CD8^+$ Antworten gegen das Krebsantigen BRAFV600E in immunisierten Mäusen detektiert werden. Mäuse, die mit einem *Salmonella* Stamm immunisiert wurden, der ein immundominantes Epitop des krebsassoziierten Prostata spezifischen Antigens

(PSA) in oberflächengebundener Form produziert, waren jedoch partiell vor PSA exprimierenden Melanomzellen geschützt. Das Tumorwachstum in diesen Mäusen verlief signifikant langsamer als in Kontrollgruppen, was indiziert, dass dieses System schützende Immunantworten gegen Krebs auslösen kann.

In einem zweiten Projekt wurde der zur Typhusimpfung zugelassene Stamm *Salmonella enterica* serovar Typhi Ty21a (Ty21a) für die Hämolysin Sekretion verbessert. Dieses aus *E. coli* stammende Typ I Sekretionssystem wurde oftmals für die Sekretion von heterologen Antigenen in lebenden bakteriellen Impfstoffträgern verwendet. In dieser Arbeit wurde gezeigt, dass eine Mutation von *rpoS* in Ty21a mit, im Vergleich zu anderen Salmonellen, verminderter Sekretionsfähigkeit von Hämolysin korreliert. Komplementation von *rpoS* oder *rfaH*, einem vermuteten Zielprotein von *rpoS* in Ty21a, verbesserte die Expression und Sekretion von heterologem Hämolysin. RfaH-rekombinante Salmonellen stimulierten höhere Serumantikörpertiter gegen Hämolysin in immunisierten Mäusen als vergleichbare Kontrollen und sogar *rpoS* komplementierte Salmonellen, vermutlich durch eine Erhöhung der Menge an freiem Hämolysin. Daher könnte dieser Stamm die Basis einer neuen Generation von Impfstämmen gegen heterologe (Krebs-) Antigene für den humanen Gebrauch bilden.

I.2. Abstract

Cancer immune therapy represents a promising alternative to conventional anti tumour therapy like radiation, surgical excision of the tumour or classical chemotherapy. The biggest advantage of cancer immune therapy is specificity, achieved by targeting tumour-associated antigens with the effector arms of the host immune system. This is believed to result in less adverse effects than standard therapy and reaches presumably also metastatic lesions at distant sites from the primary tumour. However, cancer immune therapy by vaccination against tumour antigens failed to translate into clinical success, yet. Furthermore, despite tremendous clinical efforts malignant disease still results in high mortalities giving rise to the need for novel vaccination-based therapies against cancer.

An interesting approach in this respect is the use of bacteria like attenuated *salmonellae* as carriers for heterologous cancer antigens. In numerous preclinical

studies *Salmonella*-based vaccines could elicit cell mediated immune responses of the $CD4^+$ and $CD8^+$ type against own and heterologous antigens which make them ideally suited for anti tumour therapy. Special delivery systems in *Salmonella* carriers like surface display or secretion of antigens were shown to be advantageous for the immunological outcome.

This work focussed on developing novel *Salmonella* carriers for immune therapy against cancer. In a first project, TolC, a multifunctional outer membrane protein of *E. coli* was utilized as membrane anchor for 3 heterologous antigens. Respective TolC fusion proteins encoded on plasmids were analysed for expression, functionality and plasmid stability in different engineered *Salmonella* strains. The amount of membrane localized recombinant TolC was enhanced in *tolC*-deficient strains. Furthermore, fusion proteins were functional and plasmid stability was very high *in vitro* and in *vivo.* Disappointingly, neither specific $CD4^+$/$CD8^+$ T-cell responses against the model antigen ovalbumin nor $CD8^+$ responses against the cancer antigen BRAFV600E were detectable in murine model systems. However, mice immunized with *Salmonella* strains displaying an immunodominant epitope of the cancer related prostate specific antigen (PSA) were partially protected from subsequent tumour challenge with a PSA expressing melanoma cell line. Tumour growth in mice immunized with the respective strain was significantly decelerated compared to controls, thus indicating that this surface display system confers protective immunity against tumours.

In a second study, the approved typhoid vaccine strain *Salmonella enterica* serovar Typhi Ty21a (Ty21a) was improved for the hemolysin type I secretion system of *E. coli*. This secretion system is widely used for heterologous antigen delivery in live bacterial vaccines. It was demonstrated throughout this work that a mutation of *rpoS* in Ty21a correlated with decreased ability for hemolysin secretion compared to other *Salmonella* strains. Complementation with *rpoS* or the presumed downstream target of rpoS, *rfaH* resulted in enhanced expression and secretion of heterologous hemolysin in Ty21a. Presumably by raising the amount of free antigen, *rfaH*-complemented Ty21a elicited higher antibody titres against heterologous hemolysin in immunized mice than controls and even *rpoS*-positive Ty21a. Therefore, *rfaH*-complemented Ty21a could form the basis of a novel generation of vaccines for human use based on (cancer) antigen secretion.

II. Introduction

II.1. Cancer immune therapy

II.1.1. Cancer

Cellular transformation is initiated by genetic and epigenetic mutations that activate oncogenes and inactivate tumour suppressor pathways (1). The path of cancer thus requires that somatic cells escape from various intrinsic tumour suppressor mechanisms that prevent cell cycle progression. In addition, transformed cells lose their responsiveness to extrinsic signals afforded by the extracellular matrix and the neighbouring cells that normally operate to maintain tissue homeostasis. Cancer thus arises when somatic cells acquire pro-mitotic signals and escape intrinsic and extrinsic suppressor mechanisms in the context of their cellular microenvironment (2). Furthermore, cancer cells can break away from the malignant solid tumour and spread to distant parts of the body (3). Cancer malignancies are among the most life-threatening diseases in industrialized countries. Although classical cancer therapies, including surgical resection of the primary tumour, radiation and chemotherapy, are well established, cancer still causes 25 % of mortalities. Annually, about 1 % of the population that has been diagnosed with cancer, die. Five-year survival rates range from 10-20 % for lung, oesophagus and stomach cancer, to 40-60 % for colon, bladder and cervix cancer, and 60–80 % for breast and prostate cancer (4). One reason for this is that dysfunctional tumour suppressor genes are selected in the course of standard chemotherapy leading to relapse of the disease highly resistant to conventional treatment (2). These facts emphasize the need for novel anti cancer therapies.

II.1.2. The idea of cancer immune therapy

One of these novel therapeutic approaches which gained attention in the past decades is referred to as cancer immune therapy. It has become evident that the development of cancer does not progress unnoticed by the immune system and immunity can prevent the occurrence of tumours. This concept has been named “immunosurveillance” and several mouse models together with clinical data have unambiguously demonstrated its role as an effective tumour suppressor mechanism

(5,6) suggesting that the effector arms of the immune system are able to mediate tumour regression. It is estimated that immune therapy has less side effects compared to standard therapies such as chemotherapy or radiotherapy (7). On the other hand, cancer immune therapy has been proposed as therapeutic intervention to boost or elicit immune responses in patients with minimal residual disease e.g. after surgical excision of the primary tumour before recurrence of cancer at metastatic sites (8). However, the first clear indication for effective immune therapy, the administration of high dose of interleukin-2 (IL-2), was able to mediate regression of even bulky, invasive tumours in selected patients with melanoma, kidney cancer and non-Hodgkin's lymphoma (9).

Multiple studies have been undertaken to clarify the nature of protective immunity observed in animal models and it could be shown that cellular rather than humoral immune responses were responsible for the rejection of transplanted tumours (10). Thus, significant effort has been made towards the identification of antigens recognized by human T-lymphocytes (11-13). Both, $CD8^+$ cytotoxic T-cells and $CD4^+$ T-helper cells recognize antigens presented as small peptides in the groove of surface human leukocyte antigen (HLA, the human analogue of the major histocompatibility complex (MHC)) molecules. $CD8^+$ cells recognize peptides of 8-10 amino acids length, derived from intracellular cytoplasmic proteins, digested in proteasomes and presented via the endoplasmatic reticulum on cell surface class I HLA (or MHC) molecules. In contrast, $CD4^+$ cells use a different intracellular pathway and present engulfed extracellular proteins, digested to peptides in intracellular endosomes and presented on cell surface class II HLA (or MHC) molecules (10). In principle, cancer immune therapy employing T-cells can be achieved in two ways: (i) the adoptive transfer of *ex-vivo* activated T-cells or (ii) *in vivo* activation by the use of cancer vaccines. As this work deals with vaccination therapy the following sections focus on vaccine-based cancer immune therapy knowing that adoptive transfer of T-cells, the administration of adjuvants for cancer immune therapy and antibodies against extracellular receptors e.g. Rituxan and Herceptin reached the clinics (14-16). The unique advantage of vaccination-based cancer immune therapy is specificity as the immune system is able to recognize epitopes of antigens that are expressed by tumour cells and target those cells for destruction without harnessing normal ones. Those antigens determining specificity were termed tumour-associated antigens.

II.1.3. The cellular basis of cancer immune surveillance

Tumour associated antigens (TAAs) can be segregated into five categories: (i) differentiation antigens e.g. melanocyte differentiation antigens (in melanoma) like Melan-A/MART-1, tyrosinase and gp-100; (ii) mutational antigens, e.g. abnormal forms of p53; (iii) overexpressed/amplified antigens, e.g. HER-2 neu (breast cancer) (iv) cancer testis antigens e.g. MAGE and NY-ESO (germ line tumours, sarcomas malignant melanomas), normally expressed only in the germ line, notably the testis, but also abundant in various cancer cells; and (v) viral antigens, e.g. EBV and HPV (cervix carcinoma) (11,17,18). Epitopes of TAAs are recognized by T-cells. The activation of antigen specific $CD8^+$ T-cells can depend on interaction with $CD4^+$ but the final effector cell in most mouse models is the $CD8^+$ cytotoxic T-cell (CTL). Perforin-mediated cytotoxicity and Fas/Fas ligand interaction are major mechanisms for $CD8^+$ T-cell-mediated effector function (19,20). After the TCR engages specific antigenic peptides presented by the MHC (or HLA) of target cells, perforin is released and causes damage to target cell membranes. Various granzymes and possibly other granule constituents co-secreted with perforin enter the target cell and induce apoptosis (21). The expression of a variety of cytokines, including Fas ligand (FasL) is also enhanced in T-cells after antigen specific activation. FasL cross-links Fas on the target cells and in many cells this interaction triggers apoptosis and cell death (20). Studies in perforin-deficient mice showed that perforin-mediated cytotoxicity is essential for resistance against injected tumour cell lines, viral and chemical carcinogenesis as well as spontaneous leukemogenesis (21-23). Although the role of Fas dependant cancer cell death was reported to play only a minor role, there are hints on FasL mediated cancer rejection of tumour cells which are responsive to this pathway (24).

Additionally, most effective antitumour immune responses in animal models depend on the efficient generation of $CD4^+$ T-helper 1 cell (Th 1) immunity that promotes CTL responses (8) in contrast to Th 2 helper cells which trigger the humoral immune system. The importance of Th 1 helper cells is also strengthened by the fact that progressive neoplastic disease is characterized by a response that is skewed towards Th 2 cells (25). Among the Th 1 cytokines, Interferon (IFN) γ seems to play a crucial role for alarming the immune system and cancer cell cytotoxicity (6).

However, classical $CD8^+$ T-cells are not the only source of IFNγ and mediators of cytolysis. Components of the innate immune system also participate in the process of immunosurveillance. NKG2D is a lectin-like receptor expressed by natural killer cells (NK), γδ T-cells, some $CD8^+$ T-cells and natural killer T-cells (NKT) (26). NKG2D ligands are often ectopically expressed in a wide range of murine tumours (27) and human carcinomas of lung, kidney, prostate, ovary, colon (28), liver (29) as well as melanoma (30). Tumour cells can therefore be recognized by immune cells via the NKG2D receptor which lead to tumour repressive functions *in vivo* e.g. by IFNγ secretion of γδ T-cells (31). Additionally, tumour necrosis factor related apoptosis inducing ligand (TRAIL) can engage the TRAIL-R2 receptor in mice and induce cytolysis via apoptosis. TRAIL is expressed on a subset of liver NK cells and is induced by interferons in NK cells, monocytes and dendritic cells (DCs) (32) representing another mechanism employed by the innate immune system to kill cancer cells.

II.1.4. Strategies, challenges and limitations for cancer vaccines

Attempts at immunotherapy of cancer have included vaccination with viral vectors (33,34), antigenic synthetic peptides (35,36), antigen loaded DCs (37,38) and DNA encoding tumour-associated antigens (39). All approaches have their own advantages and disadvantages but the biggest challenge for every type of anti-cancer vaccination is tolerance as all cancer antigens are self antigens and even large numbers of tumour-infiltrating, specific T-cells may fail to mediate tumour regression (40,41). This “anergy” needs to be overcome and therefore basically all cancer vaccines employ adjuvants, whether they are composed of whole cells, defined proteins or peptides and other molecules (8). Adjuvants can activate antigen presenting cells to stimulate T-cells more efficiently or activate NKs and other cells of the innate immune system to produce cytokines or promote survival of antigen specific T-cells (8). For example, cytokines like IL-2, granulozyte-macrophage colony stimulating factor (GM-CSF), IL-12, IL-4 and several others have been used as adjuvants in cancer vaccines (42). Two classes of adjuvants even facilitate delivery of antigens into the cytoplasmic compartment of antigen presenting cells (APCs) to trigger predominantly $CD8^+$ responses: (i) PLGA microspheres and virus like particles (43) as well as (ii) immunostimulatory complexes (ISCOMs), a mixture of QuilA and cholesterol that forms micelles (44).

A phenomenon that could form possible obstacles for efficient immune therapy was referred to as cancer "immunoediting". This term describes the fact that the immune system is sculpting tumours which in turn could lead to immune escape of cancer cells. Tumour immune escape mechanisms involve cellular and molecular processes. Analysis of human tumour specimens has shown that many display losses of HLA class I proteins (45,46). In addition, tumour and tumour stroma cells were identified to secrete immune suppressive factors like IL-10 and TGF-β (47) or soluble forms of NKG2D ligands (28) which attenuate innate and adaptive immune responses against cancer cells. Recently, populations of immunosuppressive cells have been identified which were generated or activated by tumour cells. Among them are IL-13 producing NKT cells (48) and $CD4^+$ $CD25^+$ regulatory T-cells (Tregs), the latter shown to be responsible for the failure to reject transplanted tumours in the murine host (49,50). A third class of immune-suppressive immune cells is represented by myeloid-derived suppressor cells (MSCs) which inhibit $CD8^+$ T-cell functions and trigger T-cell apoptosis in response to IFNγ release (51). An often critical but underappreciated point deals with the aging immune system. As most cancer patients are of advanced age, many years after the thymus stopped producing naïve T-cells, immune responses may be hindered. It was clearly shown in mouse models that generation of the primary response to antigen and the conversion to memory is compromised with age (52,53). Finally, apart from melanoma therapeutic cancer vaccination till now failed to translate into convincing clinical response in late stage trials (54,55), further underscoring the need for novel immune-stimulating therapies.

II.1.5. Bacteria and cancer: hallmarks for bacterial cancer immune therapy

As tumours or metastases develop, they stimulate angiogenesis to promote the formation of new blood vessels. However newly formed vessels are highly disorganised with incomplete endothelial linings and blind ends, resulting in inefficient nutrient and oxygen delivery to those neoplastic tissues (56). This leads to multiple regions of hypoxia and anoxia within tumours (57). Furthermore, these areas are believed to represent niches where immunological clearance mechanisms are absent (56), thus creating a favourable environment for the growth of obligate and facultative anaerobic bacteria. Indeed, it was shown that different bacteria like *Clostridia*,

Salmonella, *Vibrio* and *Bifidobacterium* can infiltrate, replicate and then preferentially accumulate in tumours (58-61). This tumour-targeting feature of bacteria gave rise to several clinical options. For example, *Clostridia* spores injected into mice harbouring transplanted sarcomas resulted in vegetative growth of those bacteria inside necrotic areas and finally oncolytic regression of the tumour (62,63). Another approach is the use of tumour-infiltrating bacteria as vehicles to deliver cytotoxic proteins or nucleic acids encoding them (61,64-66) to allow cancer targeted therapy with the aim of reducing adverse effects and achieving treatment "*in situ*".

More interesting with respect to cancer immune therapy are historical findings of the surgeon W.B. Coley. He observed that when patients suffering from sarcomas developed acute streptococcal infections, their tumours regressed, due to the stimulation of the innate immune system (67). More recently, despite their known oncolytic activity, *Clostridia* were found to eradicate tumours due to induction of the inflammatory response and generation of $CD8^+$ T-cells reactive to cancer cells in experimental animal models (68). Furthermore, bacterial products, like lipopolysaccharide (LPS) or unmethylated CpG dinucleotide-containing bacterial DNA have been used for many years as effective adjuvants. These bacterial compounds are particularly good in activating CTLs, thus being of interest for tumour immunologists (69). Bacterial products stimulate pattern recognition receptors expressed by DCs, macrophages and perhaps NK cells and other cells of the innate system. This induces their maturation, activation and production of pro-inflammatory cytokines (8). Many of these receptors belong to the family of Toll-like receptors (TLRs) that are located either on the surface or inside cells and recognize invading pathogens (70).

For those reasons, the use of attenuated live bacterial vaccines (LBV) represents an interesting alternative for cancer immune therapy. They stimulate innate responses as well as adaptive immunity. This and the ability to express foreign proteins make them ideal vectors for vaccination against tumour-associated antigens. Features of live bacterial vaccines and immune responses elicited by those vectors are discussed in depth in the following sections.

II.2. Live bacterial vaccines

II.2.1. General features of live bacterial vaccines

Live attenuated bacterial vaccines are able to elicit immune responses against own antigens and confer protection towards challenge with virulent bacteria. As mentioned above, LBV can also be used for the delivery of heterologous antigens and these vectors offer many potential clinical advantages: (i) they are easy and relatively inexpensive to produce (ii) they are also well-suited to large scale manufacture and potentially stable without refrigeration (via lyophilization), (iii) are able to carry large or multiple antigens or adjuvants (iv) and can be eradicated with antibiotics should the need arise (71). Delivery of vaccine antigens by LBV via the mucosal route results in the elicitation of effective humoral and cellular responses at the level of both systemic and mucosal compartments, not only at the specific inductive site but also at remote mucosal sites (72-74). Mucosal (oral) delivery comprises further advantages like high acceptance, easy administration and amplification of the carrier at mucosal sites leading to persistent antigen production (75). LBV induce the production of multiple cytokines, including Th 1 cytokines like TNFα, IFNγ and IL-12 and inflammatory mediators such as nitric oxide which enhance early innate immunity and create a local environment favourable to antigen presentation (76). There are currently three live bacterial vaccines licensed for human use: (i) *Salmonella enterica* serovar Typhi Ty21a (Ty21a), (ii) *Vibrio cholerae* CVD 103-HgR and (iii) *Mycobacterium bovis* BCG (BCG). These strains among others have been used as vectors to express heterologous antigens and were evaluated in numerous animal models, albeit there are no clinically used LBV in this respect (71).

II.2.2. *Salmonella typhi* Ty21a: vaccine and vaccine vector

Attenuated *Salmonella* strains were among the first bacteria used as recombinant vectors for antigen delivery (77). A prominent example is Ty21a which was isolated in the early 1970s by chemical mutagenesis of its respective wildtype *S. typhi* Ty2 as vaccine against typhoid fever in humans. It has a GalE and Vi negative phenotype (78). The mutation of the *galE* gene results in a complete deficiency of the enzyme uridine diphosphate (UDP)-galactose-4-epimerase, which is responsible for the conversion of UDP-glucose to UDP-galactose and vice versa. Since galactose is

incorporated into the lipopolysaccharide (LPS) core moiety via UDP-galactose, the absence of *galE* leads to the formation of rough LPS, devoid of parts of the core and the O-antigen. As the O-antigen is the main antigenic determinant on the cell surface, Ty21a is supplied with a source of external galactose during production of the vaccine. This enables the bacteria to generate UDP-galactose by an alternative route, thereby expressing complete immunogenic LPS. Albeit the immunogenic capacity is maintained under appropriate growth conditions, the *galE* phenotype contributes to strain attenuation *in vivo* (76). As a result of the mutagenesis method used, Ty21a acquired further spontaneous mutations including *via* and *ilvD* genes, leading to the loss of Vi polysaccharide and an auxotrophic phenotype for isoleucine, respectively and a mutation precluding H_2S utilisation (79).

Interestingly, an additional mutation in the *rpoS* gene, which also contributes to the avirulence of the Ty21a strain (80) was inherited from the wildtype parental strain Ty2. This mutation is apparently one of the reasons for the poor capacity of Ty21a to survive starvation conditions and resist various environmental stress conditions (81). This, combined with the low shedding rate (82), reduces the environmental risks posed by use of Ty21a. Ty21a conferred protection against typhoid fever in 70-80 % of vaccinees (83) accompanied by only mild and infrequent adverse effects (84). Additionally, the multiple mutations render Ty21a genetically stable, thus reversion to virulence has neither been observed *in vitro* nor *in vivo* (85). The well documented safety makes Ty21a an attractive carrier for antigen delivery and this has been undertaken in several clinical trials encompassing heterologous bacterial antigens (86-89).

Salmonella typhi strains are human pathogens and restricted to the human host. Mice are intrinsically resistant to *S. typhi*, particularly if administered orally (90). However, mice are susceptible to serovar Typhimurium infection developing a typhoid-like disease (91), therefore murine *Salmonella enterica* serovar Typhimurium infection serves as model for *S. typhi* pathogenesis. Furthermore, serovar Typhimurium attenuated mutants were assessed as vaccine carriers in various mouse models for antigens derived from other bacteria, viruses, parasites and tumours, being able to stimulate strong systemic and local immune responses against the corresponding antigens (reviewed in (92)). Nevertheless, Ty21a was found to be immunogenic in mice when applied intranasally (93), thus this model can be used for preclinical evaluation of Ty21a based vaccines.

II.2.3. Immune responses elicited by *Salmonella*

Development of immunity to *Salmonella* infection relies on the cellular, humoral and mucosal arms of the immune system (94). The concerted action of several cytokines like TNFα, IFNγ, IL-12, IL-15 and IL-18, cellular compounds of the innate immune system like DCs, macrophages and NK cells but also humoral and T-cell responses are essential for the adaptive phase of the immune response and for controlling later stages of *Salmonella* infection (reviewed in (95)). Initiation of an immune response usually involves DCs, the most important professional antigen presenting cells, which are capable of priming naïve T-cells (96). *Salmonella* infect DCs *in vivo* and *in vitro* and can induce activation of and cytokine production by those cells (97-99).

Following immunization with protective live attenuated *Salmonella* vaccines, long-lasting immunological memory develops in animals and humans (95). Cellular responses towards *Salmonella* vaccines are of the Th 1 type (100,101). Ty21a in particular, induced strong $CD4^+$ Th 1 responses in human vaccinees, characterized by the production of IFNγ in the absence of IL-4 but also humoral responses like potent induction of IgG and mucosal IgA type antibodies (102). Vaccination with Ty21a also elicited strong $CD8^+$ CTL responses which persisted for at least 2 years after immunization. A strong correlation was found between the CTL activity and the frequency of IFNγ-secreting $CD8^+$ T-cells (103). When administered intranasally, serovar Typhi vaccines were also found to elicit $CD8^+$ responses in the murine host (104). Initial studies with live attenuated *Salmonella typhimurium* showed a clear elicitation of CTL responses against a passenger circumsporozoite antigen of *Plasmodium berghei* after oral immunization in mice, furthermore these responses were protective (105). $CD8^+$ T-cell responses due to *Salmonella* encounter are surprising as these bacteria reside within defined cellular compartments of professional APCs rendering the classical MHC class I pathway unlikely for antigen presentation. However, a process termed cross-presentation explains $CD8^+$ responses after *Salmonella* infection by an alternative class I processing pathway for exogenous phagocytic antigens (106,107).

Taken together, live attenuated *salmonellae* exhibit exactly the immune-stimulatory properties which are needed for a TAA-directed immune therapy of cancer (see II.1.3). Efficient stimulation of immune responses against heterologous antigens relies on proper transfer of antigen into APCs. This is achieved by LBV utilizing special delivery systems introduced in the following.

II.2.4. Antigen localisation and antigen delivery systems

Earlier studies have shown that the localization of the antigen within a live bacterial vaccine plays a critical role for the immunological outcome. Secretion of an antigen into the extracellular medium or expression on the outer surface of a vaccine carrier appears to be advantageous in eliciting immune responses. Kang *et al*. showed that the secreted form of *Streptococcus pneumoniae* PspA elicited a 10^4 fold increase in IgG antibody titres compared with cytoplasmic expressed antigen when delivered via attenuated *Salmonella typhimurium* in mice (108). Several other studies proved this superior immunological capacity of secreted versus cytoplasmic antigen delivery (109-111). One of the best studied secretion systems for antigen delivery is the *E. coli* α-hemolysin secretion system (112). This transport machinery is the prototype of type I secretion systems and consists of three different components: HlyB, HlyD and TolC. The HlyA carries a secretion signal, 50-60 amino acids in length at its C-terminus ($HlyA_s$) which is recognized by the HlyB/HlyD/TolC translocator leading to direct secretion of the entire protein into the extracellular medium without periplasmic intermediates. Dozens of antigens of bacterial, parasitic and viral origin have been fused to the $HlyA_s$ and efficiently secreted within *Salmonella* vaccine vectors. These strains showed promising results in diverse animal model systems referring to protection against pathogen challenge or induction of humoral and cell-mediated immunity (112,113). Recently, our group demonstrated in two separate preclinical studies that live attenuated *Salmonella typhimurium* were able to induce protective, antigen-specific CTL responses against different tumour-associated antigens delivered via the hemolysin secretion system (114,115).

Another antigen delivery mechanism utilizes the type III secretion system in *Salmonella* vaccine carriers which allows translocation of proteins into the cytoplasm of APCs and therefore directly to the MHC class I restricted antigen processing pathway (116,117). Rüssmann *et al*. demonstrated that a hybrid protein encompassing the type III secreted effector protein SptP and the $CD8^+$ epitope of the murine lymphocytic choriomeningitis virus (MCLV) nucleoprotein was efficiently secreted into the host cytosol and induced potent CTL responses leading to complete protection against a lethal intracerebral challenge with the virulent virus in orally immunized mice (118). This system was also successfully applied in a murine fibrosarcoma model (119).

Display of proteins or peptides on the bacterial surface can be mediated in several ways (for review see (120)) and became interesting for vaccinology as it was shown that surface-anchored antigens also elicit stronger immune responses than antigens expressed in cytoplasmic fashion. For this purpose, antigens were fused into different outer membrane proteins or membrane attached autotransporter domains. For example, the autotransporter domain of the *E. coli* adhesion molecule AIDA-I was applied for surface display of nearly full length or T-helper epitopes of the *H. pylori* UreA protein on a *Salmonella* carrier. These strains were more protective towards challenge with *H. pylori* than *Salmonella* expressing UreA antigens in the cytoplasm (121). The outer membrane-localized ice nucleation protein of *Pseudomonas syringae* was successfully utilized as carrier for hepatitis antigens in Ty21a and found to elicit higher antibody responses in mouse serum than a similar system relying on intracellular antigen expression (122). Other methods similar to these approaches were based on fusions to outer membrane proteins like the flagella protein FliC of *Salmonella* (123,124), *E. coli* derived p87 fimbriae (125), LamB (126) and TolC (127).

II.3. Objectives – part I

II.3.1. Surface display of antigens via TolC

The first project of the present thesis aimed at developing new recombinant *Salmonella* vaccine strains for immune therapy of cancer. Therefore, we decided to apply an existing plasmid-encoded vaccination system based on surface display of antigens, for its capacity in a tumour vaccination setting. In the mentioned study (127), the outer membrane protein TolC of *E. coli* was utilized as membrane anchor for $CD4^+$ T and B-cell epitopes of the p60 protein from *Listeria moncytogenes* and transferred into the *Salmonella enteriditis* SM6T strain, lacking endogenous *tolC*. The TolC fusion protein (TolC-LisTB) was stably expressed and functional in SM6T and elicited immune responses that were protective against lethal *Listeria* challenge in mice.

TolC is a trimeric pore-forming protein in the outer membrane of *E. coli* (6) with structurally conserved homologs in virtually all gram-negative bacteria (for a list see (128)). TolC is one of the most multifunctional proteins known in gram-negative bacteria: it is essential for type I secretion of hemolysin and colicin V, import of colicin E1 and serves as receptor for phages U3 and TLS (for review see (129,130)). A

clinically relevant role of TolC is its involvement in multidrug efflux, rendering bacteria insensitive towards a wide variety of non-related noxious compounds. The most important efflux system in *E .coli* and *Salmonella* consists of the inner membrane complex AcrA/AcrB recruiting TolC as outer membrane channel. This tripartite machinery expels antimicrobials directly into the extracellular compartment inhibiting intracellular accumulation of toxic drugs (131-133). Apart from multidrug efflux, a role for TolC in pathogenesis of gram-negative bacteria is discussed (134). The crystal structure of TolC, elucidated by Koronakis *et al*. (135), shows that a TolC homotrimer forms a channel tunnel 140 Å in length, that comprises a 100 Å long α-helical barrel (the tunnel domain) anchored in the outer membrane by 12 stranded β-barrel spans (the channel domain). The larger part of the protein is located in the periplasm; only two small loops (amino acid residues (aa) 52–61 and 257–279) are exposed on the surface of the bacteria.

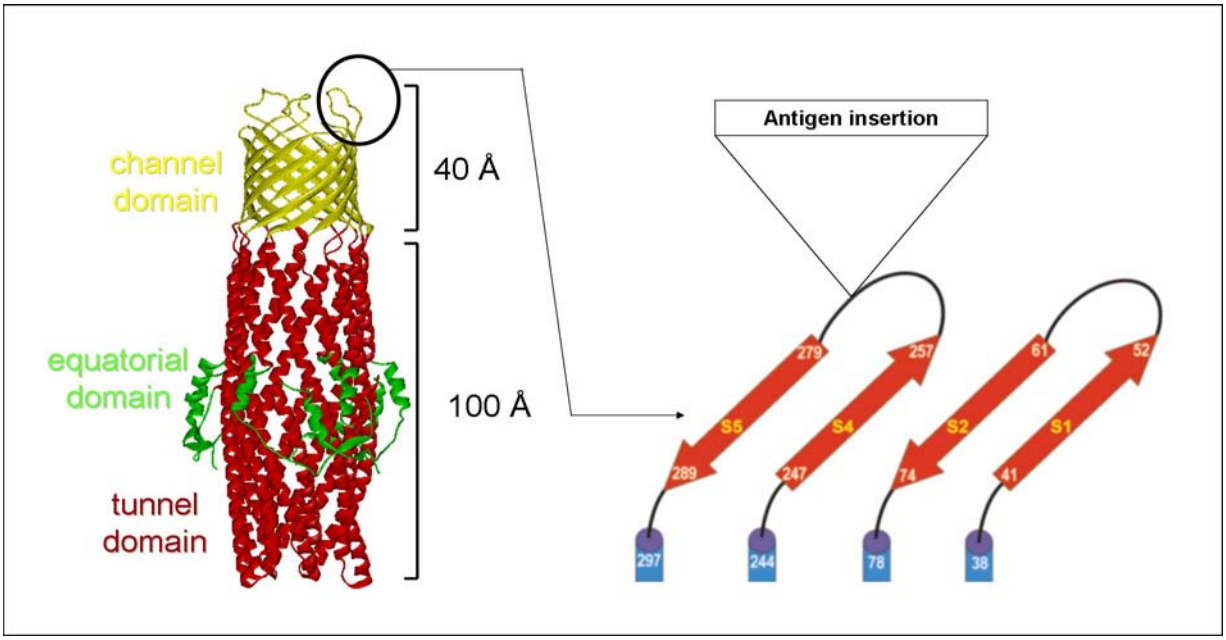

Figure II.1 Overview of TolC structure and antigen insertion site (taken from (135) and Andersen, C unpublished, abridged)
Left panel: TolC forms a homotrimer with the larger part projecting into the periplasm; the channel domain (yellow) is located in the outer membrane. Two loops are protruding outside the bacterium (ring-tagged and lower right panel)
Right panel: Schematic view on surface exposed loops of one TolC monomer. The investigated antigens were inserted within the loop comprising aa 257-279.

The loop comprising aa 257-279 contains a unique *Kpn*I restriction site allowing genetic insertion of antigens into this permissive, surface-exposed structure (127). A structural view of TolC trimers and a schematic picture of the antigen insertion site

within the surface exposed loop are depicted in Figure II.1. To investigate the established system for its capacity to protect from neoplastic disease, novel attenuated *Salmonella* strains were constructed. Ty21a and *S. enterica* serovar Typhimurium aroA SL7207 (Stm) were deleted for their chromosomal *tolC*. Three different antigens were subject of the present work, among them, chicken ovalbumin which served as model antigen, furthermore BRAFV600E and PSA that represent tumour-associated antigens. Construction of plasmids encoding ovalbumin and BRAF epitopes fused to the TolC loop are described in this study. Construction and characterization of the TolC-PSA fusion was accomplished earlier (136). Recombinant TolC proteins were assessed for functionality in the different *Salmonella tolC* phenotypes to explore viability of those novel engineered vaccine strains. Finally, recombinant strains were tested in various animal model systems for their capacity to induce T-cell responses and to confer protection against antigen expressing tumour xenografts. The respective antigens and their cognate epitopes are described in more detail in the following sections.

II.3.2. Displayed antigens – Ova

In a first approach, we aimed to elucidate the kind of T-cell response elicited by recombinant *Salmonella* employing the TolC-based antigen expression system. Chicken ovalbumin (Ova) was selected to serve as model antigen due to its defined epitope structure for mice of the H-2^b haplotype and existence of mouse models transgenic for TCRs recognizing cognate Ova epitopes (137,138). As the loop structure is spatially limited, the class I and class II restricted epitopes of ovalbumin were inserted into *tolC* and assessed in adoptive transfer models well established for investigation of T-cell responses after pathogen encounter (139-142). Furthermore, Ova expressing tumour cell lines are available and a tumour challenge experiment was conducted in mice immunized with Stm carrying Ova epitopes.

II.3.3. Displayed antigens – BRAFV600E

The first real tumour-associated antigen included in this study was derived from the proto-oncogene BRAF. *BRAF* alleles were identified as somatic mutations in 70 % of melanomas and a minority of other cancers including lung, colon and ovary carcinomas but not normal cells (143,144) Nearly all (92 %) *BRAF* mutations in

melanoma contain a single amino acid substitution, valine on position 600 to glutamic acid (V600E), which is believed to mimic activating phosphorylation of serine 598/ threonine 601 of wildtype BRAF. This mutation leads to constitutive activation of the protein. BRAFV600E transforms cells *in vitro* through activation of the mitogen activated protein kinase (MAPK) pathway (143) which consists of the kinases RAS-RAF-MEK-ERK-MAPK mediating cellular responses to growth signals (145). Because of its tumour specificity and expression in the majority of melanomas, mutated BRAF is a potentially promising target for cancer immunotherapy as it represents a TAA of the type II (see II.1.3). Recently, a mutation-specific, HLA-B27 restricted epitope of BRAFV600E was discovered with spontaneous CTL responses in melanoma patients (146). The respective epitope was cloned in triplicate into the *tolC* loop, transferred into Stm and assessed for its capacity to elicit $CD8^+$ responses in vaccinated mice transgenic for the human HLA-B27 allele.

II.3.4. Displayed antigens – PSA

The last investigated antigen in this work is represented by the prostate-specific antigen (PSA). PSA is a 34 kDa glycoprotein, primarily produced by the prostate ductal and acinar epithelium (147). Interestingly, in prostate cancer cells expression of PSA is markedly increased compared to normal prostatic cells (148). Furthermore, its expression is restricted to the prostate and not found in normal cells, rendering PSA a tumour-associated antigen of class III (see II.1.3). Prostate cancer is the most common form of cancer affecting men in the United States, and is the second leading cause of cancer deaths among men each year (149). Although several curative therapies exist for localized disease, such as radical prostatectomy, radiation therapy, and cryotherapy, approximately 20-40 % of treated patients will relapse (150,151). Since prostate cancer is dependent upon androgens for growth, treatment for advanced, metastatic disease is achieved by systemic androgen deprivation (152). Albeit initial success, androgen independent clones arise in most patients making the disease untreatable (153). For these reasons, immune therapy targeting PSA is an attractive alternative for both hormone-refractory and non-refractory cancers. Various approaches have been developed in this respect including DNA vaccines, viruses and DC-based vaccines to elicit PSA-specific immune responses (154-156). However, due to unknown reasons, results have been very disappointing when modalities were extended to clinical trials (157). Recent findings of our group

demonstrated that vaccination with Stm secreting PSA in combination with cholera toxin subunit B (CtxB) as adjuvant significantly decelerated tumour growth in a PSA expressing tumour xenograft model (115). This protection was correlated with CTL responses that were elicited emphasizing the promising perspectives for prostate cancer immune therapy based on LBV. Moreover, an immunodominant murine, H-2D^b restricted CTL epitope of human PSA was discovered recently (158) allowing to investigate PSA directed T-cell response in H-2^b mice. The selected epitope contains two putative H-2D^b binding peptides: a 9-mer, encompassing aa 65-73 and a 10-mer consisting of aa 65-74 of the PSA protein. In this work, a recombinant Stm strain expressing TolC inserted with the 10-mer PSA epitope was subject of a tumour challenge experiment employing a melanoma cell line expressing PSA.

II.4. Objectives – part II

II.4.1. The hemolysin secretion system in Ty21a

A second project of the present thesis aimed at improving the hemolysin secretion system in Ty21a for vaccination purposes. In an earlier study, the feasibility of this system in Ty21a was demonstrated (159). For this initial work, full length HlyA was utilized as heterologous antigen and successfully expressed and secreted by Ty21a. Furthermore, mice immunized with secretion-competent Ty21a developed serum antibodies against hemolysin, demonstrating that the type I secretion system is functional in Ty21a and could form the basis for combination vaccines for human use e.g. in cancer immunotherapy.

In order to enhance immunogenicity of this system we sought to increase secretion efficiency in Ty21a as the amount of free antigen is linked with immunogenicity. For this purpose a closer look at the regulation of type I secretion of hemolysin needs to be taken. As aforementioned, the secretion apparatus consist of three components, namely TolC/HlyB/HlyD (160) secreting the RTX toxin haemolysin (HlyA), a virulence factor of extra-intestinal *E .coli* (161). HlyC modifies pro-HlyA to its hemolytically active form in the cytoplasm due to its function as fatty acid acyltransferase (162). The *tolC* gene is located on the chromosome, being part of the *mar-sox* regulon (163), while the other factors are encoded on one polycistronic mRNA derived from the *hlyCABD* (164) operon located on transmissible plasmids or chromosomal pathogenicity islands (165,166). The operon is transcribed from a promoter located

upstream of *hlyC* in a strongly polar manner due to the presence of a rho-independent terminator in the *hlyA-hlyB* intergenic region (167). This termination is suppressed by RfaH which interacts with a cis-acting 5' DNA sequence termed operon polarity suppressor (ops) element, allowing efficient transcription of the entire *hly* operon by inhibiting transcriptional pausing (168-170). The regulatory cascade is schematically envisaged in Figure II.2.

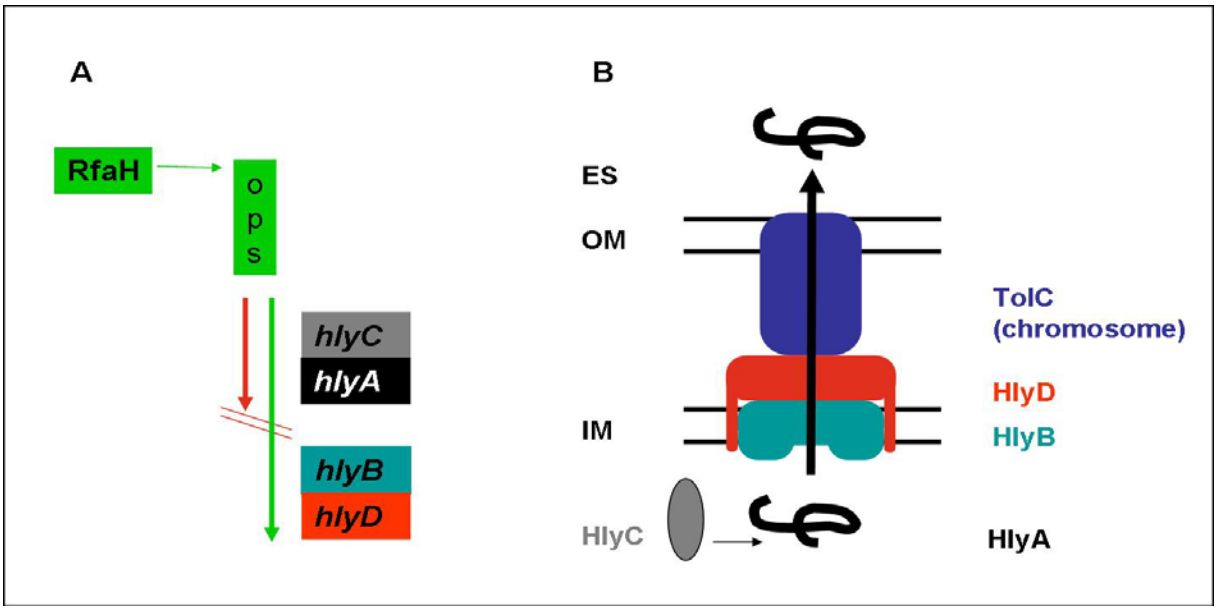

Figure II.2 Genetic regulation of the hemolysin operon (*hlyCABD*) and type I secretion of hemolysin (HlyA) in *E. coli*
A: Transcription of *hlyCABD* shows strong polarity due to a terminator in the *hlyA-hlyB* intergenic site. RfaH binds to the ops element and suppresses termination leading to transcription of the full length polycistronic mRNA and efficient secretion like depicted in **B**: HlyC activates HlyA in the cytoplasm followed by direct secretion of mature hemolysin via the tripartite type I secretion machinery HlyB/HlyD/TolC into the extracellular space (ES). ops: operon polarity suppressor element, OM: outer membrane, IM: inner membrane

In this part of the work, Ty21a was analysed for its secretion efficiency compared to other *Salmonella* strains. By analysing known Ty21a mutations and genetic complementation, it was tried to find factors positively regulating secretion of hemolysin in Ty21a and evaluate their contribution to immunogenicity in a mouse model by assessing serum antibody titres against hemolysin. This in turn could lead to the development of new generation vaccines based on this secretion system facilitating enhanced immunogenicity against secreted heterologous antigens.

III. Material and Methods

III.1. Material

III.1.1. Bacterial strains

Name	Relevant characteristics	Source or reference
E. coli DH5α	F^-, ø80d*lacZ* M15, (*lacZYA-argF*) U169 *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Invitrogen, Karlsruhe, Germany
Listeria monocytogenes EGDe *ΔtrpS*	*trpS,* balanced lethal plasmid system	(171)
Salmonella enterica serovar Dublin *aroA* SL5928	*aroA, fliC*::Tn10	Stocker, B. A. D
S. enterica serovar Typhi Ty21a (Ty21a)	*S. typhi* Ty2, *galE*, *rpoS*, *viaB*	Berna Biotech Ltd., Berne, Switzerland
S. enterica serovar Typhi Ty21aTC (Ty21aTC)	*S. typhi* Ty2, *galE*, *rpoS*, *viaB, tolC*	this study
Salmonella enterica serovar Typhimurium *aroA* SL7207 (Stm)	*his*G46, DEL407 [*aroA*544::Tn10 (Tc^s)]	Stocker, B. A. D
Salmonella enterica serovar Typhimurium *aroA* SL7207TC (StmTC)	*his*G46, DEL407 [*aroA*544::Tn10 (Tc^s)] *tolC*	this study

III.1.2. Cell lines

Name	Relevant characteristics	Source or reference
B16-Ova	mouse melanoma cell line expressing chicken ovalbumin	Juergen Hess, Erlangen
Caco-2	human colon carcinoma	ATCC
EG.7	derivative of EL-4 thymome expressing chicken ovalbumine	ATCC
RAW 264.7	murine leukaemic macrophage-like cells	ATCC
P815-BRAFV600E	murine lymphoblast-like mastocytoma cell line expressing human BRAFV600E and HLA-B2705	C. Yone, MSZ, Würzburg

III.1.3. Animals

Name	Relevant characteristics	Source or reference
C57Bl/6JO1Hsd	H-2^b haplotype	Harlan-Winkelmann
C57Bl/6-TG(TcraTcrB) 1100Mjb (OT-I)	transgenic T-cell receptor specific for $OVA_{257-264}$ in context of H2-K^b MHC I molecule	(137), obtained from Max Planck Centre for Infection Biology, Berlin
C57Bl/6-TG(TcraTcrB) 425Cbn (OT-II)	transgenic T-cell receptor specific for $OVA_{323-339}$ in context of I-A^2 MHCII molecule	(138), obtained from Dept. Virology, University Würzburg
Balb/c HLA-B2705	H-2^d haplotype, expressing human HLA-B 2705 molecule	Prof. Weiss, Munich

III.1.4. Plasmids

Name	Relevant characteristics	Source or reference
pACYC184	Cm^R, Tet^R	(172)
pANN202-812	Amp^R, *hlyR, C, A, B, D,* derivate of pBR322	(173)
pCP20	Amp^R, Cm^R, TS, encoding Flp-recombinase	(174)
pKD3	Amp^R, Cm^R, priming sites for generation of KO fragments	(175)
pKD46	Amp^R, TS, encoding λ-RED system for homologous recombination,	(175)
pMKhly1	Kan^R, derivative of pMOhly1	(115)
pRfaH	Cm^R, derivate of pACY184, encoding *rfaH* gene of *S. typhi* Ty21a	this study
pRpoS	Cm^R, derivate of pACY184, encoding *rpoS* gene of *S. typhimurium* SL7207	this study
pRSC2	Cm^R, *hlyR, C, A, B, D,* derivate of pACYC184	(176)
pSP118-PS_{acta}OVA	Ery^R, derivative of pUNK, encoding *actA* signal sequence-*ovalbumin* fusion	(139)
pTC	derivative of pTolC, Kan^R deleted, encoding *tolC* from *E. coli*	this study
pTC-OVA	derivative of pTolC-OVA, Kan^R deleted, encoding *tolC*-$OVA_{257-264}$ and $OVA_{323-339}$ epitopes fusion	this study
p*tolC*	Amp^R, derivative of pBR322, encoding *tolC* gene from *E. coli*	(127)
pTolC	Kan^R, derivative of pMKhly1, encoding *tolC* gene from *E. coli*	this study

Name	Relevant characteristics	Source or reference
pTolC-BRAF	Kan^R, derivative of pTolC, encoding *tolC*-3xB-RAFV600E GRFGLATEK epitope fusion	this study
pTolC-OVA	Kan^R, derivative of pTolC, encoding *tolC*-$OVA_{257-264}$ and $OVA_{323-339}$ epitopes fusion	this study
pTolC-PSA	Kan^R, derivative of pTolC, encoding *tolC* PSA_{65-74} epitope fusion	(136)

Amp^R: Ampicillin resistant; Cm^R Chloramphenicol resistant; Ery^R: Erythromycin resistant; Tet^R: Tetracycline resistant; TS: temperature sensitive replication

III.1.5. Primers

Name	Sequence	Characteristics
BRAF-linker1	5'CGGCGATTTTGGCCTGGCGACCGAAAAAGCGGGCGATTTTGGCCTGGCGACCGAAAAAGCGGGCGATTTTGGCCTGGCGACCGAAAAAGGTAC3'	linker for hybridisation of BRAF fragment
BRAF-linker2	5'CTTTTTCGGTCGCCAGGCCAAAATCGCCCGCTTTTTCGGTCGCCAGGCCAAAATCGCCCGCTTTTTCGGTCGCCAGGCCAAAATCGCCGGTAC3'	linker for hybridisation of BRAF fragment
c1_down	5´TTTTCACCATGGGCAAATAT3´	detection of Cm^R cassette
Cat RT (F)	5´ACGTTTCAGTTTGCTCATGG3´	Chloramphenicol transacetylase mRNA
Cat RT (R)	5´CCGGCCTTTATTCACATTCT3´	Chloramphenicol transacetylase mRNA
DhF	5'GCTTAATGTCCAAGATGCCTAC3'	multiplex PCR
DhR	5'GAGCAACGCCAGTACCATCTG3'	multiplex PCR
ectolCClaI_up	5'GGGAGAGCATCGATTAACGCCAACC3'	cloning of *tolC*
ectolCSalI_down	5'GGCATCGGTCGACTCGAAATTGAAG3'	cloning of *tolC*
HlyA RT (F)	5´CAGCTGCAGGTAGCTTCG3´	bicistronic *hlyCA* mRNA and polycistronic *hlyCABD* mRNA
HlyA RT (R)	5´TATGCTGATGTGGTCAGGGT3´	bicistronic *hlyCA* mRNA and polycistronic *hlyCABD* mRNA
HlyD RT (F)	5´ATTCTTACCCGCTCATCTGG3´	polycistronic *hlyCABD* mRNA
HlyD RT (R)	5´GTGGCAACAATTTCCACTTG3´	polycistronic *hlyCABD* mRNA
htrB_up	5'GCGAGAATACGGAGAATTG3'	detection of *htrB*
htrB2_down	5' GAGGGGAAAAATTGCAG3'	detection of *htrB*
InvAF	5'CGAGCAGCCGCTTAGTATTGAG3'	multiplex PCR
InvAR	5'CCATCAAATTAGCGGAGGCTTC3'	multiplex PCR
k1_down	5'CAGTCATAGCCGAATAGCCT3'	detection of Kan^R cassette

Name	Sequence	Characteristics
OVA-linker1	5'CTCCATTATTAACTTTGAAAAACTGGCCATTTCCC AGGCGGTGCATGCGGCGCATGCGGAAATTAATGA AGCCGGCCGTGCCGGTAC3'	linker for hybridisation of OVA fragment
OVA-linker2	5'CCGGCACGGCCGGCTTCATTAATTTCCGCATGCG CCGCATGCACCGCCTGGGAAATGGCCAGTTTTTCA AAGTTAATAATGGAGGTAC3'	linker for hybridisation of OVA fragment
PrtF	5'CGTTTGGGTTCCTTGGATCACG3'	multiplex PCR
PrtR	5'CTATAATGGCGGCGGCGAGTTC3'	multiplex PCR
rfaH_up	5'GAGGATCCACAGGAAGCTTGATGCGTTTTAG3'	cloning of *rfaH*
rfaH_down	5'CCTTATGGATCCCTAATGATGATGATGATGATG AATCTTGCGAAAACCGGTG3'	cloning of *rfaH*
rpoS_up	5'CATCGCCTGGATCCCCGGGAACG3'	cloning of *rpoS*
rpoS_down	5'GACGCAAAAAGCTTTTGATGACGCGCC3'	cloning of *rpoS*
RfaH RT (F)	5´AACGTACCTTCGTCAGCGA3´	*rfaH* mRNA
RfaH RT (R)	5´GTGGCGTTGATTGTAGTGGT3´	*rfaH* mRNA
tolCFR_up	5'CCTCGCCACTCATTTCTCCG3'	detection of *tolC* deletion
tolCFR_down	5' CGCTTACCAGACCTACAAGGGC3'	detection of *tolC* deletion
tolCKO_up	5'GCGCTAAATACTGCTTCACAACAAGGAATGCAA ATGAAGAAGTGTAGGCTGGAGCTGCTTC3'	chromosomal deletion of *tolC*
tolCKO_down	5'CGTTATTGCTGTTGGCGCGAGCGGCGGTCGGC TGTACTGCTGCATATGAATATCCTCCTTA3'	chromosomal deletion of *tolC*
tolSEQ1	5'GGATTTAACGGCTTCTACC3'	detection of *tolC* insertion
tolSEQ2	5'CGGCAGCGAGAAGCTCAGG3'	detection of *tolC* insertion
ViaBF	5'CACGCACCATCATTTCACCG3'	multiplex PCR
ViaBR	5'AACAGGCTGTAGCGATTTAGG3'	multiplex PCR

multiplex primers taken from (177), c1_down and k1_down taken from (175)

III.1.6. Media

Name	Composition	Manufacturer
Brain Heart Infusion (BHI)	ready made powder, 37 g/L	Difco
CY medium	12 mg/ml Yeast extract , 20 mg/ml Hy-Case, 12 mg/ml pepticase, 1.25 mg/ml sodium hydrogen phosphate, 3.3 mg/ml sodium chloride, 2 mg/ml glucose	Sigma, Applichem, Roth
DMEM	DMEM, 10 % fetal bovine serum	GIBCO
Luria Bertani (LB)	ready made powder, 20 g/L	Sigma

Name	Composition	Manufacturer
RP10	RPMI1640, 10 % fetal bovine serum 1x MEM non-essential amino acids , 1 mM sodium pyruvate, 2 mM L-glutamine 50 mM β-mercaptoethanol	GIBCO

Media for bacterial culture (LB, BHI and CY) were autoclaved for 20 min at 121°C. Antibiotics and other temperature sensitive supplements were added after autoclaving and cooling of the media. For plates, media were solidified with 1.5 % Agar (Difco).

III.1.7. Buffers and solutions

Name	Composition	Manufacturer
Acrylamide/Bis solution	ready made solution, 40 % Acrylamide/Bis 37.5:1 (2.6 % C)	Bio-Rad
Ampicillin stock	100 mg/ml Ampicillin (Amp) in water	Sigma
Bile salt stock	200 mg cholate/deoxycholate in LB medium	Sigma
Blocking buffer	1 % BSA in PBS	Sigma, Applichem
Carbonate buffer	7 g/L sodium hydrogen carbonate	Applichem
Chloramphenicol stock	20 mg/ml Chloramphenicol (Cm) in ethanol	Sigma
Coating buffer, pH 9.6	4.2 g/L sodium hydrogen carbonate 5.3 g/L di-sodium hydrogen carbonate	Applichem
Conjugate buffer	0.05 % TWEEN20®, 1 % BSA in PBS	Applichem, Roth, Sigma
Coomassie Protein assay reagent	ready made solution	Thermo Scientific
dH_2O	deionized water	in-house desalting device from Millipore
D-PBS	ready made solution, w/o calcium and magnesium chloride	GIBCO
Erythrocyte-lysisbuffer	100 ml 50 mM Tris, pH 7.65 900 ml 155 mM ammonium chloride, pH 7.2	Applichem
Erythromycin stock	50 mg/ml Erythromycin (Em) in ethanol	Sigma
G418	50 mg/ml G418 sulphate in water	Sigma
Gentamycin	ready made solution, 50 mg/ml Gentamycin in water	Sigma
Kanamycin stock	25 mg/ml Kanamycin (Kan) in water	Sigma
Laemmli buffer	50 mM Tris-HCl pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol, 2 % SDS, 0.05 % bromophenol blue	Sigma, Applichem, Roth

Name	Composition	Manufacturer
Loading dye	ready made solution, 10 mM Tris-HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol, 60 mM EDTA	Fermentas
Novobiocin stock	100 mg/ml Novobiocin (Novo) in ethanol	Sigma
PBS	ready made powder, 9.55 g/L	Applichem
PBS milk	5 % non-fat dried milk powder in PBS	Applichem
PBS TWEEN	0.05 % TWEEN20® in PBS	Applichem, Roth
Pen-strep	ready made solution, 10,000 U/ml penicillin G, 10,000 µg/ml streptomycin in normal saline	GIBCO
Ponceau S solution	ready made solution, 0.1 % Ponceau S in 5 % acetic acid	Sigma
SDS PAGE buffer	3 g/L Tris, 14.4 g/L glycine, 1 g/L SDS	Applichem
Tris borate EDTA buffer (TBE)	ready made solution (10 x), 20 ml/L	Applichem
Towbin buffer	3 g/L Tris, 14.4 g/L glycine, 20 % methanol	Applichem
Trypsin/EDTA	ready made solution, 0.05 % trypsine 0.02 % EDTA in PBS w/o calcium and magnesium	PAN^{TM} Biotech

Bis: N,N' methylene-bis-acrylamide, EDTA ethylene diamine tetra acetic acid, PBS: phosphate buffered saline, Tris: tris (hydroxy methyl) aminomethane, Tris-HCl: tris base-hydrogen chloride buffer system, SDS: sodium dodecyl sulphate

III.1.8. Antibodies and FACS multimers

Name	Specificity	Manufacturer
25D-1.16	$OVA_{257-264}$ /H-2K^b	(178)
$IgG_{1,\kappa}$-PE	isotype control	BD Pharmingen
$IgG_{2a,\lambda}$-FITC	isotype control (anti KLH)	BD Pharmingen
$IgG_{2a,\kappa}$-CyChrome^{TM}	isotype control	BD Pharmingen
$IgG_{2a,\kappa}$-FITC	isotype control	BD Pharmingen
$BRAFP2_{GRFGLATEK}$HLA-B2705-Dextramer-FITC	TCR specific for BRAF P2/HLA-B2705	DakoCytomation
$BRAFP3_{GRFGLATVK}$HLA-B2705-Dextramer-FITC	TCR specific for BRAF P3/HLA-B2705	DakoCytomation
$OVA_{SIINFEKL}$ –H-2Kb-Tetramer-PE	TCR specific for $OVA_{257-264}$/H-2K^b	Beckman Coulter
α-CD4-CyChrome^{TM} (RM4-5)	murine CD4 (L3T4)	BD Pharmingen
α-CD4-FITC (GK1.5)	murine CD4 (L3T4)	BD Pharmingen
α-CD8a-CyChrome^{TM} (53-6.7)	murine CD8a (Ly2)	BD Pharmingen
α-CD8a-FITC (53-6.7)	murine CD8a (Ly2)	BD Pharmingen

Name	Specificity	Manufacturer
α-CD16/32	murine FCγIII/II receptor	BD Pharmingen
α-CD62L-FITC (MEL14)	murine CD62 (Ly22)	BD Pharmingen
α-IFNγ (R4-6A2)	murine γ-Interferon	BD Pharmingen
α-IFNγ-biotin (XMG 1.2)	murine γ-Interferon	BD Pharmingen
α-$HlyA_s$	HlyA secretion signal	(115)
α-mouse IgG-AKP	murine IgG	Dianova
α-mouse IgG+IgM-AKP	murine IgG and IgM	Dianova
α-mouse IgG-FITC	murine IgG	BD Pharmingen
α-rabbit IgG-HRP	rabbit IgG	GE Healthcare
α-TolC	TolC from gram negative bacteria	(179)
α-Vα2 TCR (20.1)	murine α2 side chain of T cell receptor	BD Pharmingen
α-Vβ5.1 TCR (MR9-4)	murine Vβ5.1 side chain of T cell receptor	BD Pharmingen

AKP: alkaline phosphatase; FITC: Fluorescein isothiocyanat, HRP: horseradish peroxidase, PE: phycoerythrin, TCR: T cell-receptor

III.1.9. Chemicals

All Chemicals not mentioned elsewhere were obtained from Sigma, Difco, Roth and Applichem.

III.1.10. Enzymes and special reagents

Name	Manufacturer/Source
4-Nitrophenyl phosphate disodium salt-hexahydrate substrate (pNPP)	Sigma
Agarose for electrophoresis	Invitrogen
BioTherm Taq Polymerase	Genecraft
BCIP/NBT, Sigma *Fast*™ substrate	Sigma
Bovine serum albumin (BSA)	Sigma
BRAF27R2E8 Peptide P2 (GRFGLATEK)	KJ Ross-Petersen ApS
BRAF27R2V8 Peptide P3 (GRFGLATVK)	KJ Ross-Petersen ApS
Calf intestine alkaline phosphatase (CIAP)	Fermentas
Desoxynucleotides (dNTPs)	Fermentas
DNA ladder, 1 kB	Fermentas
Lipopolysaccharide (LPS) from *Salmonella typhi* Ty21a	Berna Biotech

Name	Manufacturer/Source
Pfu Polymerase, cloned	Stratagene
Phusion Taq	Finnzymes
$OVA_{257-264}$ SIINFEKL peptide	Dept. Microbiology, Würzburg
Polynucleotid kinase	Fermentas
Protein ladder, BenchMark	Invitrogen
rATP	Fermentas
recombinant Interleukin-2 (IL-2)	Sigma
Restriction endonucleases	Fermentas
Streptavidin alkaline phosphatase	R&D Systems
T4-Ligase	Fermentas

III.1.11. Consumable material

Name	Manufacturer
96-well plates	Nunclon, Greiner bio-one
96-well nitrocellulose plates; millititer HA	Millipore
Cell culture flasks; 75 cm^3 and 125 cm^3	Greiner bio-one
Cell culture plates; 24 wells	Greiner bio-one
Cell scraper	Greiner bio-one
Gene pulser cuvette; 0.1 cm electrode gap	Bio-Rad
Cryo tubes; 1.5ml	Greiner bio-one
Insulin syringe	Braun
Micro tube; 1.5 ml, 2 ml	Sarstedt
Nitrocellulose; Hybond ECL	Amersham
Parafilm	Hartenstein
Pasteurpipettes	Hartenstein
Petri dish; 12 cm	Greiner bio-one
Pipette tips	Sarstaedt
Sterile filter Millex-GS; 0.22µm	Millipore
Polypropylene tubes; 15 ml and 50 ml	Greiner bio-one
Whatman paper 3 mm 190 g/m^2	Schleicher & Schuell
X-ray films	Amersham

III.1.12. Instruments

Name	Manufacturer
Bacterial incubator	Heraeus Instruments
Bacterial shaker	Scientific Innova
Bacterial shredder, Fast Prep FP120	Thermo Electron Corporation
Cell culture incubator	Heraeus Instruments
Cell culture microscope	Leica
Cell counting chamber, Bürker	Marienfeld
Clean bench	Heraeus
Developing cassette for Western Blot	Dr. Goos-suprema GmbH
Developing machine for Western Blot	Agfa
DNA Sequencer, ABI PRISM 373	ABI
Electrophoresis power supply	Bio-Rad
Electrophoresis unit, MiniProtean II for SDS PAGE	Bio-Rad
Electrophoresis unit, sub-cell GT for DNA electrophoresis	Bio-Rad
Elisa reader	TECAN
ELISPOT reader	Autoimmun Diagnostika GmbH
Flow Cytometer FACSCalibur	Becton Dickinson
Freezer (-20°C)	Liebherr
Freezer (-80°C)	Nunc Advantage
Fridge (4-8°C)	Liebherr
Gene pulser	Bio-Rad
Glassware	Schott
Heating block	Liebisch
Ice machine	Scotsman
Mega centrifuge J-6B	Beckman
Megafuge 1.0 R:	Heraeus
Microwave	Siemens
Mini centrifuge, 5417R, Biofuge 15	Eppendorf; Heraeus
pH-electrode, SenTix 61	WTW
pH-meter, inolab 720	WTW
Pipettes	Eppendorf
Real Time PCR machine, Rotor Gene 2000	Corbett
Semi-dry blotting device, Trans-Blot SD	Bio-Rad
Sonicater SonoPlus HD70	Bandelin
Thermocycler, T-Gradient Thermoblock	Biometra
Timer	Roth
Scale	SCALTEC
Vortex genie	Scientific Industries

III.1.13. Kits

Name	Manufacturer
DNeasy® Mini Kit	QIAGEN
DNAfree™ Kit	Ambion
DyNAmo™ HS SYBR® Green qPCR Kit	Finnzymes
ECL™ Western Blotting detection Kit	Amersham
First Strand® cDNA Synthesis Kit	Fermentas
RNase-Free® DNase Kit	QIAGEN
RNeasy® Mini kit	QIAGEN
QIAquick® Gel Extraction Kit	QIAGEN
QIAquick® PCR Purification Kit	QIAGEN
QIAquick® Plasmid Mini Kit	QIAGEN

III.1.14. Software

Name	Manufacturer
CellQuest Pro 4	Apple
GraphPad Prism 4	GraphPad Software, Inc
MS Excel 2003	Microsoft
MS Word 2003	Microsoft
MS Power Point	Microsoft
Photoshop CS2	Adobe
Rotor-Gene Analysis Software V4.6.70	Corbett

III.2. Methods

III.2.1. Microbiology

III.2.1.1. General culture

For overnight (o/n) cultures, one single bacterial colony was picked from a plate and resuspended in 5-10 ml of BHI or LB medium containing the appropriate antibiotics (usually diluted 1/1000 from the stock solution) and incubated o/n with vigorous shaking at 30°C or 37°C. Overnight cultures usually grow to the stationary phase within 18 h. For logarithmic cultures, overnight cultures were diluted 1/25 to 1/200 in fresh medium and incubated until the desired optical density at 600 nm (OD600) was reached.

III.2.1.2. Electrotransformation of bacterial cells

For electrotransformation of bacteria with plasmid or linear DNA, cell suspensions need to be salt free to avoid current flux due to the high field pulse. Cultures with an OD600 of approximately 0.6-0.8 were harvested by centrifugation (3,345 × *g* for 30-60 min) and washed two times with ice-cold 10 % glycerol, concentrated 100 × in 10 % glycerol and used immediately for transformation or stored at -80°C. 1-5 µl of DNA was mixed with 100 µl cell suspension and DNA was introduced into the bacteria by electroporation using a Bio-Rad Gene Pulser at 2.5 kV, 25 µF and 200 Ohm in a 0.1-cm electroporation cuvette. Transformed cells were dissolved in 900 µl BHI incubated at 37°C for 1 h and then plated on LB agar plates containing the appropriate antibiotic for selection of the resistance gene.

III.2.1.3. Preparation of infection aliquots

Immunization aliquots were prepared by cultivating strains overnight at 37°C in 50 (i.v. injection) to 1000 (oral and intranasal immunization) ml BHI or LB medium containing appropriate antibiotics. The next day, cells were harvested by centrifugation in a Beckmann-Coulter centrifuge, washed in PBS and concentrated 10 (i.v.) or 200 (oral, nasal) fold in PBS containing 10-20 % Glycerol and aliquoted in 500-1000 µl portions. Aliquots were stored at least 24 hours at -80°C before the bacterial titre was determined. The number of colony forming units (CFU) was

obtained by plating serial dilutions on BHI or LB agar plates and incubation at 37°C until bacterial colonies were visible. Plates containing between 30 and 300 colonies were counted to determine the CFU. The vials were thawed on ice 30 min prior to use.

III.2.1.4. On-plate hemolysin assay

Plates containing blood erythrocytes were prepared as follows: Erythrocytes were collected by centrifugation of 30 ml of sheep blood at 300 × *g.* Cells were washed 3-4 times with 0.9 % NaCl solution until the supernatant was clear. Finally, 25 ml of the erythrocyte suspension was mixed with 1 L of warm LB agar to prepare blood agar plates. For determination of the hemolysin secretion ability, single colonies of assayed strains were picked, resuspended in 20 µl of sterile water. 1 µl portions of these solutions were spotted on LB blood agar plate and incubated for 24 h at 37°C. Secretion-competent colonies can be identified by a corona of lysed erythrocytes.

III.2.1.5. Oxidative stress test

For the oxidative stress test, bacterial strains were incubated in CY-medium to the stationary phase. The cells were harvested by centrifugation, washed with 0.9 % NaCl and equally separated into two tubes. Bacteria were then suspended in CY medium with none, 3 mM or 30 mM H_2O_2. Survival was assessed after 20 min incubation at 37°C by plating 0.1 ml cell suspension on BHI-agar and o/n incubation at 37°C. The percentage of surviving bacteria was determined by comparing the numbers of colony forming units (CFU) of H_2O_2 treated and untreated cells.

III.2.1.6. Determination of the MIC of antimicrobial compounds

The MIC (minimal inhibitory concentration) is defined as the lowest concentration of an antimicrobial agent which is sufficient to inhibit growth of a given bacterial culture. For determination of the MIC value 100 µl of LB was applied to a 96-well plate and a gradient of the assayed agent was established by twofold dilution steps of an initial concentration of this compound over the plate. Mid-logarithmic bacterial cultures were diluted to a concentration of 200 cells per µl and 100 µl of this suspension was applied in each well of one row per strain. The plate was incubated for 24 h at 37°C

and then read-out in an ELISA reader at 620 nm. Cultures with OD620 values below 0.06 were determined as growth repressed.

III.2.1.7. Determination of plasmid stability *in vitro*

To investigate if plasmids are stably replicated without antibiotic selection a plasmid stability tests were performed *in vitro*. Cultures were grown in 5 ml LB to the logarithmic phase with selection antibiotics (AB). Afterwards, 50 µl of the culture were harvested in a microcentrifuge at 2,000 × *g* and then resuspended in either medium only or medium containing selection antibiotic. Both cultures were grown overnight with vigorous shaking at 37°C. The next day, serial dilutions of the cultures were plated on LB and LB agar plates and further incubated at 37°C until colonies were visible. Plates containing between 30 and 300 colonies were counted. The plasmid stability P was calculated with the following equations:

$P\ (\%) = (CFU_{LB\ AB}/CFU_{LB})_{raised\ w/o\ AB} * 100 * F_P$; Def.: $P \leq 100$;

$F_P = (CFU_{LB}/CFU_{LB\ AB})_{raised\ with\ AB}$; Def.: $F_P = 1$ if $(CFU_{LB\ AB}/CFU_{LB})_{raised\ w/o\ AB} \geq 1$ or $(CFU_{LB}/CFU_{LB\ AB})_{raised\ with\ AB} < 1$

III.2.2. Molecular Biology

III.2.2.1. PCR for cloning

Polymerase chain reaction (PCR) is an enzymatic method for in vitro synthesis of multiple copies of specific sequences of DNA. For cloning purposes, high fidelity polymerases, namely Pfu and Phusion Taq, were used. For PCR products facilitating disruption of chromosomal genes according to the method of Datsenko and Wanner (175), Biotherm™ Taq polymerase was used similar to the Pfu protocol. Typical protocols are shown in the following tables:

1 × mix Pfu/Taq polymerase			program		
5 µl	Dimethylsulfoxid (DMSO)	10 %	95°C	5 min	
5 µl	10 × buffer Pfu/ Biotherm™ Taq	1 ×	95°C	1 min	} 33x
0.5 µl	100 µM primer upstream	1 µM	50°C	1 min	
0.5 µl	100 µM primer downstream	1 µM	72°C	1 min/1kb	
1 µl	10 mM dNTPs	0.2 mM	72°C	10 min	
1 µl	template DNA		4°C	∞	
1 µl	Pfu (2.5 U/µl)/Taq (5 U/µl)	2.5 U/5 U			
ad 50 µl	dH_20				

1 × mix Phusion polymerase			program		
10 µl	5 × buffer Phusion	1 ×	98ºC	30 s	
0.5µl	100 µM primer upstream	1 µM	98ºC	10 s	} 33x
0.5 µl	100 µM primer downstream	1 µM	60ºC	20 s	
1 µl	10 mM dNTPs	0.2 mM	72ºC	10 s /1kb	
1 µl	template DNA		4ºC	∞	
1 µl	Phusion polymerase (2 U/µl)	2 U			
ad 50 µl	dH_20				

III.2.2.2. Colony PCR

Colony PCR was used for screening purposes facilitated by BioTherm Taq polymerase. With this method, single bacterial colonies or plasmids could be analysed for the existence of specific DNA sequences.

1 × mix Taq polymerase			program		
2.5 µl	10 × buffer BioTherm Taq	1 ×	95°C	5 min	
0.25 µl	100 µM primer upstream	1 µM	95°C	1 min	} 30x
0.25 µl	100 µM primer downstream	1 µM	50°C	1 min	
0.5 µl	10 mM dNTPs	0.2 mM	72°C	1 min/1kb	
1 µl	template DNA/single colony		72°C	10 min	
0.3 µl	Taq polymerase (5U/µl)	1.5 U	4°C	∞	
ad 25 µl	dH_20				

III.2.2.3. Multiplex PCR

Multiplex PCR is used for a rapid detection and discrimination of *Salmonella* strains (in abridged form of (177)). The reaction mix was prepared as follows:

1 × mix multiplex PCR			program		
5 µl	10 × buffer BioTherm Taq	1 x	95°C	5 min	
1 µl	100 µM DhF	2 µM	95°C	1 min	} 38x
1 µl	100 µM DhR	2 µM	50°C	1 min	
1 µl	100 µM InvAF	2 µM	72°C	1 min/1kb	
1 µl	100 µM InvAR	2 µM	72°C	10 min	
1 µl	100 µM PrtF	2 µM	4°C	∞	
1 µl	100 µM PrtR	2 µM			
1 µl	100 µM ViaBF	2 µM			
1 µl	100 µM ViaBR	2 µM			
1 µl	10 mM dNTPs	0.2 mM			
1 x	single colony				
1 µl	Taq polymerase (5 U/µl)	5 U			
ad 50 µl	dH_20				

III.2.2.4. Direct hybridisation of DNA linkers

For production of small-sized DNA fragments, direct hybridization of two complementary DNA linkers is an attractive alternative to PCR. For this purpose, oligos were synthesized like common PCR primers, phosphorylated and subsequently hybridized in a temporal temperature gradient. As the oligos contain 5' overhangs that mimic restriction by *Kpn*I endonuclease, the hybridization construct can be directly ligated in a vector digested with *Kpn*I. Reactions were performed like listed below:

phosphorylation			program	
10 µl	100 µM DNA linker 100 µM	66 µM		
1.5 µl	10 × PNK buffer B	1 x	37°C	30 min
1.5 µl	10 mM rATP	1 mM	70°C	20 min
0.5 µl	T4 PNK (10 U/µl)	15 U		
ad 15 µl	dH_20			

hybridisation			program	
3.5 µl	66 µM phosphorylated linker 1	23 µM		
3.5 µl	66 µM phosphorylated linker 2	23 µM	90°C	10 min
1 µl	formamide	10 %	gradient*	2 h
ad 10 µl	dH_20			

*The temperature gradient was achieved by switching-off the heating block at 90°C and allowing it to cool down while leaving samples in.

III.2.2.5. Gel electrophoresis

DNA samples were mixed 1/10 with loading dye solution and subjected to electrophoresis in 1 % (fragments over 500 bp) or 2 % (fragments below 500 bp) agarose gels in an electrophoresis unit at 180 V. DNA bands were visualized under UV light in a gel imaging system. Agarose gels were prepared in a special gel chamber with 10 or 20-well combs as follows:

agarose gels

1 or 2 g	agarose for electrophoresis	1 or 2 %
8 µl	10 mg/ml ethidium bromide solution	4 µg/ml
ad 200 ml	TBE buffer	

III.2.2.6. DNA purification

All DNAs were extracted or purified using Qiagen purification kits according to manufacturers' protocols. In general, DNA purification is based on selective binding to silica columns, washing and final elution in weak alkaline Tris-HCl buffer. Chromosomal DNA was extracted from bacteria with the DNeasy® Mini Kit with slight modifications for gram-negative species. Plasmid DNA was obtained using QIAquick® Plasmid Mini Kit, PCR products were purified with the QIAquick® PCR Purification Kit and the QIAquick® Gel Extraction Kit was used to purify DNA fragments after gel electrophoresis. The latter methods were carried out strictly following manufacturer's protocols.

III.2.2.7. DNA restriction and ligation

DNAs were digested using restriction endonucleases from Fermentas facilitated in their respective buffers or according to manufacturers' recommendations as suggested by the DoubleDigest® algorithm (180).

In order to construct new DNA molecules the desired fragments were ligated with T4-DNA ligase. For the ligation reaction, restricted and therefore sticky ended DNA fragments were mixed at a molar ratio of vector to insert 1:3-1:10, respectively. Ligation was performed as follows:

ligation mix			program	
16 µl	vector-insert mix (1:3-1:10)			
2 µl	10 × T4 ligase buffer	1 x	16°C	12 h
2 µl	T4 ligase (5 U/µl)	10 U	65°C	20 min
ad 20 µl	dH_20			

III.2.2.8. Sequencing

DNA sequencing was performed in house with an ABI PRISM 373 sequencer. Therefore, approx. 1 µg of DNA was mixed with 10 pmol of primer in a total volume of 14.5 µl and subjected to sequencing according to manufacturers' protocols.

III.2.2.9. RNA isolation and cDNA synthesis

Cultures were grown in BHI medium containing the appropriate antibiotics to the early logarithmic (OD600 = 0.4) or stationary phase (OD600 = 2.5). 5×10^9 from the early and 1×10^{10} cells from the late time-point were collected by centrifugation and shock-frozen in liquid nitrogen at -80°C for storage. Bacterial cells were lysed with a FastPrep shredder and total RNA was extracted with the RNAeasy Mini Kit according to manufacturers' protocol. On column DNA digestion with RNase-Free DNase kit was performed for 20 min at room temperature. Presence of chromosomal DNA was analyzed by PCR with the primers htrB_up and htrB2_down. Residual DNA was digested with DNAfree kit, 0.5 µg of total RNA were applied for cDNA synthesis with random hexamers using First Strand cDNA Synthesis Kit.

III.2.2.10. Semi-quantitative Real-Time PCR (qRT-PCR)

Semi-quantitative Real-Time PCR was applied to measure the relative level of transcript of certain genes compared to a control strain. The primers Cat RT (F) and Cat RT (R) were used to amplify a 136 bp fragment specific for the cat gene. The primers HlyA RT (F) and HlyA RT (R) were used to amplify a 121 bp fragment specific for the *hlyA* gene. The primers HlyD RT (F) and HlyD RT (R) were used to amplify a 135 bp fragment specific for the *hlyD* gene. The primers RfaH RT (F) and RfaH RT (R) were used to amplify a 91 bp fragment specific for the *rfaH* gene. qRT-PCR was performed on the Rotor-Gene 2000 using DyNAmo™ HS SYBR® Green qPCR Kit. In a total volume of 20 µl each sample was analysed in triplicate, each qRT-PCR was performed in duplicate. 1 µl of tenfold diluted cDNA was used for qRT-PCR. The presence of the primer specific amplicon was determined by detection of one melting-temperature peak and a single band at the expected size on a 2 % agarose gel after electrophoresis. The Ct values were determined with the Rotor-Gene Analysis Software V4.6.70. By raising 2 by the power of the corresponding Ct value a relative unit for comparison of the initial RNA amount was calculated. The relative changes in gene expression between different strains were calculated after normalization with the cat gene as internal control. Significances of regulation were calculated using Students T-Test. Conditions applied for qRT-PCR are listed below:

1 × mix qRT-PCR			program			
			95°C	15 min		
10 µl	2 × Sybr green Mastermix	1 x	94°C	10 s	}	40x
1 µl	10 µM primer upstream	1 µM	57°C	20 s		
1 µl	10 µM primer downstream	1 µM	72°C	30 s		
1 µl	cDNA		72°C	5 min		
ad 20 µl	dH_2O		70-95°C	melt curve		

III.2.3. Protein analysis

III.2.3.1. Preparation of bacterial cellular proteins

Bacteria were grown in BHI or LB medium containing appropriate antibiotics to the desired growth phase. 1-20 ml of culture were harvested by centrifugation and lysed in an appropriate amount of Laemmli buffer, cooked for 5 min and subjected to SDS polyacrylamide gel electrophoresis (PAGE). This fraction was referred to as cellular proteins or pellet proteins, as these samples contain the whole proteome of bacterial cells.

III.2.3.2. Preparation of bacterial supernatant proteins

Supernatant proteins were obtained by precipitating bacterial extracellular medium. Cultures were raised in BHI or LB medium containing appropriate antibiotics. After certain time points, 20 ml culture were taken, bacterial cells were collected by centrifugation and discarded. The resulting supernatants were precipitated with 10 % trichloric acid overnight at 4°C. The next day, the precipitate was collected by centrifugation at 3,345 × *g* and 4°C for 1 h, washed with 1 ml ice-cold acetone and carefully resuspended in 200 µl Laemmli buffer by rinsing the walls of the centrifugation tube. Finally, the pH was adjusted by adding 1 µl of saturated Tris solution; samples were cooked for 5 min and subjected to SDS PAGE.

III.2.3.3. Preparation of bacterial membrane proteins

To investigate the abundance of proteins in bacterial membranes, a quick protocol to separate this fraction from the cytosol was used. Cultures were grown in 20 ml LB medium containing appropriate antibiotics until an OD 600 of 0.9-1.1 was reached. Roundabout 7.5 × 10^9 cells were harvested by centrifugation, washed, and concentrated in 1 ml of PBS. Subsequently, the cells were sonicated 6-8 times with

15 s pulse length at 70 % output until a clear lysate was visible. Viable cells were collected by centrifuging for 3 min at 2,000 × *g* and discarded. The obtained supernatant was centrifuged at 10,000 × *g* and 4°C for 30 min. The resulting pellet was lysed in 200 µl Laemmli buffer w/o β-mercaoptoethanol, half was cooked and half was left untreated to investigate heat-labile, multimeric proteins. The pellet contains the membrane fraction comprised of inner and outer membrane. 100 µl of the supernatant was mixed 1:1 with Laemmli, cooked and referred to as cytoplasmic fraction.

III.2.3.4. SDS PAGE

To separate and analyze proteins, SDS polyacrylamide electrophoresis (PAGE) was used. SDS binding enables proteins to be electrophoretically separated according to their size in a polyacrylamide gel matrix. 10-40 µl of protein samples and 10 µl BenchMark mass standard were loaded onto gels. Electrophoresis was performed in a SDS PAGE running buffer containing MiniProtean II device at 180 V until the bromphenol front ran out of the gel. Gels were prepared as follows:

Resolving gel (10 %)		Stacking gel	
5 ml	40 % acrylamide/Bis solution	1.1 ml	40 % acrylamide/Bis solution
2.5 ml	3 M Tris pH 9.0	1.25 ml	1 M Tris pH 6.8
100 µl	20 % SDS solution	50 µl	20 % SDS solution
20 µl	TEMED	10 µl	TEMED
200 µl	10 % APS	100 µl	10 % APS
ad 20 ml	dH_2O	ad 10 ml	dH_2O

APS: ammonium persulfate, TEMED: N, N, N', N'-tetramethylethylenediamine

III.2.3.5. Western Blot

After electrophoresis, SDS gels were equilibrated in Towbin buffer and subsequently transferred onto nitrocellulose membranes using a semi dry blotting device at 25 V for 40-75 min depending on the target protein size. To evaluate if equal amounts of proteins were applied on SDS PAGE, blotted membranes were stained with Ponceau S solution. Then, membranes were blocked in PBS milk for 1 h and incubated with the primary antibody (1/1000-1/3000) in PBS milk) overnight with gentle agitation at 4°C. The remaining gels were stained with Coomassie blue to control total protein

amount applied for SDS PAGE. The next day, membranes were washed 3 × with PBS TWEEN and incubated with secondary HRP coupled antibodies (1/1000 in PBS milk) for at least 1 h. After additional 3 washing steps, blots were developed with the ECL™ Western Blotting detection Kit.

III.2.4. Eukaryotic cell culture

III.2.4.1. General culture

RAW 246.7 macrophages were cultured in RP10; Caco-2 cells were maintained in D-MEM and B16-Ova cells were grown in RP10 supplemented with 2 mg/ml G418 to ensure expression of ovalbumin. All cell lines were kept under 5 % CO_2 atmosphere at 37°C and subcultured when they reached 90 % confluence. Subcultures of Raw cells were obtained by scraping the cell layer and transferring $1/10^{th}$ of the suspension in fresh medium. Subcultures of Caco-2 and B16-Ova cells were prepared as follows: cellular monolayers were washed with PBS, treated with Trypsin/EDTA to obtain non-adhering single cells and propagated also 1:10 in fresh medium.

III.2.4.2. Infection experiments with RAW 264.7 and Caco-2 cells

For infection studies of eukaryotic cells with live bacteria, 5×10^5 Raw or Caco-2 cells were seeded in 24-well TC plates 24 h prior to infection. Bacteria were washed in PBS, diluted in RPMI 1640 medium (w/o FCS) and then added to the cells at a multiplicity of infection of 100 in triplicates. Bacterial cell counts were determined by measuring the OD600 and confirmed by plating serial dilutions on LB agar plates. Bacteria were centrifuged onto cells at 500 × *g* for 5 min and then incubated for 2 h at 37°C in an atmosphere of 5 % CO_2. After infection, cells were washed two times with PBS and then incubated in RP10 (w/o FCS) containing 100 µg of gentamicin/ml After 2 and/or 4 h of incubation, cells were washed two times with PBS and lysed with 1 % Triton X-100. For enumeration of intracellular bacteria, serial dilutions were plated on LB agar plates. CFU were counted after 24 h incubation at 37°C. Differences of bacterial entry and survival inside cells were analyzed using Students T-Test.

III.2.4.3. Plasmid stability test in macrophages

To test stable maintenance of plasmids within macrophages, 5×10^5 Raw cells were seeded 24 h prior to infection in 24-well TC plates. The next day, cells were infected with bacteria as described in III.2.4.2 but at an MOI of 10. After 2 h, extracellular bacteria were killed by incubation in RPMI containing 100 µg/ml Gentamycin for 2 h. Subsequently, the Gentamycin concentration was lowered to 10 µg/ml and infected cells were incubated for 24 in the presence of FCS before cells were lysed in 1 % Triton and serial dilutions were plated on LB or LB Cm plates to assess the number of intracellular bacteria which maintained Cm^R conferring plasmids.

III.2.4.4. Flow cytometry (fluorescent activated cell sorting, FACS)

Flow cytometry is a qualitative method to determine expression of protein markers in cell populations at the single-cell level. For this purpose, approximately 1×10^6 cells were collected by centrifugation at 250 × *g* in a microcentrifuge from in vitro culture or single-cell splenocyte suspensions (see also III.2.5.3). Cells were washed with PBS BSA and incubated with either fluorochrome-conjugated or primary antibodies (1/100-1/600 in PBS 1 % BSA) at 4°C for 30 min. In the latter case, incubation with a fluorochrome-conjugated secondary antibody targeting the primary antibody is needed. Finally, cells were washed, eluted in 400 µl of PBS BSA and analyzed in a BD FacsCalibur machine. Specificity of antibody mediated fluorescence was controlled by staining with fluorochrome-conjugated IgG isotypes or by staining cells with the conjugated secondary antibody only. The abundance of a specific population of marker positive cells in a total population can be determined by this method.

III.2.5. Immunology and in vivo methods

III.2.5.1. General animal handling and breeding

All animal experiments were performed according to the German “Tierschutzgesetz”. OT-I mice were obtained from the Max-Planck Institute for Infection Biology in Berlin and bred for further studies in the MSZ animal facility. Therefore, one male and three female mice were kept together in one cage for 3 weeks. Then, mice were separated and 4 weeks after birth, the offspring were separated from the mother. OT-II mice were kindly provided by the department of Virology in Würzburg.

III.2.5.2. Immunization of mice with bacteria

Three methods for immunizing mice were applied in this work. For oral immunization, mice were given 50 µl of carbonate buffer intragastrically 5 min prior to immunization to lower the pH of the stomach. Then, bacteria from infection aliquots (see III.2.1.3) were applied in 100-150 µl liquid by the same route. For i.v. injection, immunization aliquots were thawed on ice and diluted to 2.5×10^6 bacteria per ml. Mice were put under infrared light prior to application and 200 µl of the cell suspension were injected into the lateral tail vein. Immunization with Ty21a was carried out intranasally. Therefore, bacteria in 10-15 µl from the infection aliquots were applied into the nares of mice without anaesthesia using a micropipette.

III.2.5.3. Isolation of murine splenocytes

To isolate and analyze splenocytes, mice were anesthetized under CO_2 atmosphere and killed via cervical dislocation. Spleens were removed aseptically, transferred into sterile D-PBS and mashed with a steel sieve to obtain a crude cell suspension. The suspension was centrifuged at 300 × *g* and 4°C; the pellet was resuspended in 3 ml Erythrocyte-lysisbuffer and incubated for 5 min until erythrocytes were lysed. The lysis buffer was neutralized by adding 10 ml of PBS and the suspension was applied onto a 40 µm nylon filter to give a single cell suspension. For adoptive transfer, splenocytes were washed and finally collected in D-PBS. For FACS analysis cells were washed and resuspended in PBS 1 % BSA.

III.2.5.4. Determination of bacterial counts in organs and plasmid stability in vivo

To determine bacterial counts in organs after infection, spleens or livers were removed and single cell suspensions were prepared like described in III.2.5.3 and lysed in 0.02 % TWEEN. Serial dilutions of lysates were plated on LB or LB Kan plates to determine plasmid stability. Stability (in %) was calculated by dividing CFU numbers grown on LB Kan by CFU numbers grown on LB multiplied with 100. To obtain plating efficiency and as an alternative to stability test by plating, grown clones on LB plates were picked and transferred to Kan plates. If all colonies grew on LB Kan, plasmid stability was also defined as 100 %.

III.2.5.5. OT-I and OT-II adoptive transfer

The adoptive transfer model was used to analyze the capacity of an immunization strategy to elicit the expansion of ovalbumin-specific CD4+ or CD8+ T-cells. Ovalbumin serves as model antigen and the adoptive transfer approach is widely used to investigate the $CD8^+$ or $CD4^+$ T-cell response mediated by bacterial challenge (139-142). On day 1, 3-5 OT-I or OT-II mice were sacrificed and splenocytes were isolated and transferred into syngenic C57Bl/6 mice by i.v injection into the lateral tail vein. The next day, 3 of these mice per group were immunized with bacteria also by i.v. injection (see III.2.5.2). Three days later, mice were sacrificed and splenocytes were analysed by flow cytometry (see also III.2.4.4) to follow the expansion of Ova specific $CD8^+$ or $CD4^+$ T-cells. After removal and washing, splenocytes were treated for 5 min with the α-CD16/32 antibody on ice to block unspecific binding of IgG molecules to the FCγ III/II receptor via their constant part. To identify $CD8^+$ cells, splenocytes were stained 30 min with the Ova SIINFEKL Tetramer (1/50) followed by an additional 30 min with α-CD8a CyChrome (1/200), α-Vα-FITC (1/600), α-Vβ-PE (1/200) and/or α-CD62-L-FITC (1/400). For detection of CD4 T-cells, splenocytes were stained with α-CD4 CyChrome (1/200), α-Vα2-FITC (1/600), α-Vβ5-PE (1/200) and/or α-CD62L-FITC (1/400). All isotypes were applied in the same dilution as their respective specific IgG antibody. Significances in expansion of T-cells were calculated with 1-way ANOVA followed by Newman-Keuls multiple comparison test.

III.2.5.6. Isolation of murine serum

Mice were anesthetized with carbon dioxide and whole blood was obtained by puncturing the left ventricle of the heart. Then, mice were killed by cervical dislocation. Serum was extracted by incubating the whole blood for 1 h at room temperature following centrifugation at 10,000 × *g* for 5 min to separate serum from the cellular part. Serum was stored at -20°C until used.

III.2.5.7. Enzyme linked immunosorbent assay (ELISA)

The titres of hemolysin or LPS antibodies present in mouse sera were determined by ELISA. For detection of LPS antibodies, 1 µg/ml Ty21a LPS was coated onto NUNC 96-well MaxiSorp plates at 4°C overnight. For detection of HlyA specific antibodies,

HlyA was precipitated from culture supernatants using strain Ty21a pANN202-806 following the protocol for preparation of supernatant proteins (III.2.3.2) with slight modifications. Instead of Laemmli-buffer, the final pellet was resuspended in PBS and neutralized with saturated Tris solution. Finally, the solution was diluted 1/500 in coating buffer and coated overnight on 96-well plates. Plates were washed twice with PBS TWEEN and blocked with 1% BSA in PBS. After washing twice, three dilutions of mouse sera (1:33; 1:100; 1:300) in 100 µl conjugate buffer were incubated in duplicates for 1.5 h at 37°C. After four washing steps, AKP-coupled sheep anti mouse IgG or anti mouse IgG and IgM diluted 1/1,000 in 100 µl conjugate buffer was added. After 1 h at 37°C and two washing steps, 50 µl of pNPP substrate in buffer was added. The reaction was incubated at room temperature and stopped after 30 min by 50 µl 1 M NaOH. Optical density was read at a wavelength of 405 nm in a microplate reader. Significant differences in absorption were analyzed through 1-way ANOVA followed by Newman-Keuls multiple comparison test.

III.2.5.8. Enzyme linked immunospot assay (ELISPOT)

EILISPOT was performed according to (181) for analyzing the frequency of B-RAFV600E specific, IFNγ-secreting $CD8^+$ T-cells after immunization. For this purpose, 3-4 HLA-B2705 transgenic mice per group were immunized orally 3 times fortnightly with 5×10^9 Stm. One week after the last immunization splenocytes were removed and restimulated *ex-vivo* to expand the specific T-cell population. Therefore, P815 B-RAFV600E cells were pulsed for 1 h with either 10 µg/ml peptide P2 (GRFGLATEK), P3 (GRFGLATVK) or Ova SIINFEKL as control and irradiated at 30 Gray to serve as target cells. 2×10^6 target cells and 1×10^7 splenocytes from individual mice were mixed and incubated for 5 days in RP10 supplemented with 60 U/ml IL-2 and Pen-strep (1/100). If 4 mice were vaccinated, 2 preparations were pooled after restimulation. Half of the medium was changed carefully after 2.5 days. On day prior to analysis, 96 well nitrocellulose plates were coated with 5 µg/ml IFNγ capture antibody (R4) o/n at 4°C. The next day, plates were blocked with 1 % BSA for 2 h at 37°C and washed with sterile PBS. Freshly prepared splenocytes from HLA-B2705 mice were pulsed with the three peptides as described above and irradiated with 10 Gray to serve as feeder cells. Either 1×10^4, 3×10^4 or 1×10^5 splenocytes were incubated with 1×10^5 feeder cells in 100 µl RP10 medium plus the usual supplements in each well of the prepared microtiter plates for 20 h at 37°C

under 5 % CO_2 atmosphere. Afterwards, plates were washed 10 × with PBS following 10 × with PBS TWEEN in a microplate washer and incubated with the secondary, biotinylated IFNγ detection antibody (XMG 1.2, 0.25 µg/ml in 100 µl PBS Tween) for 2 h at 37°C. After washing 10 × with PBS TWEEN, plates were incubated with 100 µl/well streptavidin-conjugated AKP diluted 1/2,000 in conjugate buffer for 1h at 37°C. Finally, plates were washed 5 × with PBS TWEEN and 50 µl of BCIP/NBT substrate (1 tablet dissolved in 10 ml dH_2O) was applied in each well to visualize IFNγ spots. Plates were washed, dried, and specific spots were counted and analyzed in an AID ELISPOT reader. Each specific spot represents an IFNγ-secreting CD8 T-cell.

In addition to ELISPOT, splenocytes restimulated with P2 were analyzed by flow cytometry. In order to follow the proliferation of specific T-cells after vaccination and restimulation, cells were incubated with HLA-B2705 P2 or P3 dextramers (1/25) for 30 min. $CD8^+$ T-cells were identified with α-CD8 CyChrome as described in III.2.5.5.

III.2.5.9. Tumour challenge in mice

To analyze the protective capacity of Salmonella based immunization against ovalbumin, 8-9 C57Bl/6 mice of 6-8 weeks of age per group were immunized 3 times with 2 week intervals with 5×10^9 bacteria. Three weeks after the third immunization, mice were challenged with the B16-Ova cell line by two s.c. injections of 5×10^5 cells into each flank of shaven abdominal skin. Mice were monitored over a period of 18 days for tumour appearance and tumour volume was assessed by measuring the largest (a) and smallest (b) tumour diameter. Tumour volume (V) was calculated as rotation ellipsoid using the following formula: $V = \pi/6 * a * b^2$; $a > b$

The results' significances were analyzed by Students T-Test and 1-way ANOVA followed by Newman-Keuls multiple comparison test. For analysis of the protective capacity of *salmonellae* presenting the PSA epitope, 8-9 C57/Bl6 mice of 6-8 weeks of age per group were immunized 3 times weekly with 1×10^{10} bacteria as described above. Two weeks after the third immunization, mice were challenged with B16-PSA by two s.c. injections of 5×10^5 cells into each flank of shaven abdominal skin. Mice were monitored over a period of 21 days for tumour appearance and tumour volume was assessed and analysed as described above. Significant differences in tumour growth were calculated with the non-parametric Mann-Whitney test as tumour volumes within the same groups were not distributed according to Gauss.

IV. Results

IV.1. Part I: surface display of antigens via TolC

IV.1.1. Construction of *Salmonella* strains expressing antigens in surface-exposed fashion

IV.1.1.1. Construction of the pTolC plasmid and derivatives

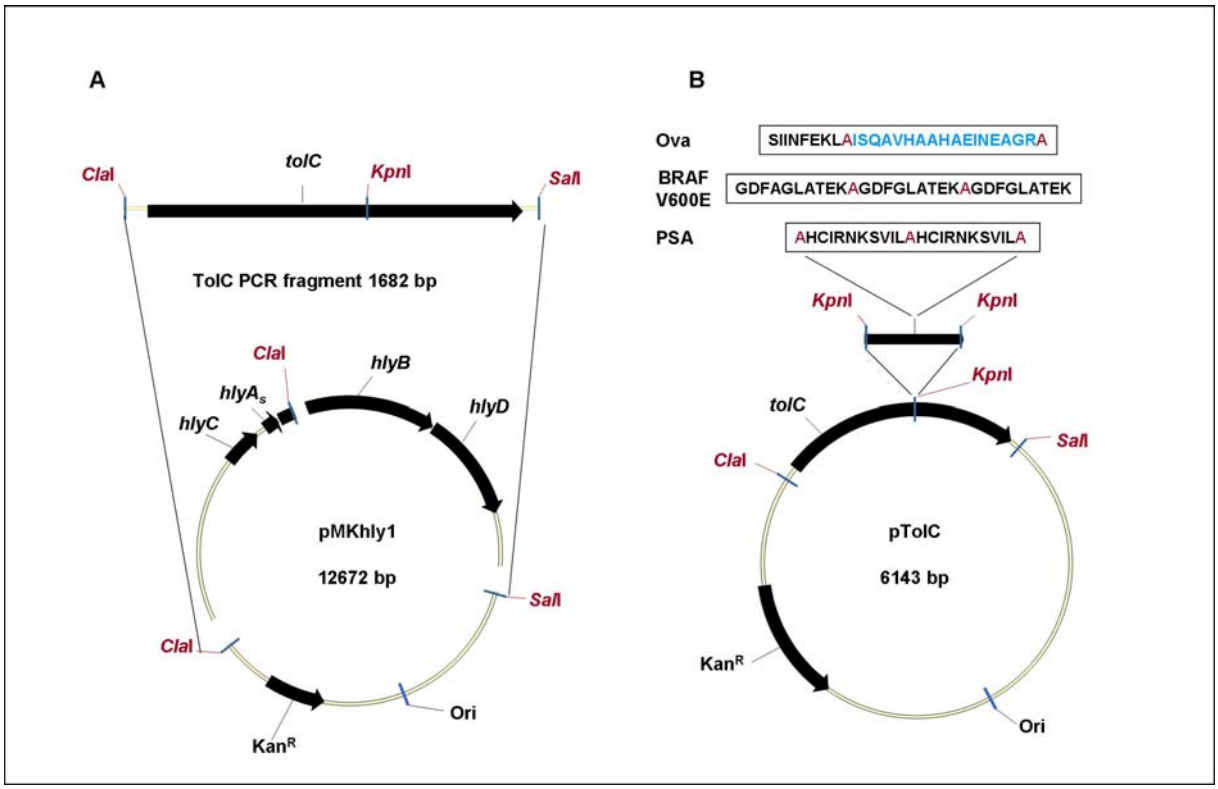

Figure IV.1 Cloning strategy for pTolC vectors
A: Construction of pTolC. The PCR fragment of *tolC* included the indicated restriction sites. Vector and fragment were digested with *Sal*I and *Cla*I and the 1.7 insert was ligated into the 4.4 kB backbone of pMKhly1 containing the Kanamycin resistance (Kan^R) and the origin of replication (ori).
B: Construction of vectors expressing antigen-fused TolC proteins. Antigens were obtained by hybridizing two polylinkers carrying 5' overhangs mimicking *Kpn*I restriction. These fragments were inserted into a single *Kpn*I restriction site of *tolC* on pTolC. In the upper panel, peptide sequences of the three investigated antigens are given. Construction of pTolC-PSA encoding the TolC-PSA protein is described elsewhere (136). Ova: chicken ovalbumin derived epitopes: CD8$^+$ epitope (black) and CD4 $^+$ epitope (blue) separated with linker alanines (red). BRAFV600E: CD8$^+$ epitope of BRAFV600E in triplicate separated by linker alanines (red). PSA: CD8$^+$ epitope of prostate specific antigen in duplicate separated by linker alanines (red).

To display epitopes on the surface of *Salmonella* vaccine strains, plasmid pTolC, encoding *tolC* from *E. coli*, was constructed. It formed the basis for later antigen

insertion into a single *Kpn*I restriction site within a permissive, surface exposed TolC loop to achieve the so called “surface display” of antigens. Ampicillin resistance is inadequate for possible human use of these vectors. Kanamycin resistance as selection marker was chosen because of higher acceptance and lower risk to the environment (182). The cloning strategy is depicted in Figure II.1. For plasmid construction, *tolC* was amplified from p*tolC* with primers ectolClaI_up and tolCSalI_down with Pfu polymerase. The resulting 1.7 kB fragment was cut with *Cla*I and *Sal*I and ligated into the 4.4 kB backbone pMKhly1 obtained by restriction with the same enzymes. The ligation mix was transformed into electrocompetent *E. coli* which were plated on LB Kanamycin plates. Clones were screened for insertion by colony PCR with primers TolSEQ2 and k1_down. To verify *tolC* insertion, plasmids of PCR-positive clones were isolated and digested with *Cla*I and *Sal*I (Figure IV.2).

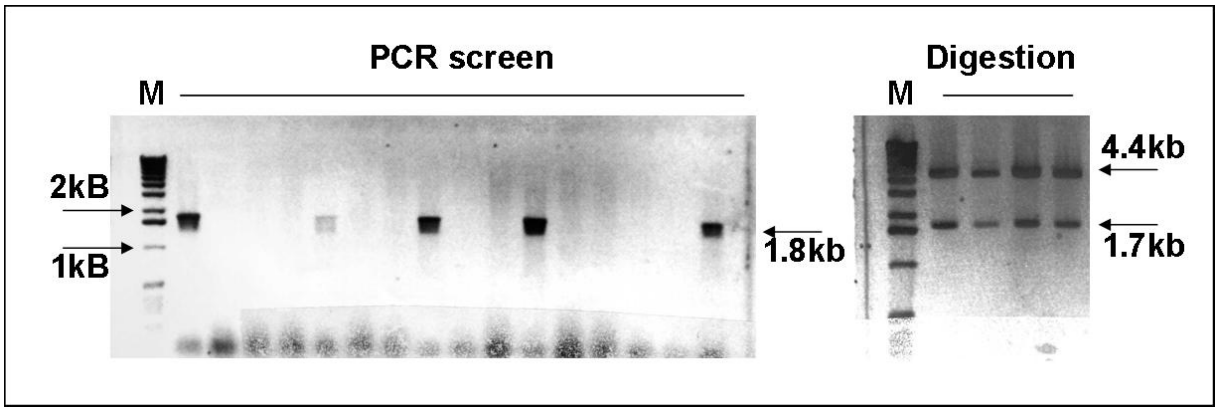

Figure IV.2 Agarose DNA gel colony PCR screen and plasmid digestion of pTolC isolates
Left panel: Colonies grown after ligation and transformation were screened by PCR with primers TolSEQ2 and k1_down to obtain a 1.8 kb fragment indicating insertion of tolC.
Right panel: pTolC plasmid isolates were digested with ClaI and SalI to visualize the *tolC* insert of 1.7 kB size. M: marker

For construction of epitope-bearing pTolC, the vector was cut with *Kpn*I and dephosphorylized with CIAP. The insertion fragments were generated by hybridizing two complementary linkers (OVA-linker 1 and 2 or BRAF-linker 1 and 2, respectively) which carry 5’ overhangs mimicking *Kpn*I digestion. Linkers were first adapted to the codon usage of *Salmonella* using the tables of the Kazusa DNA research institute (183). Those fragments were ligated into the prepared vector and clones were screened by PCR with primers tolSEQ1 and 2. The resulting plasmids were named pTolC-OVA, which carries the H-2K^{b} restricted Ova CD4^{+} and CD8^{+} epitopes, and pTolC-BRAF encoding the HLA-B2705 restricted B-RAFV600E epitope in triplicate

(Figure IV.1). As *tolC* is distributed almost ubiquitously in gram-negative bacteria, expression of these plasmids might result in a binding competition of chromosomal encoded TolC and plasmid-borne, recombinant TolC in the outer membrane of the carrier strains. Therefore, the chromosomal copy was deleted with the aim to maximize abundance of the recombinant isoforms at the bacterial surface.

IV.1.1.2. Disruption of chromosomal *tolC*

First, the two different Salmonella strains, Ty21a and Stm, were screened by multiplex PCR according to (177). *S*. *typh*i strains can be identified by a typical PCR pattern as all four primer pairs (*invA*, *viaB*, *fliC-d*, *prt*) bind and lead to amplification, whereas in serovar *typhimurium* only one specific PCR fragment (*invA*) is generated. Figure IV.3 A depicts the results of the PCR identification. Both strains showed the expected pattern. Chromosomal gene disruption in these strains was performed with the method of Datsenko and Wanner (175). An overview of this knock-out approach is depicted in Figure IV.3 B.

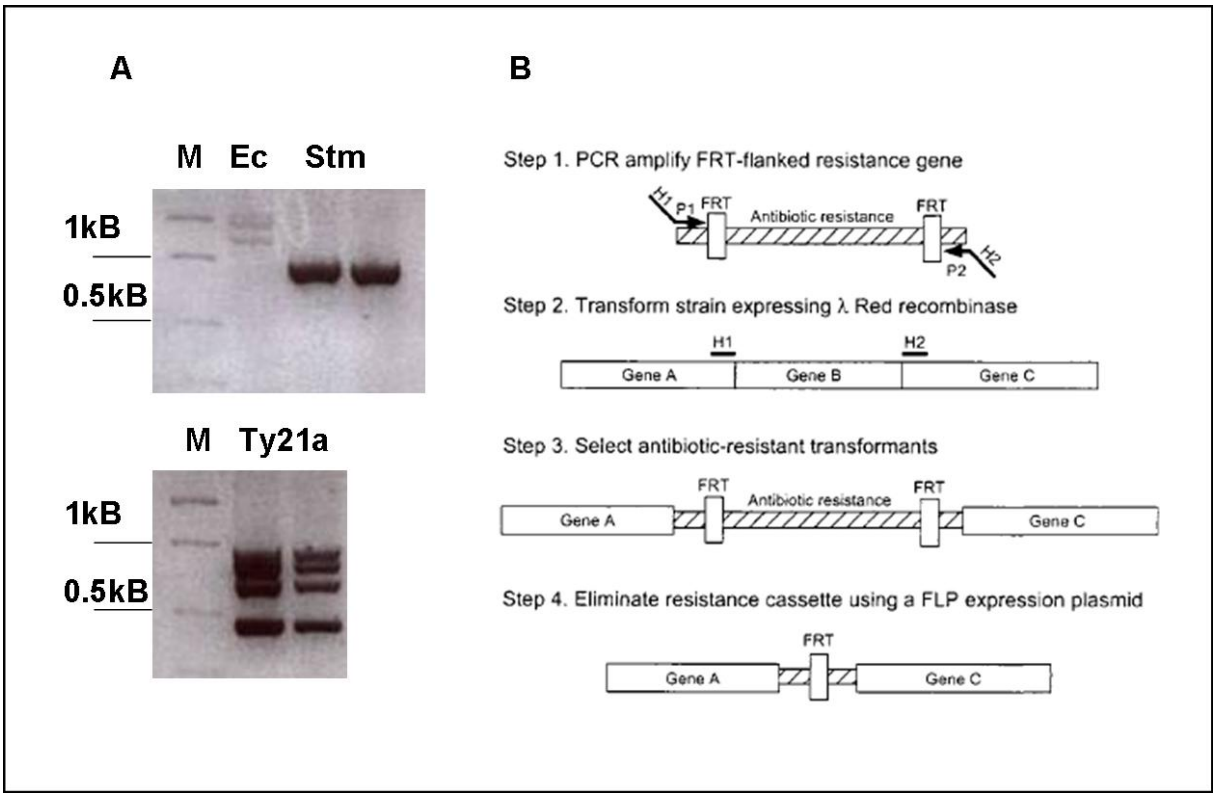

Figure IV.3 Strain identification and overview of chromosomal disruption of bacterial genes
A: Multiplex PCR with Ty21a and Stm, 4 primer pairs were used to detect typhi specific genes, 1 band (*invA*) is common among *Salmonella* strains. Ec: *E. coli* negative control
B: Overview of the chromosomal deletion of *tolC* in *Salmonella*, taken from (175)

For disruption of *tolC*, Ty21a or Stm were transformed with plasmid pKD46 and grown in LB medium supplemented with 0.2 % L-(+)-arabinose for induction of gam, bet and exo, the gene products on the plasmid conferring the ability for homologous recombination with linear PCR products. These strains were incubated at 30°C to stably replicate the temperature-sensitive plasmid pKD46 until an OD600 of 0.6 was reached. Cells were washed and concentrated 200 × in PBS 10 % glycerol to make them electrocompetent.

PCR products were amplified with Taq polymerase from pKD3 (for *tolC* KO in Stm) or pKD4 (for *tolC* KO in Ty21a) containing antibiotic resistance cassettes. Primers were composed of 5' and 3' overhangs homologous to the *tolC* locus which was the target of disruption and priming sites p1 and p2 which annealed to pKD plasmids. The final PCR products contained the 5' homology region followed by an FRT site flanked Cm^R (pKD3) or Kan^R (pKD4) cassette and a homology region at the 3' end. Knock-out fragments were generated from 400 µl of PCR product, purified, digested with *Dpn*I for 3 h, purified again and eluted in 30 µl of EB buffer. Finally, 5 µl of PCR product were used to transform 100 µl electrocompetent cells mentioned above. Clones were selected on LB agar plates containing 0.2 % arabinose and 10 µg/ml Cm for Stm or 25 µg/ml Kan for Ty21a at 30°C. Insertion of the PCR fragment into the *tolC* locus was controlled by colony PCR using primers c1_down (Stm) or k1_down (Ty21a) and tolCFR_up (Figure IV.4).

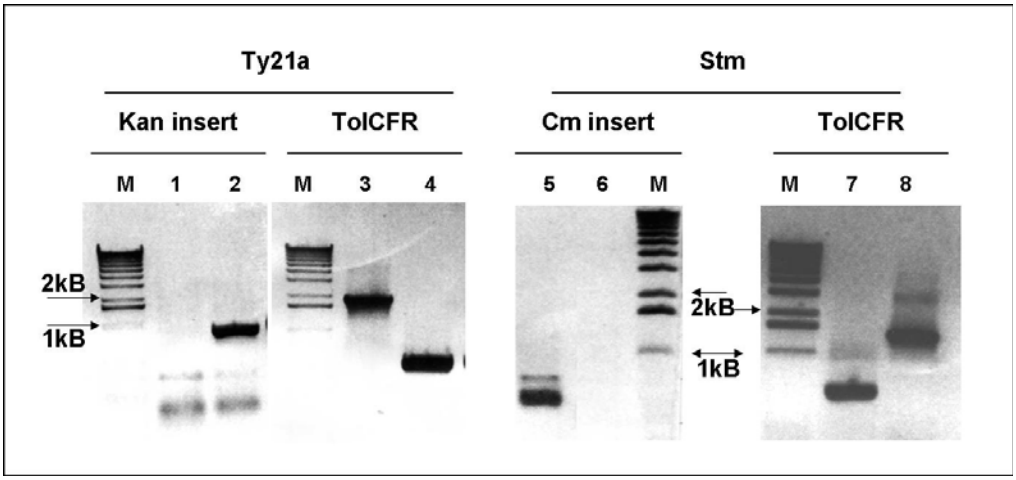

Figure IV.4 Agarose DNA gels of colony PCRs to confirm *tolC* deletion in Ty21a and Stm
Insertion of Kan^R and Cm^R cassettes into the tolC locus was confirmed with primers k1_down and TolCFR_up (Kan insert) or c1_down and TolCFR_up (Cm insert), respectively (lanes 2 and 5). Deletion of the resistance cassettes were verified with tolCFR primers (TolCFR) leaving a 500 bp scar sequence (lanes 4, 7). M: marker, 1: Ty21a, 2 and 3: Ty21a *ΔtolC*::Kan^R, 4: Ty21aTC, 5 and 8: Stm *ΔtolC*::Cm^R, 6: Stm, 7: StmTC:

To eliminate the antibiotic resistance, insertion-positive clones were cultivated at 37°C and screened for Amp sensitivity (loss of plasmid pKD46). Those clones were transformed with pCP20, a plasmid encoding Flp recombinase which targets the FRT sites for recombination. Incubation at 37°C activates the recombinase which mediates recombination of FRT sites resulting in Cm^R loss. Furthermore, pCP20 is eliminated as it is not stably replicated at temperatures above 30°C. Clones were screened for Amp and Cm sensitivity and the loss of the Cm^R cassette in the *tolC* locus by PCR, using primers tolCFR_up and tolCFR_down (Figure IV.4). The resulting *tolC*-deficient strains were named Ty21TC and StmTC, respectively. Plasmids were introduced into the strains by electroporation.

IV.1.1.3. Expression and localization of recombinant TolC

After construction of the carrier and plasmids, recombinant protein expression and localiztion was analyzed. Therefore, bacterial cells were disrupted and separated into cytoplasmic and membrane fractions. To analyze trimer formation, which indicates insertion of TolC into the outer membrane, half of the membrane fraction was left uncooked during sample preparation to ensure stability of heat-labile multimers. All samples were subjected to SDS PAGE and Western Blot using the α-TolC antibody (1/3000 in PBS milk). To control the efficacy of the fractionation process, blotted gels were stained with Coomassie blue and the protein pattern was analyzed.

The left panel of Figure IV.5 shows the protein pattern after fractionation, SDS PAGE and Coomassie staining. Cytoplasmic and membrane fraction differ in their protein content as shown by Coomassie staining (Figure IV.5, left panel) which confirms proper separation of bacterial compartments by this method. When stained with the α-TolC antibody (Figure IV.5 and Figure IV.6), both, endogenous and recombinant TolC, can be detected in their monomeric form in the Ty21a and Stm strains. The *tolC*-deficient strains do not possess the chromosomal copy, so the 48 kDa band was lacking. Furthermore, recombinant TolC could be found in the cytoplasm as in the membrane fraction and built-up trimers in respective transformed Ty21a and Stm strains. Most strikingly, the amount of trimeric TolC was much higher in the *tolC*-deficient strains and this was true for both investigated strains and both recombinant proteins. It is unclear whether the antibody can detect trimeric endogenous TolC as the overall trimer abundance in the *tolC* wildtype strains was low. Nevertheless, it

seems that there exists indeed a binding competition between TolC isoforms for a limited number of membranous TolC insertion sites. As a result, deletion of chromosomal *tolC* leads to saturation of the outer membrane with recombinant TolC.

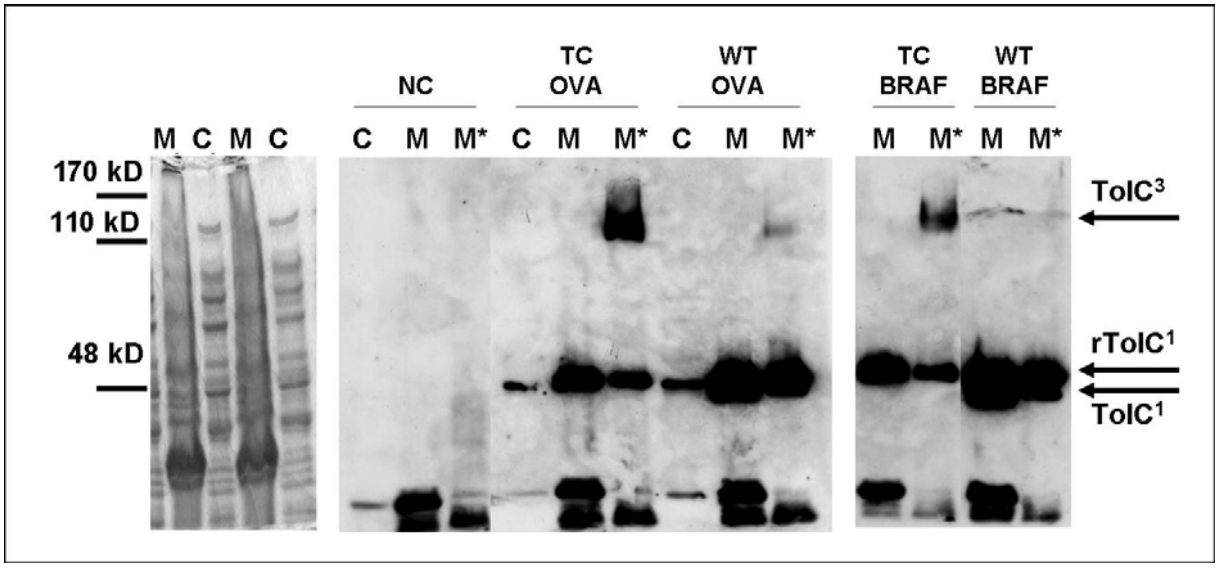

Figure IV.5 SDS PAGE and Western Blot of Ty21a strains after membrane preparation
Membrane protein samples of 2 ml or cytoplasmic proteins of 0.2 ml culture were loaded in each lane.
Left panel: Coomassie staining of a SDS gel after blotting.
Middle panel: Western Blot of strains expressing TolC-OVA detected with the α-TolC antibody
Right panel: Western Blot of strains expressing TolC-BRAF detected with the α-TolC antibody
NC: Ty21aTC w/o plasmid, OVA: pTolC-OVA, BRAF: pTolC-BRAF, WT: Ty21a, TC: Ty21aTC
C: cytoplasmic fraction, M: membrane fraction, M*: uncooked membrane fraction, $TolC^3$. trimeric TolC, $TolC^1$: monomeric endogenous TolC, $rTolC^1$: monomeric recombinant TolC

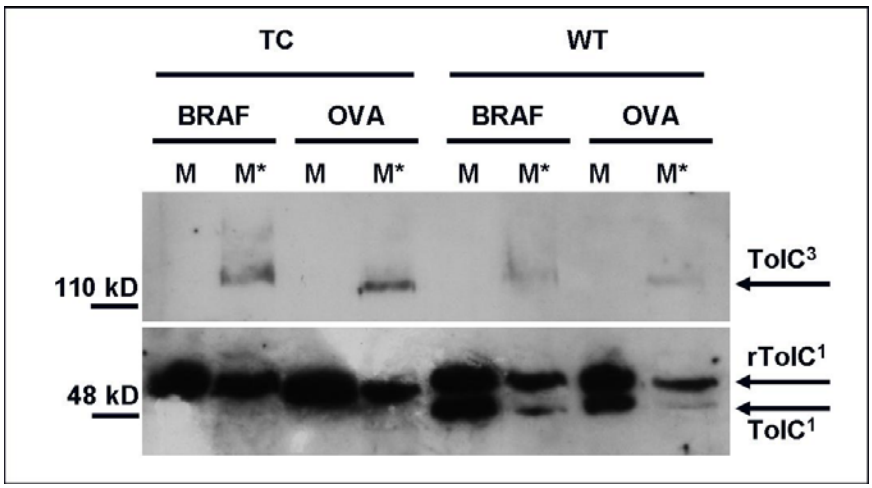

Figure IV.6 Western Blot of Stm strains after membrane preparation
Membrane protein samples of 2 ml or cytoplasmic proteins of 0.2 ml culture were loaded in each lane. and TolC was detected with the α-TolC antibody
OVA: pTolC-OVA, BRAF: pTolC-BRAF, TC: StmTC, WT: Stm, C: cytoplasmic fraction, M: membrane fraction, M*: uncooked membrane fraction, $TolC^3$. trimeric TolC, $TolC^1$: monomeric endogenous TolC, $rTolC^1$: monomeric recombinant TolC

IV.1.1.4. Plasmid stability in vitro

Plasmid stability is an important prerequisite for plasmid-based, recombinant live vaccines. Therefore, plasmid stability was assessed in *Salmonella* as described (*in vitro*, III.2.1.7). Furthermore, the Kan^R of plasmids pTolC and pTolC-OVA were eliminated by recombination and analyzed for expression and stability in StmTC. This strategy was followed to create antibiotic resistance-free vaccines that may be advantageous for later human use e.g. in Ty21a.

The Kan^R of plasmid pTolC was inherited from pMKhly1 but originally is derived from plasmid pKD4, thus the Kan^R is flanked by FRT sites. Analogous to the deletion of resistance cassettes from the chromosome (see IV.1.1.2) this selection marker was removed by homologous recombination using Flp from plasmid pCP20. StmTC containing plasmids pTolC or pTolC-OVA were transformed with pCP20 and incubated at 37°C for simultaneous induction of the recombinase and loss of the temperature-sensitive plasmid pCP20. Clones were screened for Kan and Amp sensitivity to confirm deletion of Kan^R on pTolC plasmids and loss of pCP20. Retained plasmids were isolated and named pTC and pTC-OVA, respectively. Those "marker-free" plasmids could be selected in the presence of 2-4 µg/ml Novobiocin as *tolC*-deficient strains are highly susceptible to this compound, thus leading to maintenance of the only functional, plasmid-encoded *tolC*.

Table IV.1 shows plasmid stability of all vectors *in vitro*, Figure IV.7 depicts expression of TolC encoded by marker-free vectors in StmTC.

Plasmid	Stm	StmTC	Ty21aTC
pTolC	97-100%	-	-
pTolC-OVA	100 %	91-100 %	100 %
pTC	-	96-100 %	86-91 %
pTC-OVA	-	94-100 %	-

Table IV.1 Stability of plasmid replication in *Salmonella* vaccine carriers.
Cultures were grown o/n in the presence or absence of selection antibiotic. The next day serial dilutions were plated on LB or LB Novobiocin (2-4 µg/ml). Plasmid stability was determined as described in III.2.1.7. All tests were performed at least 2 times; therefore a range of stability is given if single results differed.

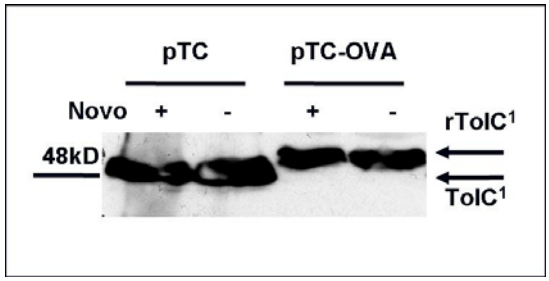

Figure IV.7 Western Blot of StmTC harbouring pTC or pTC-OVA
Cultures were grown o/n in the presence (+) or absence of (-) 2 µg/ml Novobiocin. Lysates of 0.1 ml culture were loaded in each lane and TolC was detected with the α-TolC antibody.
$TolC^1$: monomeric TolC, $rTolC^1$: monomeric recombinant TolC

These results demonstrate that plasmids pTolC and pTolC-OVA are highly stable in *Salmonella* vaccine strains. Plasmid stability ranged from 90-100 % in vitro for Kan^R plasmids. In addition, resistance cassettes were not essential for expression and maintenance of plasmids as plasmids pTC and pTC-OVA were as stable as their parental vectors and allowed efficient expression of (recombinant) TolC in Stm. However, the Kan^R-bearing plasmids were used for further studies due to easier selection with Kanamycin.

IV.1.2. Recombinant TolC proteins are functional

IV.1.2.1. Investigation of major TolC functions with recombinant proteins

TolC is a multifunctional protein, involved in type I secretion of hemolysin (160), multidrug resistance of *Salmonella*, invasion/survival of *Salmonella* in macrophages (184,185) and invasion into epithelial cells (186) To assess the capacity of the recombinant TolC proteins to substitute TolC functionally in *tolC*-deleted strains, resistance against antimicrobial agents, the ability for hemolysin secretion, invasion into Caco-2 cells and survival inside macrophage-like RAW cells were tested.

IV.1.2.2. Resistance against antimicrobial agents

AcrAB forms a tripartite efflux pump by recruiting TolC as outer membrane pore thus mediating resistance against a wide range of noxious, chemically non-related compounds (187). The ability for multidrug efflux of different *Salmonella* strains was tested by determining the MIC of bile salts.

Strain	**Stm**	**StmTC**				**Ty21a**	**Ty21aTC**			
Plasmid	-	-	pTolC	pTolC OVA	pTolC BRAF	-	-	pTolC	pTolC OVA	pTolC BRAF
MIC (mg/ml)	>50	0.2	>50	>50	50	>50	0.05	>50	50	50

Table IV.2 Bile salt MIC values of *Salmonella* strains
Strains were cultivated 24 h in LB medium on 96-well microtiter plates containing twofold serial dilutions of bile salts. The MIC value represents the lowest concentration which repressed growth completely.

As Table IV.2 shows, *tolC* deficiency rendered the bacteria highly susceptible to the tested compound. All TolC isoforms were able to rescue this phenotype when introduced into *Salmonella* via plasmids. Albeit pTolC encodes *tolC* from *E. coli* it was fully functional in *Salmonella* with respect to multidrug efflux. Furthermore, insertion of epitopes into the outer loop did not negatively influence the efflux function underlining the permissive character of the antigen insertion site.

IV.1.2.3. Ability for hemolysin secretion of recombinant TolC proteins

Strains were transformed with plasmid pRSC2 which encodes the *hly* operon of *E. coli* and secretion was investigated employing two different methods. First, an on-plate hemolysin assay was performed. Secreted hemolysin is diffusing into blood agar cells lysing erythrocytes thus resulting in corona formation around secretion-competent cells. As visible in Figure IV.8 A, all strains lysed red blood cells independent of the TolC isoforms. In contrast, the *tolC*-deficient strain was not able to secrete hemolysin. In the second approach, hemolysin was precipitated from supernatants and analyzed by Western Blot with an α-$HlyA_s$ antibody (Figure IV.8 B)

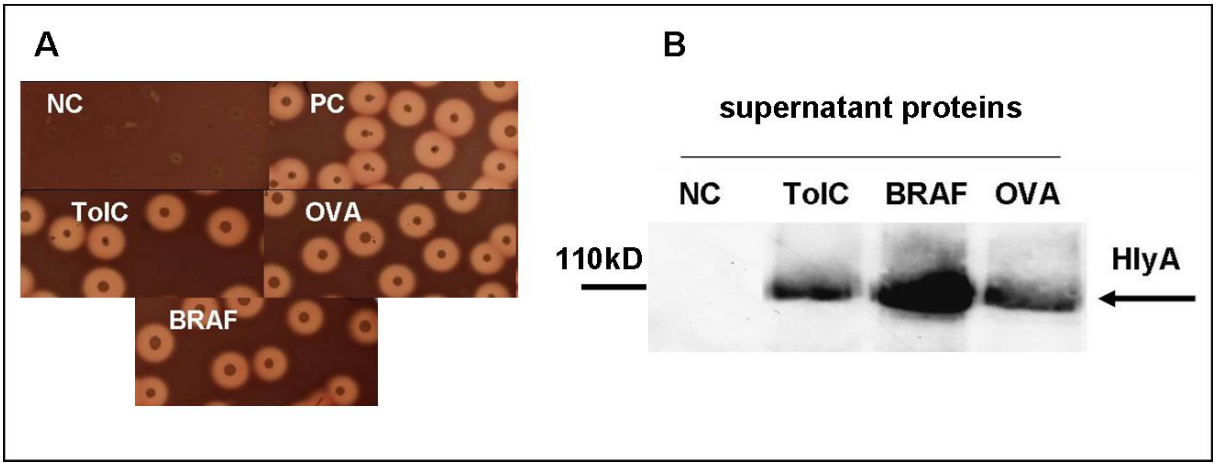

Figure IV.8 HyA secretion on Salmonella strains
A On-plate hemolysin assay**.** Cell suspensions were dropped onto blood agar and incubated 24 h at 37°C. Coronas indicate lysis of erythrocytes due to secreted hemolysin. NC: StmTC/pRSC2, PC: Stm/pRSC2, OVA: StmTC/pRSC2/ pTolC-OVA, BRAF: StmTC/pRSC2/pTolC-BRAF
B: Western blot of precipitated supernatants of *Salmonella* strains
Proteins of 2.5 ml TCA precipitated culture supernatant were applied in each lane. HlyA was detected with the a-$HlyA_s$ antibody. NC: StmTC/pRSC2, OVA: StmTC/pRSC2/pTolC-OVA, BRAF: StmTC/pRSC2/pTolC-BRAF

Western Blot data confirmed the results described above. Again, all (recombinant) TolC proteins built-up functional secretion machineries with Hly proteins leading to

secretion of hemolysin. Similar results in both assays were obtained with Ty21a strains (not shown).

IV.1.2.4. Invasion/survival in epithelial and macrophage-like cells

In order to test the ability of recombinant *Salmonella* strains to invade into and survive inside epithelial cells, CACO-2 cells were infected with bacteria at an MOI of 100. After uptake (2 h) and gentamicin mediated killing of extracellular bacteria (2 h), cells were lysed and the amount of intracellular bacteria was determined by plating serial dilutions of this lysates and counting CFU. The results are shown in Figure IV.9. Interestingly, Ty21a invaded and survived in Caco-2 cells approx. 1 log magnitude less efficient than Stm. Furthermore, the *tolC* mutant exhibited decreased numbers of intracellular bacteria indicating a role of *tolC* in the process of invasion or the ability to survive in Caco-2 cells. Both, TolC and TolC-OVA were able to restore the overall poor invasion ability of Ty21a in the *tolC*-deficient strain. Stm strains showed a similar behaviour but the yield of intracellular bacteria was much higher, even though the *aroA* mutation renders Stm replication defective when grown intracellularly (188).

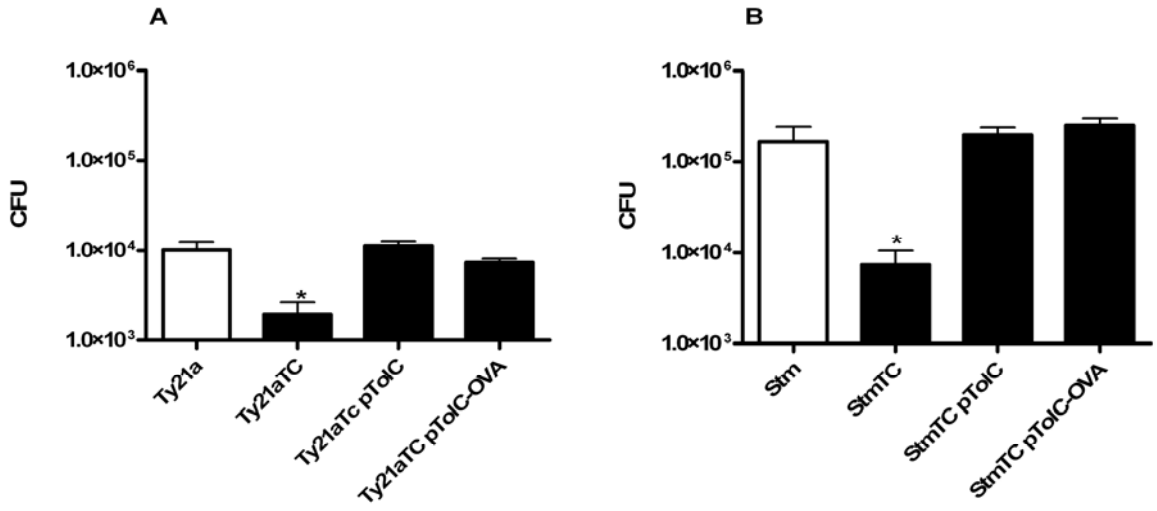

Figure IV.9 Invasion/survival of bacteria in Caco-2 cells
2 h post infection, Caco-2 cells were lysed and plated for determination of the number of intracellular bacteria.
A: Ty21a strains and **B**: Stm strains. Significances were calculated by logarithmic transformation of CFU values followed by Students T-Test comparing each strain with its respective wildtype (in white).
* p-value < 0.05

When investigating survival behaviour of *Salmonella* in RAW macrophages employing an analogous experimental setting, contrary observations were made. In this case, intracellular yield of Ty21a was higher than for Stm strains (Figure IV.10).

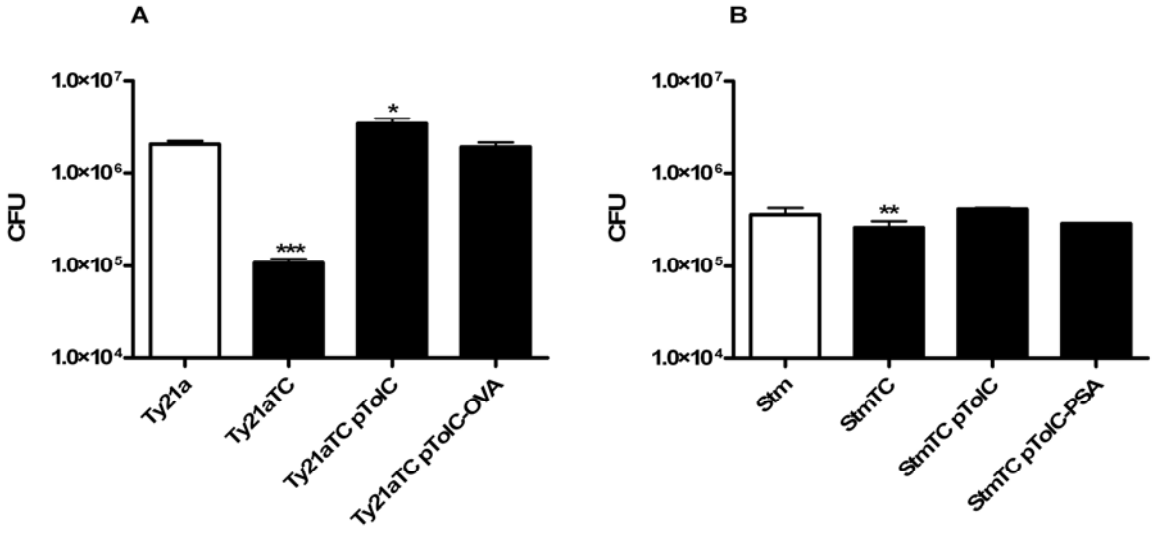

Figure IV.10 Invasion/survival of bacteria in RAW cells
2 h post infection, macrophage-like cells were lysed and plated for determination of the number of intracellular bacteria.
A: Ty21a strains and **B**: Stm strains. Significances were calculated by logarithmic transformation of CFU values followed by Students T-Test comparing each strain with its respective wildtype (in white). * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001

Again, a clear dependency on *tolC* for intracellular survival was observed for Ty21a. Complementation with TolC-OVA rescued the *tolC* phenotype, while TolC alone even increased the yield of intracellular bacteria significantly (p-value = 0.047). Surprisingly, *tolC* deletion exerted only a little but significant effect on intracellular numbers of Stm cells within macrophages. All *S. typhimurium* strains tested exhibited similar yields of intracellular bacteria. Howsoever, *tolC* deletion displayed at least no substantial negative effect on survival behaviour of Stm when infecting RAW cells.

IV.1.3. Stm strains expressing surface-associated OVA failed to activate Ova specific CD4$^+$ and CD8$^+$ T-cells

IV.1.3.1. OT-I transfer

To assess the capacity of the newly constructed strains to prime the adaptive immune system adoptive transfer models were chosen. In a first attempt the expansion of CD8$^+$ T-cells upon stimulation with Stm(TC) pTolC-OVA strains was investigated. Therefore, CD8$^+$ T-cells specific for the Ova SIINFEKL peptide in the

context of MHCI molecules were purified from OT-I mice and transferred into syngenic C57Bl/6 mice. The next day, these mice were immunized with Stm displaying SIINFEKL via TolC or control strains. Three days later, mice were sacrificed and splenocytes were analysed by FACS for the expansion of OT-I cells. The Ova SIINFEKL Tetramer binds specifically to the OT-I T-cell receptor, the type of cell was confirmed with anti CD8 staining and CD62L served as activation marker as CD62L expression is lost in activated T-cells (CD62Lhi to CD62Llo). Figure IV.11 illustrates the staining of splenocytes with the different markers. Figure IV.12 shows the results of the assay indicated by the mean percentage of OT-I cells in the population of splenocytes of 3 mice.

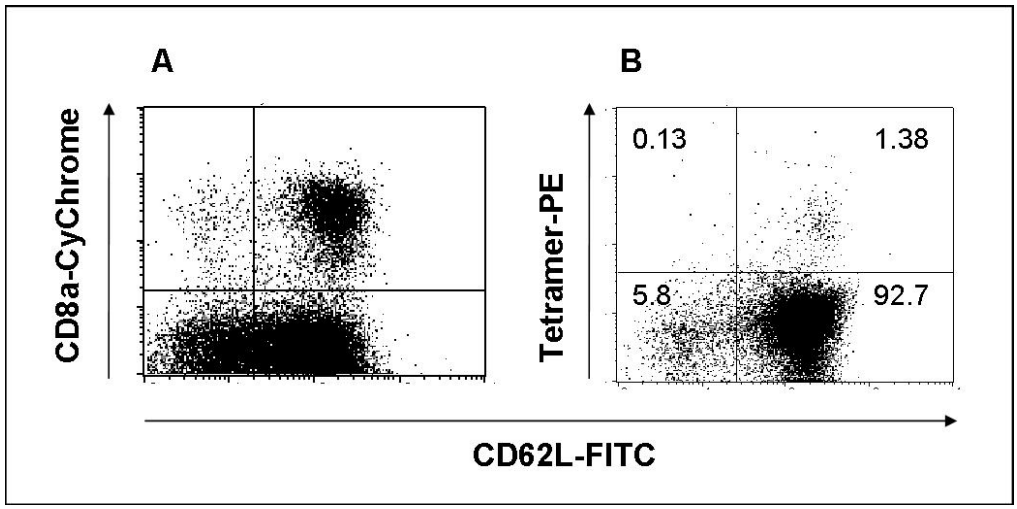

Figure IV.11 FACS staining of splenocytes after immunization to determine frequencies of OVA specific CD8 T-cells.
OVA SIINFEKL specific T cells (OT-I cells) were transferred into Bl/6 mice, mice were immunized, and T-cell frequencies was assessed by FACS.
A: Splenocytes were stained with α-CD8a CyChrome and α-CD62L-FITC antibodies
B: CD8a positive cells as indicated in A were gated and investigated for the presence of the OT-I TCR (OVA SIINFEKL Tetramer-PE) and activation state (α-CD62L-FITC). Numbers indicate the frequencies of cells in %.

As visible, immunization did not result in substantial proliferation of OT-I cells *in vivo*. The overall frequency of these Ova SIINFEKL/MHC-I specific $CD8^+$ cells was low. Figure IV.12 A depicts frequencies of non-activated T-cells after immunization. The background staining level was rather low as indicated by analysis of splenocytes from mice which did not receive OT-I cells. StmTC pTolC-OVA induced significant higher frequencies of OT-I cells compared to all other groups. However, the difference was marginal and was not reproducible in another experiment (not shown). Furthermore, most of the OT-I cells were not activated as the frequency of CD62Llo Tetramer

positive cells ranged often far below 0.5 % of the whole splenocyte population (Figure IV.12 B). Additionally, no significant differences in expansion of activated T-cells in between the groups were observed.

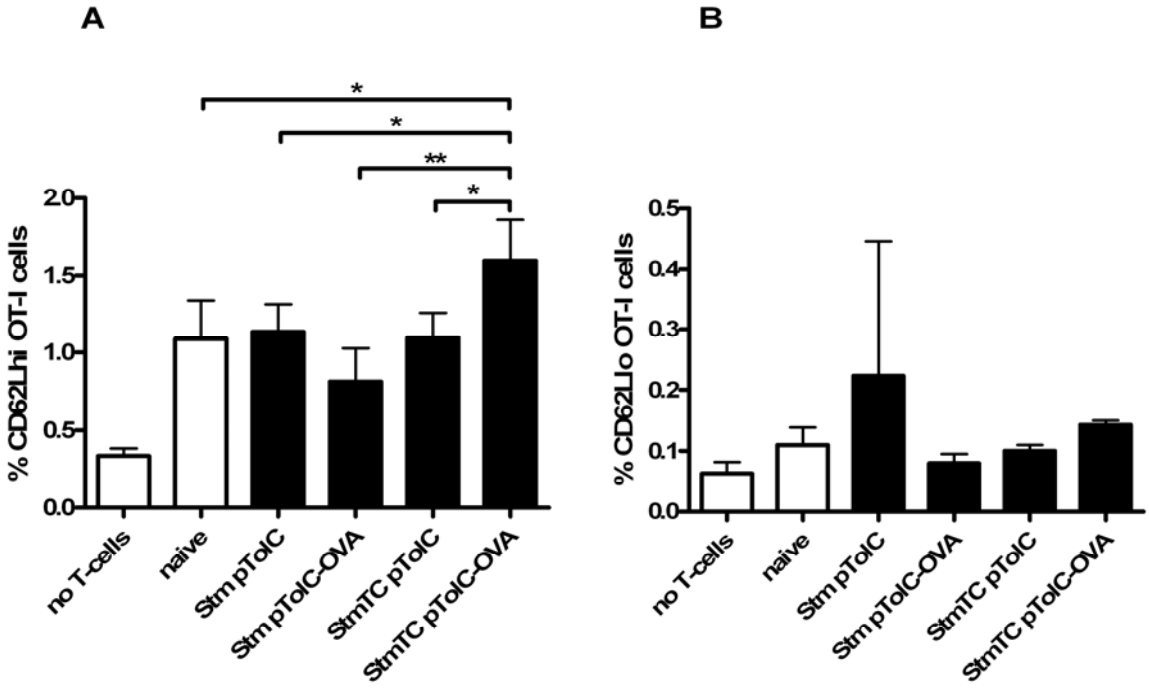

Figure IV.12 OT-I cell expansion after immunization with Stm strains.
OVA SIINFEKL specific T cells (OT-I cells) were transferred into C57Bl/6 mice, mice were immunized, and T-cell expansion was assessed by FACS.
The frequency of **A**: non-activated (CD62Lhi) and **B**: activated (CD62Llo) OT-cells is given as mean percentage of these cells of the whole splenocyte population (n = 3 mice). Significances were calculated by 1-way ANOVA followed by Newman-Keuls multiple comparison test. * p-value < 0.05, ** p-value < 0.01

To confirm that Salmonella were able to colonize lymphatic tissue and to rule out that the inability to induce proliferation of T-cells was due to defects in infection ability or loss of antigen expression, CFU in livers of infected mice and plasmid stability were determined. Therefore, livers in parallel to analyzed spleens were extracted, homogenised and plated on LB and LB Kan plates. CFU was counted after 24 h of incubation at 37°C and plasmid stability was assessed. If plasmid stability was below 100 %, 200 clones grown on LB agar w/o selection were streaked out on LB Kan plates. The next day, clones containing plasmids were assessed by the ability to grow in the presence of Kanamycin. This was performed to investigate the plating efficiency as clones that did not grow in the presence of Kanamycin may still harbour plasmids but did not express the resistance gene initially.

Table IV.3 summarizes the results of these control experiments. As visible, the yield of bacteria in the liver was similar in different strains, except StmTC pTolC-OVA which colonized this organ approx. 2-fold better than the other strains. Nevertheless, this difference was not significant (p-value = 0.156 compared to StmTC pTolC) and may also be attributed to a higher inoculation titre. Furthermore, plasmid stability proved to be very high in this setting, even when bacteria were passaged *in vivo* followed by 24 h incubation on LB agar plates, no colony lost the plasmid in strain StmTC pTolC-OVA. To sum up, poor efficacy of stimulating proliferation and activation $CD8^+$ T-cells by Stm strains was neither due to loss of antigen nor poor capacity to colonize secondary lymphatic organs within 3 days in the murine host.

Strain	CFU/liver	Plasmid stability	Plasmid stability*
Stm pTolC	$1.34 \times 10^5 \pm 8.54 \times 10^4$	100 %	-
Stm pTolC-OVA	$1.47 \times 10^5 \pm 3.14 \times 10^4$	82 %	-
StmTC pTolC	$9.8 \times 10^5 \pm 2.78 \times 10^4$	100 %	-
StmTC pTolC-OVA	$2.62 \times 10^5 \pm 7.6 \times 10^4$	72 %	100 %

Table IV.3 Colonization of the liver and plasmid stability of Stm strains used in the OT-I transfer
Mice were immunized with 5 × 105 Stm strains, 3 days later mice were sacrificed, spleens and livers were removed and homogenised. Splenocytes were analysed as described above, serial dilutions of livers were plated on LB or LB Kan agar to determine CFU and plasmid stability. CFU values are indicated as means plus/minus standard deviation, differences were not statistically significant. Plasmid stability: assessed by plating; Plasmid stability*: assessed by streaking clones on LB Kan plates after 3 days passage in mice and 24 h incubation on LB agar.

IV.1.3.2. OT-II transfer

Analogous to the OT-I approach, $CD4^+$ T-cells specific for $OVA_{323-339}$ can be adoptively transferred into syngenic mice. The schedule was kept identical to the OT-I approach with exception of the positive control *Listeria monocytogenes* EGDe *ΔtrpS* (Lm) harbouring plasmid pSP118-OVA. This strain was found to induce the expansion of OT-I and OT-II cells in an adoptive transfer setting (139). Specific $CD4^+$ T-cells were identified by the presence of CD4 and the OT-II T-cell receptor, indicated by binding of α-Vα2 and α-Vβ5 antibodies. Again, activated T-cells were discriminated from non-activated by loss of CD62L expression. As a result, no Stm strain was able to increase frequency of neither activated nor non-activated T-cells compared to controls, in contrast to *Listeria monocytogenes* pSP118-OVA (Figure IV.13). Of note, the frequencies of activated OT-II cells (Figure IV.13 B) seemed to be

higher than of non-activated (Figure IV.13 A) OT-II cells. This was possibly due to the staining procedure, as activated cells were only stained with α-Vβ5 and α-CD4 antibodies whereas the total cell fraction was identified additionally by Vα2 expression. Nevertheless, Stm expressing surface bound OVA were not able to prime specific $CD4^+$ T-cells and no proliferation or activation of those cells occurred as detectable with this system.

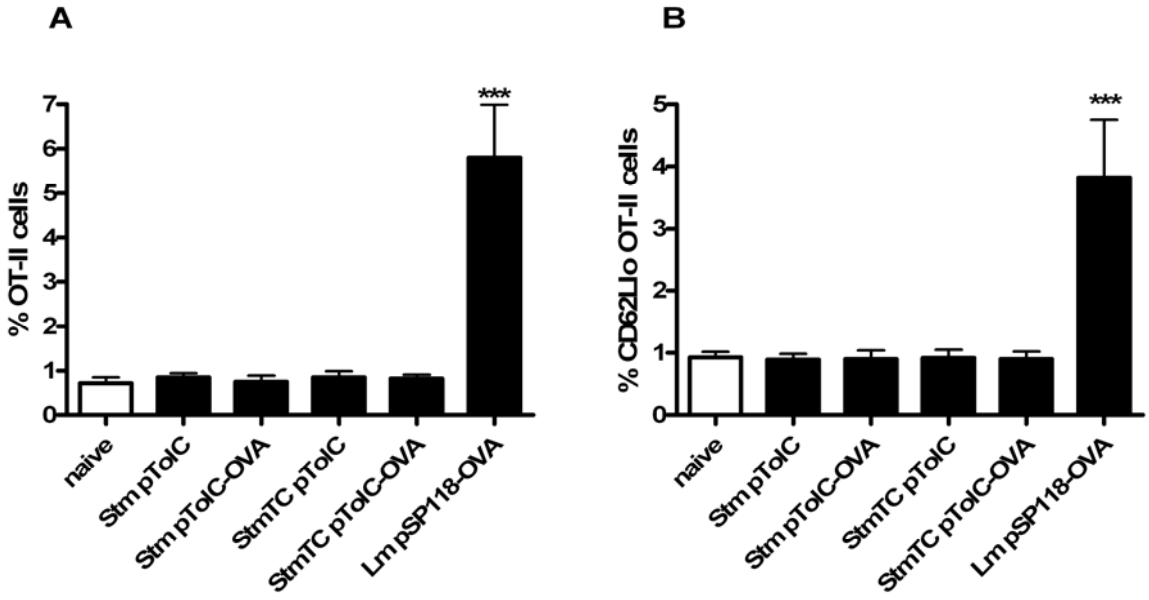

Figure IV.13 OT-II cell expansion after immunization with Stm and Lm
$OVA_{323-339}$ specific T cells (OT-II cells) were transferred into Bl/6 mice, mice were immunized, and T-cell expansion was assessed by FACS.
The frequency of **A** total OT-II cells and **B**: activated (CD62lo) OT-II cells is given as mean percentage of these cells of the whole splenocyte population (n = 3 mice). Significances were calculated by 1-way ANOVA followed by Newman-Keuls multiple comparison test. *** p-value < 0.001 as compared to all other groups

IV.1.4. Stm strains expressing surface associated B-RAFV600E epitope failed to induce BRAF specific $CD8^+$ T-cell response

IV.1.4.1. IFNγ ELISPOT

To determine specific immune responses mediated by vaccination of mice with *salmonellae* expressing the BRAFV600E epitope on the bacterial surface, an ELISPOT assay was applied. Briefly, 3-4 mice/group were immunized 3 times fortnightly with 5×10^9 Stm. One week after the last immunization splenocytes were removed and restimulated *ex-vivo* for 5 days in the presence of IL-2 and target cells pulsed with different peptides to expand specific T-cell populations. P2 and P3 comprised B-RAF derived epitopes which were modified for stricter binding to HLA-

B27 (D2R exchange) whereas P2 contains the V600E mutation and P3 represents the non-mutated wildtype peptide. In addition, an unrelated peptide, namely Ova SIINFEKL (P0), was included in the study to serve as negative control. After restimulation, cells were co-incubated with feeder cells pulsed with the described peptides on ELISPOT plates coated with IFNγ capture antibody. Detection was carried out with IFNγ detection antibody in a sandwich-like approach and a final phosphatase reaction. Each spot represented one IFNγ secreting T-cell stimulated by peptide-HLA complex recognition. The resulting plates were read-out in an AID ELISPOT reader device and analysed with the corresponding software. As illustrated in Figure IV.14, vaccination with Stm strains expressing the B-RafV600E epitope did not result in substantial activation or proliferation of specific T-cells since spot counts did not increase when restimulated cells of vaccinated mice were analysed compared to controls.

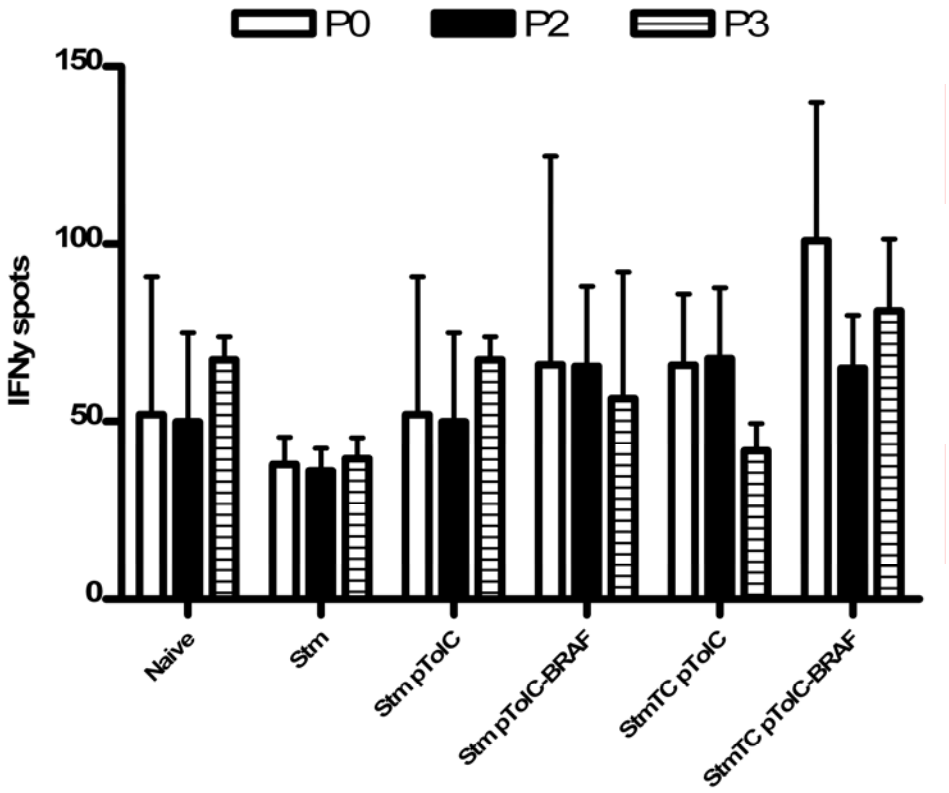

Figure IV.14 IFNγ ELISPOT for detection of B-RAF specific CD8 T-cells after immunisation of HLA-B27 transgenic mice with Stm
HLA-B2705 transgenic mice were immunized fortnightly with 5 × 10^9 Stm. One week after the last immunization mice were sacrificed and splenocytes were restimulated ex-vivo with B-RAF derived peptides and controls. P0 = Ova SIINFEKL, P2 = mutated B-RAF and P3 = wildtype vBRAF. ELISPOT was performed by detecting cells secreting IFNγ in response to stimulation by feeder cells presenting different peptides in the context of HLA/MHC-class I molecules. Spot counts were analysed by 1-way ANOVA to evaluate significance of differences between immunization groups.

FNγ cell counts derived from naïve mice were rather high. However this was due to the effector/target cell ratio selected for the final analysis, which leads to the conclusion that IFNγ-positive cell counts were very low in the immunization groups. Furthermore, no significant difference in numbers of IFNγ-secreting T-cells was observed when stimulated with different peptides. For a positive result spot counts of T-cells stimulated with peptide P2, which contains the V600E mutation, should have had increased, as the HLA-B2705 molecule should be able to present the epitope on the surface of professional APCs. Taken together, immunization with Stm strains failed to induce detectable levels of P2 specific T-cells.

IV.1.4.2. FACS analysis with B-RAF specific dextramers

To confirm the ELISPOT data, frequencies of P2 and P3 directed $CD8^+$ cells were determined by FACS in different splenocyte fractions restimulated with P2. Therefore, cells were stained with α-CD8-CyChrome and dextramers of peptides P2 and P3, respectively. The frequencies of double positive cells are depicted in Figure IV.15 indicated as $CD8^+$ T-cells with a TCR specific for peptide P2 (GRFGLATEK, assigned D-P2+CD8+) or peptide P3 (GRFGLATVK, assigned D-P3+CD8+) in the context of HLA-B2705.

The overall frequencies of dextramer-positive $CD8^+$ T-cells were low, meaning that specific T-cells did not expand extensively after restimulation. In addtion, no differences in numbers of specific T-cells were observed when comparing the individual immunization groups, which is consent with the ELISPOT data. However, frequencies of D-P2 positive are higher than D-P3 positive $CD8^+$ T-cells in these populations of P2 restimulated cells. This difference was significant in two immunization groups albeit these observations could not be attributed to B-RAF mediated immunization as StmTC pTolC-BRAF failed to induce a significant shift towards D-P2+ CD8+ T-cells. Taken together, in these experimental settings *Salmonella*-based antigen presentation within surface exposed TolC loops was not able to mediate detectable expansion and activation of antigen specific $CD4^+$ or $CD8^+$ T-cells *in vivo*. This was valid for ovalbumin and B-Raf derived epitopes. Nevertheless, T-cell responses induced by *Salmonella*-based immunization might ranged under the detection limits of the diverse assays but may be sufficient to protect from challenge with antigen expressing tumours. To address this possibility, two xenograft models were established as described in the following.

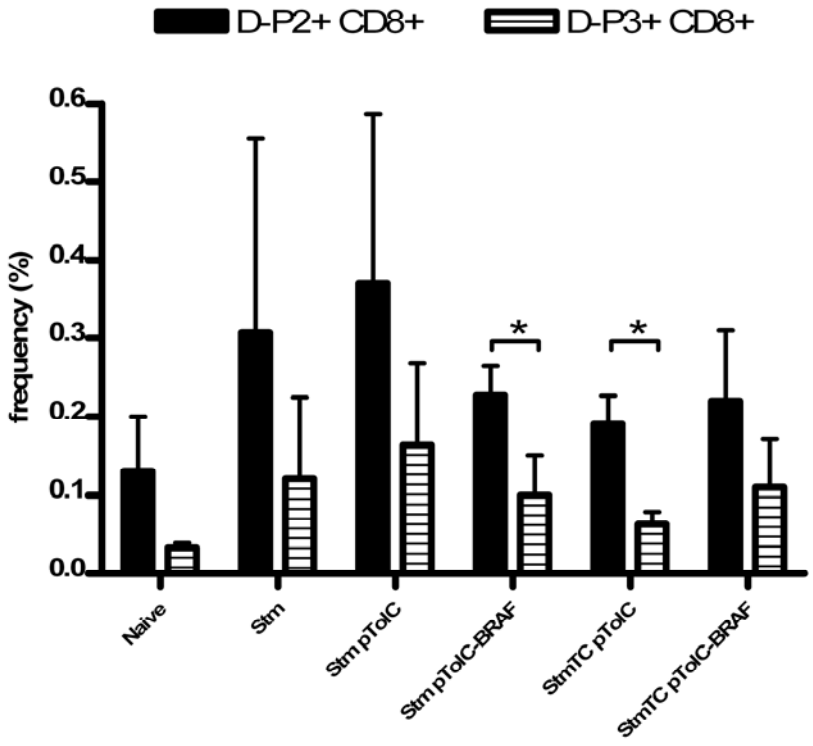

Figure IV.15 FACS analysis for detection of B-RAF specific CD8 T-cells after immunisation of HLA-B27 transgenic mice with Stm
HLA-B2705 transgenic mice were immunized fortnightly with 5 × 10^9 Stm. One week after the last immunization mice were sacrificed and splenocytes were restimulated *ex-vivo* with B-RAF derived mutated peptide P2 (GRFGLATEK). T-cells specific for the different peptide/HLA-B2705 complexes were assessed by staining with α-CD8 antibody and dextramers P2 (D-P2) and P3 (D-P3), respectively. Frequencies of peptide specific T-cells were analysed by 1-way ANOVA to evaluate significances of differences between immunization groups. Students T-Test was performed to analyse differences of frequencies comparing T-cells positive for D-P2 and D-P3 within one immunization group. * = p-value < 0.05

IV.1.5. Vaccination of mice with *Salmonella* expressing surface bound antigen was protective against PSA- but not Ova- positive tumour cell challenge

IV.1.5.1. B16-Ova challenge

To investigate the capacity of *Salmonella*-based immunization to protect from tumour challenge an ovalbumin-positive xenograft model was applied. First, ovalbumin expressing cells had to be identified. Two independent cell lines were investigated in a Western Blot setting. Surprisingly, neither in B16-Ova nor in EG.7 cells expression of ovalbumin was detectable (not shown). Therefore, the ability of cell lines to present Ova SIINFEKL peptide in the context of MHC class I molecules on the surface of cells was assessed. This assay displays increased sensitivity and ensures already that those cells can be targeted by CTLs with their cognate TCR. To address this

issue, different cell lines were incubated with the 25D-1.16 antibody that recognizes Ova SIINFEKL bound to MHC-I. Detection was carried out by FACS analysis after staining with a FITC-labelled secondary antibody. EG.7 cells were pulsed o/n with 25 µg/ml Ova SIINFEKL peptide to serve as positive control. Figure IV.16 shows the results, depicted by the shift in florescence when stained with both antibodies compared to staining with primary antibody alone. EG.7 cells showed a very low staining intensity (Figure IV.16 A) indicated by a marginal shift compared to staining with primary antibody alone. Additionally this shift was also present when stained with secondary antibody alone (not shown). As conclusion, EG.7 cells did neither show expression of Ova nor presentation of Ova SIINFEKL on MHC-I molecules.

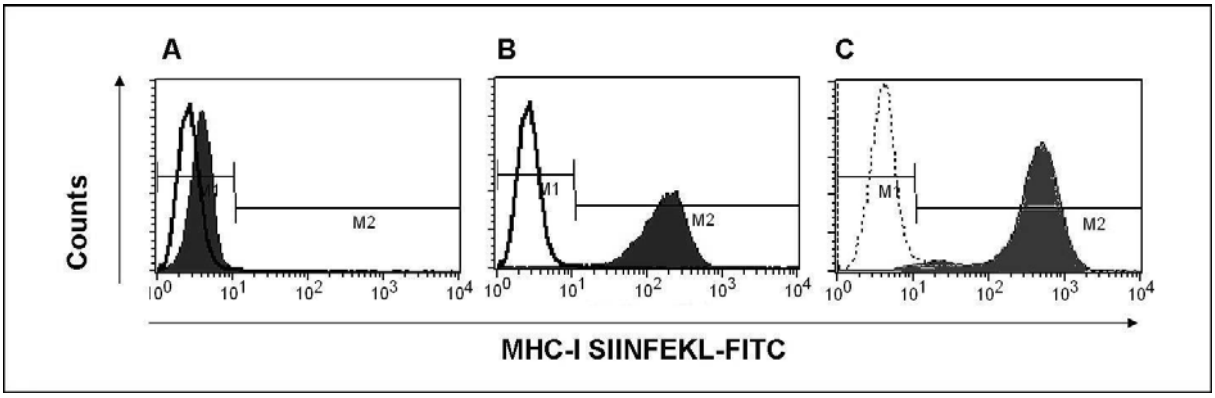

Figure IV.16 FACS analysis for Ova SIINFEKL presentation in the context of MHC-I molecules
Cells were stained with 25D-1.16 antibody recognizing Ova SIINFEKL in the context of MHC-I molecules. Empty curves indicate cells stained with primary antibody only. **A**: EG.7 cells, **B**: EG.7 cells pulsed o/n with 25 µg/ml SIINFEKL peptide, **C**: B16-Ova cells

To exclude that MHC-I molecules were absent, EG.7 was pulsed with SIINFEKL peptide and a clear shift was observed, meaning that this cell type is able to present exogenous SIINFEKL (Figure IV.16 B). B16-Ova cells in contrast were able to present endogenous Ova SIINFEKL (Figure IV.16 C). Therefore, this cell line was used for further tumour challenge studies.

For the protection assay, 7 C57BL/6 mice per group were immunized 3 times fortnightly with 5×10^9 Stm. Three weeks after the last immunization, mice were challenged with B16-Ova tumours by s.c. injection of 5×10^5 cells in each flank of shaven abdominal skin. Tumour growth was monitored over a period of 16 days. On day 18 post injection all mice were sacrificed because of high tumour burden. Throughout the whole experiment mice immunized with OVA-presenting Stm possessed smaller tumours than other groups (Figure IV.17) of which StmTC pTolC-

OVA immunized mice harboured the smallest. However, these differences were not significant as evaluated with 1-way ANOVA and Newman-Keuls multiple comparison test. Immunization therefore failed to translate into efficient deceleration of tumour growth in a B16-Ova tumour xenograft model.

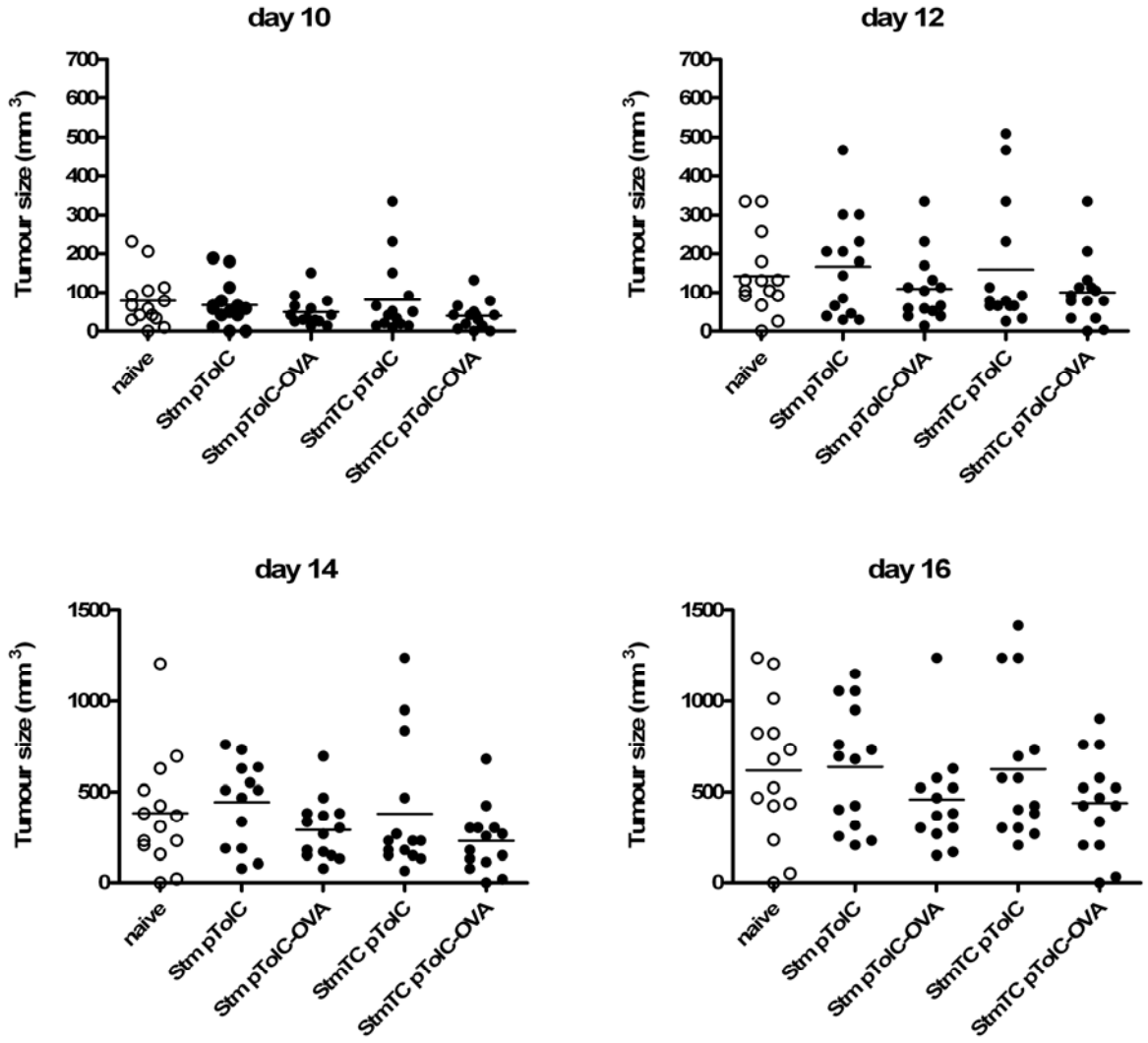

Figure IV.17 Tumour growth of B16-Ova xenografts in *Salmonella*-vaccinated mice
Mice (n=7/group) were immunized 3 times fortnightly with different *Salmonella* strains (5×10^9 bacteria) and challenged 3 weeks after the last immunization with 5×10^5 B16-Ova cells by s.c injection into each abdominal flank. The results for days 10, 12, 14 and 16 post injection are depicted here. Tumour sizes were obtained by measuring diameters of palpable neoplasias and calculating the volume for rotating ellipsoids. Tumour volumes were analysed by 1-way ANOVA followed by Newman-Keuls multiple comparison test for significant differences.

IV.1.5.2. B16-PSA challenge

As a result of the challenge experiment with the B16-Ova cell line, group numbers were decreased and immunization was performed weekly due to time constraints.

Furthermore, the number of applied bacteria for immunization was raised to 1×10^{10} CFU.

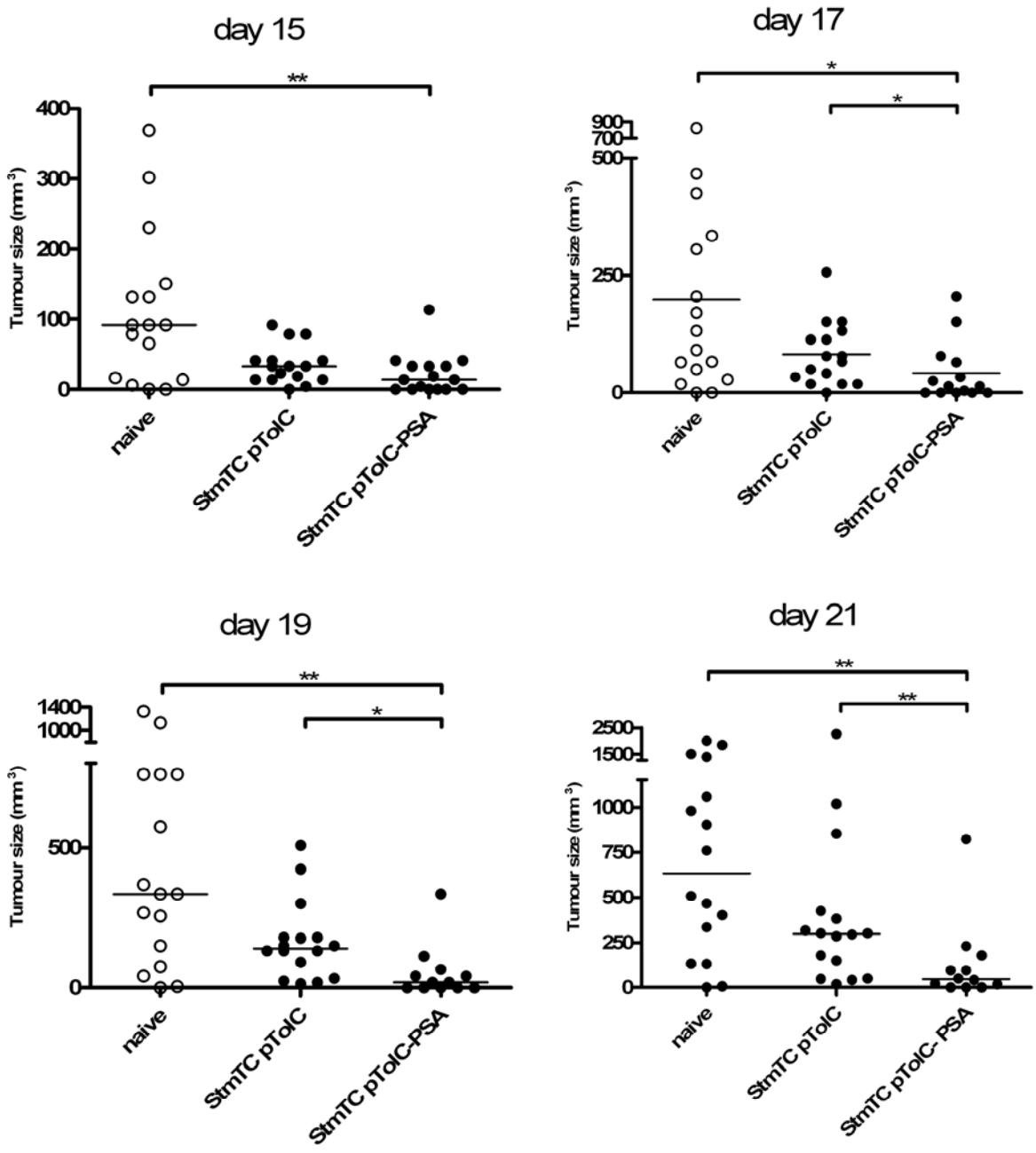

Figure IV.18 Tumour growth of B16-PSA xenografts on *Salmonella*-vaccinated mice
Mice (n=8-9/group) were immunized 3 times with one week intervals with 1×10^{10} *Salmonella* intragastrically. Two weeks after the third immunization, mice were challenged with B16-PSA by two s.c. injections of 1×10^{5} cells into each flank of shaven abdominal skin and tumour sizes were measured for 21 days. Differences of tumour volumes were analyzed for significance using the non-parametric Mann-Whitney test. *: p-value < 0.05, **: p-value < 0.01

Functional characterisation of the *Salmonella* carrier and generation of a B16 cell line stably expressing PSA are described elsewhere (136). In summary, 7-9 Bl/6 mice

were immunized weekly with 1×10^{10} Stm and 2 weeks after the last immunization these mice were challenged with 5×10^5 B16-PSA cells by s.c. injection into both abdominal flanks. Tumour growth was monitored over a period of 21 days.

Tumour volumes measured at days 15–21 are illustrated in Figure IV.18. On day 16 and 18 one mouse of the StmTC pTolC-PSA group died. This was not due to obvious tumour burden, since tumour volumes were rather low in these mice. Nevertheless, tumour volumes in StmTC pTolC-PSA immunized mice were significantly lower than in the naïve control observed from day 11 on (not shown) until the experiment was stopped. *Salmonella* alone also seem to elicit growth repressive effects on tumours, as StmTC pTolC immunized mice suffered from lower tumour sizes than naïve ones. However, significances for this observation were only given on day 15 and 19. Most strikingly, tumour growth in PSA vaccinated mice was also decelerated significantly compared to the *Salmonella* control group from day 15 on. Therefore, immunization with epitope displaying *salmonellae* facilitated specific and unspecific immune stimulation, leading to protection against tumour challenge. It is noteworthy that most tumours in this vaccinated group stayed very small throughout the whole experiment proving a strong cancer suppressing effect facilitated by an epitope specific immune reaction. However, the nature of this specificity remains to be elucidated.

IV.2. Part II: Improvement of the hemolysin secretion system in Ty21a for vaccination purposes

IV.2.1. The hemolysin secretion system of *E. coli* is less efficient in Ty21a than in other *salmonellae*

IV.2.1.1. Analysis of hemolysin (HlyA) secretion by Western Blot

To improve the approved strain Ty21a for antigen delivery via the hemolysin secretion system, different *Salmonella* strains were investigated for their ability to secrete hemolysin. For this purpose, 3 different serotypes were transformed with plasmid pANN202-812 encoding the *hly* operon, raised in BHI medium and supernatant and cytoplasmic proteins were prepared from stationary phase cultures as described in III.2.3.2. Figure IV.19 illustrates the results of this assay. As visible, Ty21a harbouring pANN202-812 was much less efficient in expressing (Pe fraction) and secreting (Sn fraction) HlyA compared to the other serotypes tested. Of course, Ty21a alone did neither express nor secrete HlyA.

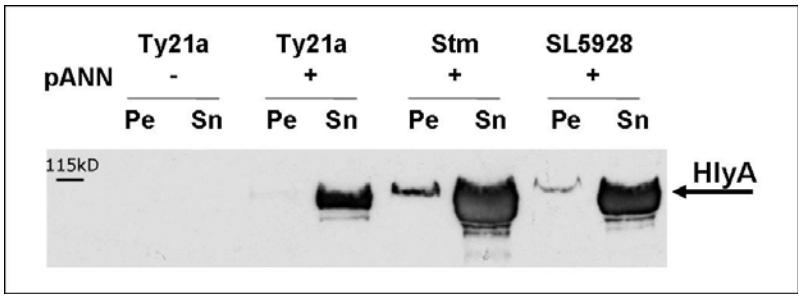

Figure IV.19 Identification of HlyA secretion ability of different Salmonella strains harbouring plasmid pANN202-812 by Western Blot.
Cellular proteins (Pe) of 0.05 ml and supernatant (Sn) proteins of 2.5 ml logarithmic culture of different *Salmonella* strains were applied on the SDS PAGE in each lane, respectively. Detection was carried out with α-HlyAs antibody. SL5928: *Salmonella dublin* SL5928, pANN: plasmid pANN202-812

IV.2.1.2. Ty21a harbours mutations possibly influencing secretion

The vaccine strain Ty21a is an attenuated mutant of *S. typhi* Ty2, achieved by multiple mutations induced by chemical mutagenesis (78). Therefore some of the mutations could be responsible for the less efficient hemolysin secretion ability. In order to test this supposition an approach for testing the effect of single mutations in Ty21a on hemolysin secretion was started. It was shown earlier that the *galE* defect

of Ty21a has no influence on its poor capacity for hemolysin secretion (Meier, S.R and Gentschev, I.). Supplementing limiting galactose (0.001 %) to the growth medium can overcome *galE* deficiency in Ty21a with respect to LPS biogenesis (79,189). However, galactose addition did not alter HlyA secretion efficiency in Ty21a pANN202-812 (Meier, S.R. and Gentschev, I.). Another prominent mutation which could contribute to the observed phenotype is a nonsense mutation of *rpoS* mediated by a base insertion within the respective gene. To investigate the influence of this alternative sigma factor on HlyA expression and secretion, *rpoS* was cloned from Stm and subjected to analysis as stated below.

IV.2.2. RpoS from Stm is functional in the *rpoS* negative Ty21a strain

IV.2.2.1. Plasmid construction

The *rpoS* gene was amplified from chromosomal DNA of Stm. The PCR fragment was generated with Pfu Polymerase with primers rpoS_up and rpoS_down and contained putative promoter regions of *rpoS* as determined by the Neural Network Promoter Prediction algorithm (190). The 1.9 kB fragment was ligated into vector pACYC184 by restriction with *BamH*I and *Hind*III. Expression of *rpoS* was assayed in the *rpoS*-negative *S. typhi* Ty21a strain by a positive catalase reaction. RpoS-positive clones could be detected by producing visible oxygen bubbles when treated with hydrogen peroxide (H_2O_2). The extent of bubbling indicates absence or reduction of catalase production in strains with differing *rpoS* genotypes (191). The resulting plasmid was termed pRpoS.

IV.2.2.2. Growth characteristics of Ty21a pRpoS

Ty21a harbouring pRpoS was analysed for its growth kinetics to rule out that substantial changes in the metabolism occurred by expressing *rpoS* from plasmid. Therefore, Ty21a pACYC184 and Ty21a pRpoS were inoculated in BHI medium containing 20 µg/ml Cm, incubated under vigorous shaking at 37°C and OD600 was measured every hour. The OD was plotted against time in Figure IV.20. No differences in growth kinetics between the two strains were observed.

IV.2.2.3. Oxidative stress test

The indicated strains (Table IV.4) were grown to the mid-logarithmic growth phase, washed and treated with different concentrations of hydrogen peroxide or left untreated. Then serial dilutions were plated on agar plates and the survival rate was assessed as described. The data (Table IV.4) demonstrate that pRpoS is functional in Ty21a and that the *rpoS*-complemented Ty21a strain is less sensitive than Ty21a alone. As expected, Stm showed the best survival rate in this assay.

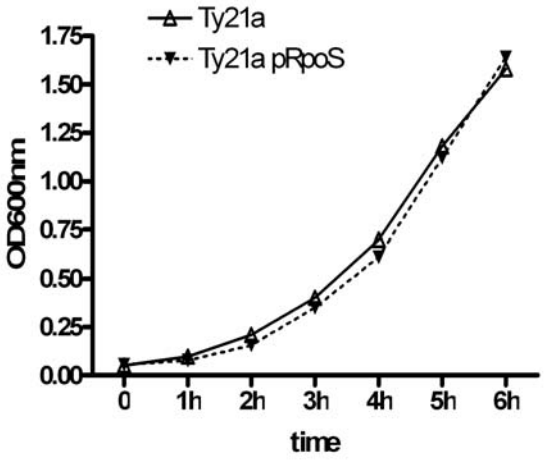

Figure IV.20 Growth kinetics of Ty21a strains with different *rpoS* phenotypes
Cultures were grown in BHI and OD600 was measured every hour. The experiment was performed at least 3 times with similar results.

Strain	3 mM H_2O_2	30 mM H_2O_2
Ty21a	0 %	0 %
T21a pRpoS	2 %	0 %
Stm	15 %	0 %

Table IV.4 Survival rate of bacterial cultures treated with H_2O_2.
Cells were treated with the indicated concentrations of H_2O_2 or left untreated and plated on BHI agar. The survival rate was determined by dividing CFU of treated by CFU of untreated cells.

IV.2.3. RpoS influences the hemolysin secretion efficiency in Ty21a

IV.2.3.1. Western Blot analysis of Ty21a strains with different *rpoS* phenotypes

First, Ty21a pRpoS and Ty21a harbouring pACYC184 as control were transformed with pANN202-812 and grown in BHI. At different time points (OD600 of 0.4, 0.7, 1.4 and 2.3) cytoplasmic and supernatant proteins were extracted and analyzed for differences in secretion of HlyA (Figure IV.21 left panel). Samples taken from the early logarithmic (OD 0.3-0.4) and stationary (OD 2.3-3.0) growth phase were found to display characteristic differences in secretion of HlyA between strains and were therefore applied for further studies. The *tolC*-deficient Ty21aTC pANN202-812 strain (NC) was not able to secrete HlyA as the outer membrane channel of the export machinery is lacking.

Figure IV.21 depicts a Western Blot detecting HlyA in Ty21a pANN202-812 supernatant and cellular fractions. Expression (right panel) and secretion (left and right panel) was enhanced in Ty21a harbouring pRpoS. This was true for the logarithmic (OD 0.4) and stationary phase (OD 2.5), even though *rpoS* represents a sigma factor induced at the end of bacterial growth.

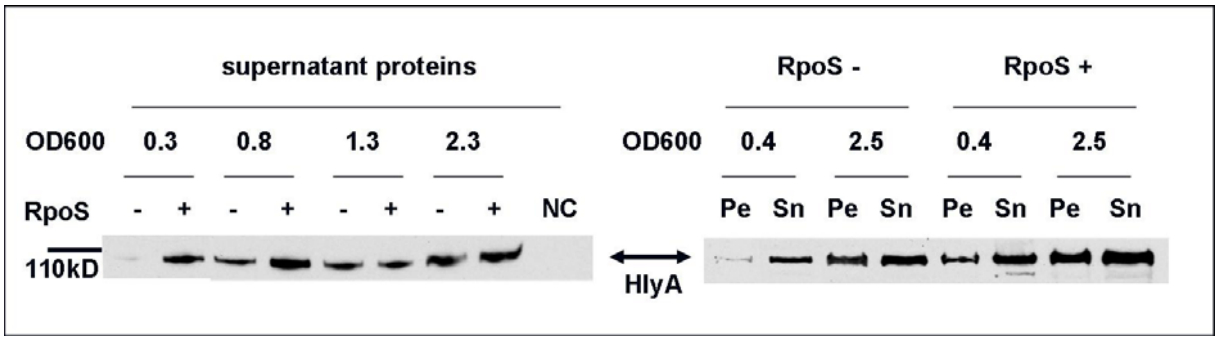

Figure IV.21 Western Blot for HlyA secretion and/or expression of Ty21a strains.
Cultures were grown in BHI and cytoplasmic or supernatant proteins were taken at indicated densities (OD600). Supernatant proteins (Sn) of 2.5 ml and cellular proteins (Pe) of 0.12 ml were loaded on each lane, respectively. Detection was carried out with the α-$HlyA_S$ antibody.
Left panel: supernatants of Ty21a pANN202-812 harbouring pRpoS (+) or pACYC184 (-) as control. NC: Ty21aTC pANN202-812
Right panel: supernatant (Sn) and cellular (Pe) proteins of Ty21a pANN202-812 harbouring pRpoS (+) or pACYC184 (-) as control.

IV.2.3.2. Transcriptional analysis by qRT-PCR

One reason the above described observation could be the fact that RpoS is involved in the growth dependent regulation of *rfaH* transcription and O-antigen expression in *S. typhi* (192). RfaH also enhances elongation of *Escherichia coli hlyCABD* mRNA most likely via antitermination (193) thus, increasing the expression and secretion of hemolysin. Therefore, the effect of *rpoS* on the transcription of *rfaH*, *hlyA* and *hlyD* was analyzed using transcription of *cat* present on plasmids pACYC184 and pRpoS as internal control. Strains were grown to the early logarithmic or stationary growth phase, RNA was isolated and reverse transcribed into cDNA. The indicated genes (Figure IV.22) were nanlyzed by qRT-PCR. Change in transcription was calculated by comparing profiles of the pRpoS-complemented strain with the control harbouring the empty vector pACYC184.

Unfortunately, results were highly variable and differed from one qRT-PCR to the other as illustrated in Figure IV.22. No clear tendency in transcriptional profiles of *rfaH* and *hlyD* was observed. Taking together the results of 6 independent

experiments, only *hlyA* transcription showed a stable 2-3-fold induction when *rpoS* was present. The nature of RpoS mediated *hlyA* upregulation in Ty21a is therefore not known and can be at least in parts distinct from antitermination via *rfaH*.

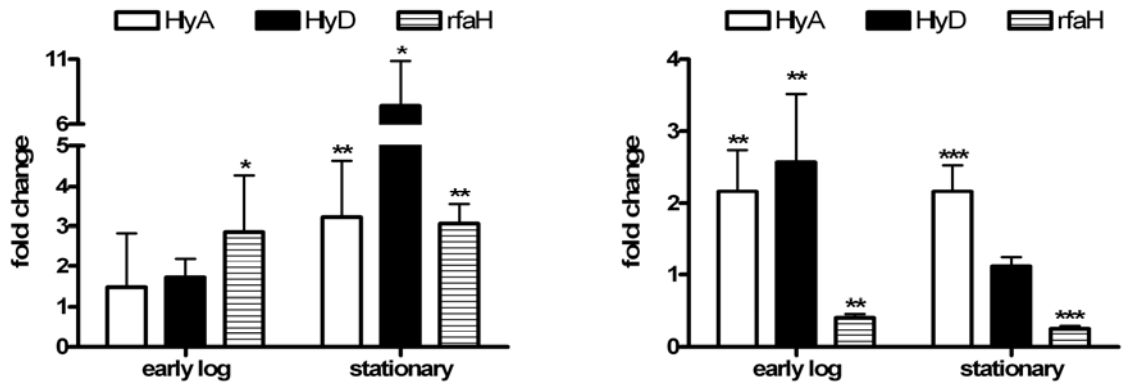

Figure IV.22 Two independent qRT-PCR experiments of the *rpoS* positive Ty21a strain secreting hemolysin
RNA was isolated from cultures of Ty21a pANN202-812 pACYC184 and Ty21a paNN202-812 pRpoS grown to the early logarithmic (OD 0.4) or stationary phase (OD 2.5) and reverse transcribed into cDNA. Indicated genes were analyzed by qRT-PCR. The relative changes in gene expression between Ty21a pANN202-812 pACYC184 and Ty21a paNN202-812 pRpoS were calculated after normalization with the cat gene as internal control. Significances in changes of transcription were calculated using Students T-Test. *: P-value < 0.05, ** : P-value < 0.01

Regarding the fact that *rpoS* regulates the expression of numerous genes, diverse regulational effects might have interfered with transcription of the investigated genes. Therefore, the influence of sole *rfaH* overexpression on *hly* transcript and protein levels was studied.

IV.2.4. RfaH regulates *hly* genes as analyzed on mRNA and protein level

IV.2.4.1. Plasmid construction

The *rfaH* gene was amplified from chromosomal DNA of Ty21a with PhusionTaq and primers rfaH_up and rfaH_down. The resulting 800 bp fragment was ligated into vector pACYC184 analogous to *rpoS*. The construct contained the Ty21a *rfaH* promoter and C-terminal 5 × His Tag to confirm expression with a His Tag antibody (not shown). Ty21a harbouring pRfaH showed similar growth kinetics than Ty21a alone (not shown)

IV.2.4.2. Western Blot analysis of Ty21a overexpressing RfaH

Ty21a pANN202-812 complemented with pRfaH was analyzed as described for Ty21a harbouring pRpoS. In Western Blot assay a markedly increased expression and secretion of HlyA was detected when multiple copies of *rfaH* were present on plasmid pRfaH compared to the control strain. The increase was similar to that achieved by *rpoS* complementation.

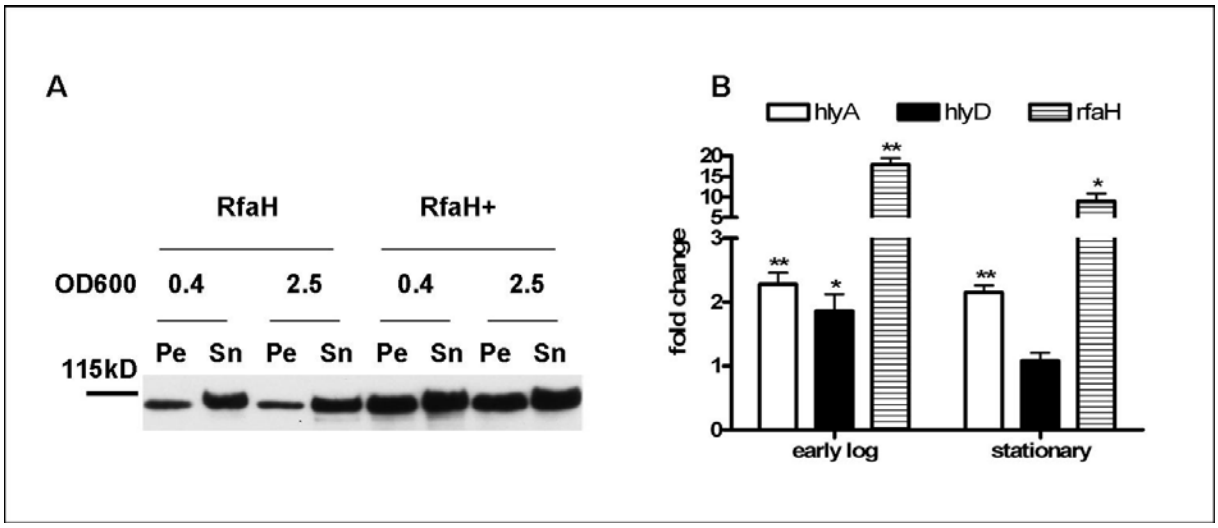

Figure IV.23 Effects of *rfaH* on transcription, expression and secretion of HlyA. WB (A), semi-quantitative RT-PCR (B).
A: Cultures of Ty21a pANN202-812 pACYC184 (RfaH) and Ty21a pANN202-812 pRfaH (RfaH+) were grown in BHI and cytoplasmic or supernatant proteins were taken at indicated densities (OD600). Supernatant proteins (Sn) of 2.5 ml and cellular proteins (Pe) of 0.12 ml were loaded on each lane, respectively. Detection was carried out with the α-$HlyA_S$ antibody.
B: RNA was isolated from cultures of Ty21a pANN202-812 pACYC184 and Ty21a paNN202-812 pRfaH grown to the early logarithmic (OD 0.4) or stationary phase (OD 2.5) and reverse transcribed into cDNA. Indicated genes were analyzed in a Rotor-gene qRT-PCR. The relative changes in gene expression between Ty21a pANN202-812 pACYC184 and Ty21a paNN202-812 pRfaH were calculated after normalization with the cat gene as internal control. Significances in changes of transcription were calculated using Students T-Test. *: P-value < 0.05, **: P-value < 0.01

IV.2.4.3. qRT-PCR

On the mRNA level, *hlyA* increased roundabout twofold when plasmid pRfaH was present in Ty21a, measurable in the early logarithmic and stationary phase (Figure IV.23 B). Furthermore, the *hlyD* mRNA level increased up to twofold in the logarithmic phase when compared with Ty21a alone, but this benefit was lost in the stationary phase. Interestingly, the level of *rfaH* mRNA is highly increased even in the early logarithmic growth phase. It was shown that transcription of *rfaH* in *S. typhi* is growth phase dependent with peak expression at the end of the logarithmic phase

(194). This tight regulation seems to be altered by introducing multiple copies of *rfaH* through pRfaH. Taken together, by complementation with both, pRpoS and pRfaH, twofold upregulation of *hlyA* mRNA was achieved. This alteration of RNA levels seemed to be sufficient for significant differences in the level of secreted HlyA protein (Figure IV.21 and Figure IV.23 A) since transcription of the *hlyD* gene was highly variable (pRpoS) or not affected in all growth phases (pRfaH).

IV.2.5. RpoS and RfaH effect invasion and survival of Ty21a in RAW 264.7 macrophages

IV.2.5.1. Invasion and survival assay

RpoS was described to improve survival of *Salmonella* in macrophages by countering the action of nitric oxide synthase (195) which in turn could influence antigen presentation and immune stimulation. Therefore, the invasion and survival behaviour inside Raw macrophages of different Ty21a strains was assessed. Briefly, cells were infected for 2 h at an MOI of 100, extracellular bacteria were killed with Gentamycin and 2 and 4 h post infection, and cells were lysed to determine the yield of intracellular bacteria by plating serial dilutions on LB agar.

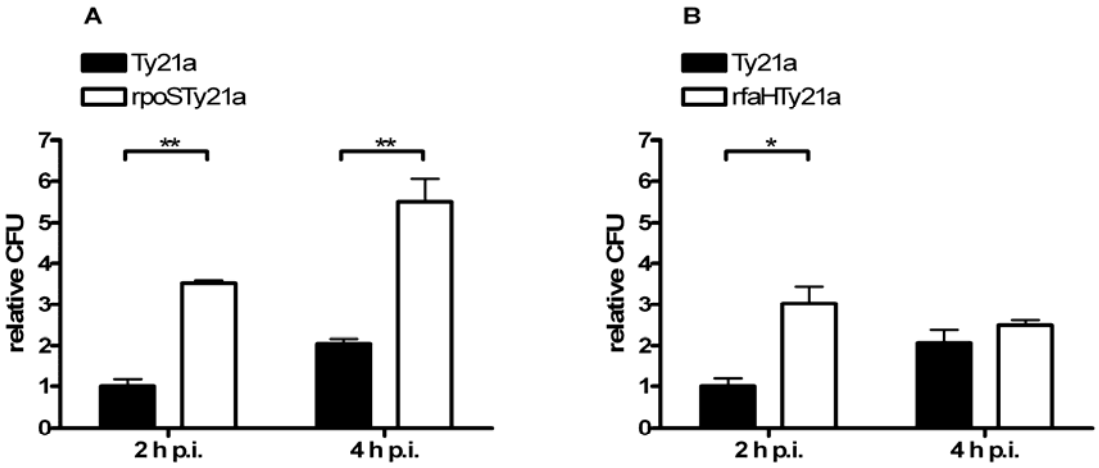

Figure IV.24 Invasion and intracellular survival of Ty21a strains in RAW 264.7 macrophage-like cells **A**: Ty21a pRpoS (rpoSTy21a) and **B**: Ty21a pRfaH (rfaHTy21a). Cells were infected at a multiplicity of infection of 100 and lysed after 2 and 4 h post infection (p.i.). CFU was determined by plating serial dilutions on LB agar plates. Relative CFU was calculated by dividing the CFU from different strains by the CFU from Ty21a at 2 h p.i.. Significant differences in relative CFU were calculated using Students T-Test. * = P-value < 0.05; ** = P-value < 0.01

Significant differences between the intracellular CFU of Ty21a and Ty21a pRpoS strains were found 2 and 4 h post infection. Plasmid pRfaH in contrast, only conferred a benefit during the early time-point. Four hours after infection, the number of intracellular bacteria was equal for Ty21a and Ty21a pRfaH. While Ty21a replicates intracellularly with a doubling time of 2 h, Ty21a pRfaH did not show significant intracellular growth (Figure IV.24 B).

IV.2.5.2. Plasmid stability test

Raw macrophages were infected for 24 h with Ty21a pRpoS and Ty21a pACYC184, respectively before cells were lysed and serial dilutions were plated either on LB or LB Cm agar. Plasmid stability was determined by dividing CFU values determined by counting colonies on Cm containing agar plates by CFU numbers on LB plates. Additionally, grown clones were tested for catalase activity by the described H_2O_2 reaction. Plasmid pRpoS was stably inherited in Ty21a whereas pACYC184 was readily lost during passage within macrophages (Table IV.5). Furthermore, *rpoS* was still functional after cultivation in macrophages since clones were able to express catalase. The data demonstrate that *rpoS* is positively selected within macrophages..

Strain	Plasmid stability	catalase expression
Ty21a pRpoS	100 %	100 %
Ty21a pACYC184	1-4 %	-

Table IV.5 Plasmid stability of pACYC based vectors in Ty21a after 24 h growth in macrophages
Raw cells were infected for 24 h with indicated Ty21a strains. Serial dilutions of lysed cells were plated on LB or LB Cm plates and CFU was determined. Plasmid stability was calculated by comparing CFU on LB Cm and LB plates. Catalase expression was determined by the emergence of visible oxygen bubbles after H_2O_2 treatment.

IV.2.6. Antibody responses against hemolysin but not LPS were enhanced after intranasal immunization of mice with Ty21a strains secreting HlyA

In order to test the immunological effect of *rpoS* and *rfaH*, the humoral immune responses against HlyA and LPS of recombinant Ty21a were assessed *in vivo*. For this purpose, four groups of C57/Bl6 mice (n = 5 per group) were immunized i.n. twice with Ty21a pANN202-812 pRpoS, Ty21a pANN202-812 pRfaH, Ty21a

pANN202-812 pACYC184, and Ty21a (control). One addtional group consisted of naïve mice. Induction of HlyA and LPS-specific immune responses was analyzed on day 49 by HlyA and LPS-specific ELISA like shown in Figure IV.25. Interestingly, immunization with the Ty21a pANN202-812 pRfaH strain revealed a significant enhancement of antibody responses against HlyA (Figure IV.25 A), but not LPS (Figure IV.25 B) in comparison to all other groups. Furthermore, the difference in HlyA-specific antibody responses between experimental groups immunized with Ty21a pANN202-812 pRpoS and Ty21a pANN202-812 pRfaH was also statistically significant ($p < 0.05$), as determined by 1-way ANOVA followed by Newman-Keuls multiple comparison test. The overall reactivity of the sera against LPS was rather low, only 4 of 25 mice were responding to this antigen, even though detection was carried out with anti IgG and IgM antibodies in this case.

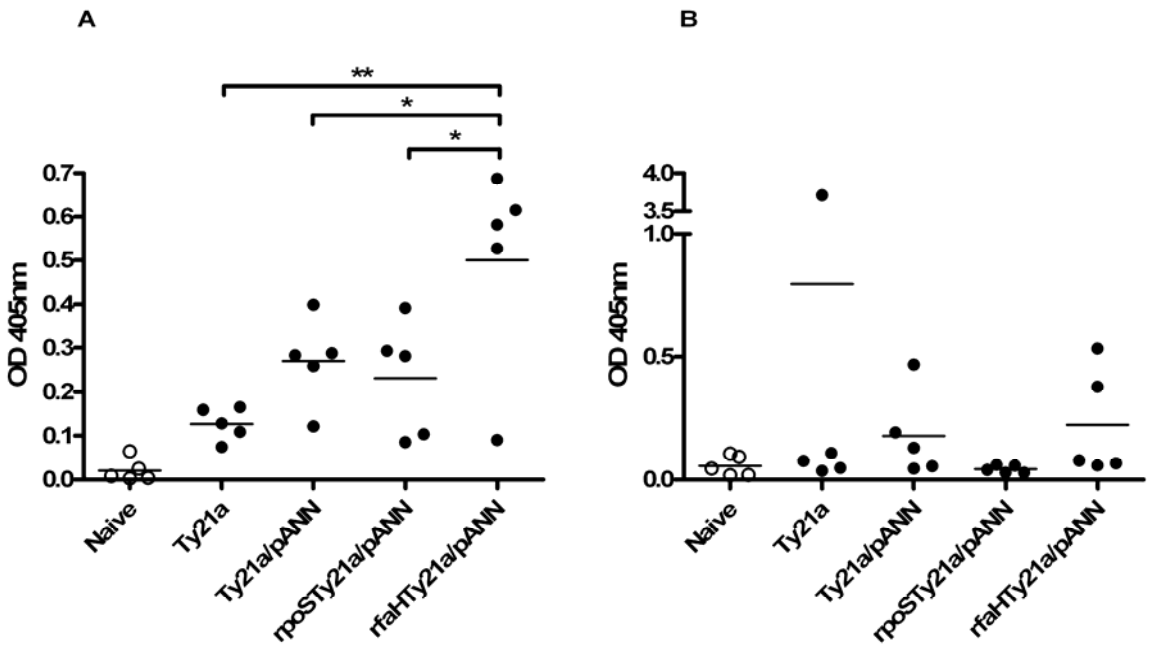

Figure IV.25 Antibody titres present in murine sera after immunization with Ty21a strains
HlyA (A) and LPS-specific (B) serum antibody responses of mice immunized with Ty21a pANN202-812 pRpoS (rpoSTy21a/pANN), Ty21a pANN202-812 pRfaH (rfaHTy21a/pANN), Ty21a/pANN202-812 (Ty21a/pANN), Ty21a (control) and naïve mice, determined by HlyA or LPS-specific ELISA with anti IgG (A) and anti IgG + IgM (B) detection antibodies. Data were analyzed by 1-way-Anova followed by Newman-Keuls multiple comparison test. * = P-value < 0.05; ** = P-value < 0.01

As a result, immunization with a strain exhibiting increased expression and secretion of hemolysin resulted in an increased efficacy of stimulation of humoral immune responses against the heterologous HlyA antigen.

V. Discussion

V.1. Part I: Surface display of antigens via TolC

In this project, a surface display system for antigen presentation via the outer membrane protein TolC was employed in two different *Salmonella* vaccine strains. The first set of experiments dealt with the construction of the carriers and investigation of viability. In the latter part of this project, quantities and qualities of immune responses were subject of investigations. Three distinct antigens were included in preclinical studies including epitopes of the model antigen Ova and of the cancer-related antigens PSA and BRAFV600E.

Several strategies to present epitopes on the surface of live bacterial vaccines were developed and it was shown earlier that surface display of antigens is superior in inducing immune responses over sole cytoplasmic expression (121,122,125). As mentioned, TolC was the membrane anchor of choice for the described antigens. The chromosomal *tolC* copy was deleted in both, Stm and Ty21a, strains to saturate TolC insertion sites into the outer membrane with plasmid-encoded epitope-fused TolC. This was performed as the level of the immune response in animals is proportionally to the amount of expressed antigen in live *Salmonella* vaccines (196,197). TolC-BRAF and TolC-OVA were expressed and assembled as stable trimers in *Salmonella* strains and indeed, abundance of recombinant TolC trimers was higher in the knock-out strains (Figure IV.5 and Figure IV.6).
It was clearly demonstrated that recombinant TolC was able to complement *tolC* deficiency in the newly constructed strains with respect to multidrug efflux and Type I secretion (Table IV.2 and Figure IV.8). The efficiency of HlyA secretion as determined by on-plate hemolysin assay was comparable in *Salmonella* strains harbouring either chromosomal-encoded, plasmid-borne *E. coli* TolC or plasmid-borne, recombinant *E. coli* TolC (Figure IV.8). Furthermore, recombinant TolC isoforms were able to restore the ability for survival within macrophages and invasion and survival in Caco-2 cells in *tolC*-deficient strains (Figure IV.9 and Figure IV.10). This was crucial as TolC plays an important role in pathogenesis of *Salmonella* strains. The exact role of TolC in this processes is discussed, ranging from direct and indirect effects on adhesion and invasion, secretion of an unknown factor, to

conferring resistance against antimicrobial peptides and bile (134,184-186,198). Surprisingly, the ability of Ty21a to invade and survive in Caco-2 cells was low compared to Stm although the latter strain has poor intracellular replication capacity due to the *aroA* mutation (188).

Invasion of intestinal cells strongly depends on expression of genes located on the *Salmonella* pathogenicity island I (SPI-I) including a type III secretion system (199). Anyway, Ty21a seems to be devoid of a functional type III secretion system (personal communication with H Rüssmann and G. Dietrich) and this fact may explain the low invasion ability into Caco-2 cells of this strain. Interestingly, a recent study showed that the invasion deficiency of a *tolC* mutant was mediated by downregulation of SPI-I genes in *Salmonella typhimurium* (186). In contrast, in this work infection of Caco-2 cells with Ty21a was shown to be dependent on *tolC* (Figure IV.10) leading to the supposition that regulation of SPI-I is not the only function of *tolC* in this serotype with respect to invasion into and survival within epithelial cells. However, TolC may preferentially be crucial for intracellular survival in this setting and not for entry, since it participates in mediating resistance against host-derived antimicrobial peptides (134). The intracellular yield of Stm after infection of macrophages, in contrast to Caco-2, was low when compared to Ty21a. Ty21a is able to replicate inside cells (see also Figure IV.24) while Stm is not due to its *aroA* mutation, thus explaining in parts the observed difference. Unexpectedly, also the effect of *tolC* on intracellular survival of Stm inside RAW cells was marginal. However, more recent investigations in our group employing Stm and its *tolC*-deficient derivative StmTC could demonstrate the crucial role of TolC for this process. The number of intracellular bacteria was 30-fold reduced when *tolC* was absent (136) which is more congruent with the published data (184).

As *Salmonella typhi* strains are typical human pathogens, Stm strains were applied for animal studies and results of these experiments are now discussed. All tested recombinant Stm strains were able to colonize the murine liver (Table IV.3) which is a target organ for *Salmonella typhimurium* aroA in mice (200). In secondary lymphatic tissues, including spleen and liver, systemic immunity develops (201) and colonization of these organs is crucial to prime the immune system. In the same setting, it was shown that pTolC plasmids were retained with an astonishing stability. No colony of StmTC was found after 3-days passage in the murine host and 18 h on LB agar plates that lost antigen-encoding vector pTolC-OVA (Table IV.3). These

observations strengthened the results obtained by *in vitro* stability tests (Table IV.1). Usually, plasmids stabilized via antibiotics *in vitro* are unstably replicated *in vivo* due to the lack of selective pressure. As many live vaccines express heterologous antigens via those plasmids, this instability might result in loss of expression and less efficient antigen presentation. This can be circumvented by insertion of the antigen into the chromosome of the carrier (115) or by using so-called balanced-lethal plasmid systems (202). The first approach has limitations as single copy expression may also decrease antigen presentation. The latter strategy is accompanied by complex genetic manipulations.

These limitations do not concern this vaccination approach. Plasmid stability and expression was high in the *tolC*-deleted strain. The reason for this stability *in vivo* may be due to: (i) high intrinsic stability of pBR322-based plasmids (203), (ii) low metabolic burden by antigen insertion into TolC, (iii) low replication rate of the carrier *in vivo* (188) and (iv) selective pressure to maintain TolC for the export of e.g. bile salts and antimicrobial peptides. This in turn leads to sustained antigen expression and presentation on the bacterial surface since these antigens are inserted into a positively selected protein. Additionally, it was accomplished to delete the Kanamycin resistance from plasmid pTolC in StmTC by homologous recombination. Selection *in vitro* was facilitated in the presence of Novobiocin which is a target for TolC-AcrAB mediated efflux (132). Plasmid stability of this construct was up to 100 % without selection for 18 h *in vitro* (Table IV.1). Additionally, TolC encoded on these vectors was expressed independently from antibiotic selection (Figure IV.7). Antibiotic resistance free plasmids are advantageous for a possible human use as they pose lower risks for the environment and are of higher acceptance for approving institutions.

In summary, the recombinant Stm carrier strain displayed all prerequisites for efficient immunotherapy leading to the second part of this project which aimed to elucidate the nature of the immune response against (tumour) antigens and protective capacity of immunization. First, the successful immunization of mice with recombinant vaccine displaying the PSA epitope will be discussed.

Tolerance is one major problem and limitation of immunotherapy of cancer: as all tumour antigens are self antigens specific T-cells might exist but may be attenuated and therefore anergic e.g. due to the action of regulatory T-cells (204,205). In

general, CTL responses are directed against only a few peptide epitopes of an antigen and this is termed immunodominance (206,207). It was suggested that dominant and subdominant epitopes might help to break immunotolerance of tumour antigens (208,209). Other attempts to override this anergy is the circumvention of immune checkpoints or the ablation of regulatory T-cells (for review see (2)) which bear the danger of collateral damage due to detrimental autoimmune responses. A recently discovered immunodominant epitope of PSA (158) was included in the TolC surface display approach. Most strikingly, the StmTC strain expressing the TolC-PSA fusion was able to confer protective immunity against challenge with a PSA expressing melanoma cell line in mice. Tumour growth was significantly decelerated in comparison to controls (Figure IV.18). It is widely known that *Salmonella* live vaccines can induce the proliferation of CTLs (105,210) but stimulate also parts of the innate immune system (211-213) which concert in the anti tumour response (6,10,214).

The latter was confirmed in our studies since *Salmonella* alone exerted inhibiting effects on B16 melanoma-driven, PSA-positive tumours. Intrinsic capacity to stimulate components of the innate immune system represents a major advantage of tumour immune therapy applying LBV. This stimulation may play a key role to overcome anergy of T-cells in the tumour microenvironment apart from using dominant and subdominant tumour-derived epitopes. However, expression of the immunodominant $CD8^+$ epitope within the TolC-loop was superior in decelerating tumour growth, suggesting that epitope specific and non-specific mechanisms act synergistically to protect from tumour challenge. To conclude, one $CD8^+$ epitope instead of a full length protein, inserted 2-fold into the TolC loop, was sufficient to promote protective effects without further need of an adjuvant besides carrier antigens.

The data strongly suggest that PSA specific CTLs are at least in parts the cause for this protection as the $CD8^+$ epitope was sufficient to mediate significant differences between tumour volumes in mice. These results become even more striking when considering that the B16 cell line alone is poorly immunogenic and displays strong immune escape mechanisms compared to other cell lines (215,216). Employing TolC mediated surface expression of antigens circumvents another major obstacle of cancer immune therapy which is cancer immune evasion (see II.1.4). We are

currently performing an IFNγ ELISPOT (Fensterle, J. and Hotz, C., results not obtained yet) to elucidate the role of PSA-specific CTLs in the observed protection.

The presented vaccination approach was shown to be protective in an infection model (127) and it was now demonstrated to mediate protection in a cancer immunotherapy approach. Therefore, TolC-based epitope display is functional in vaccination against infectious and neoplastic diseases. Because of these promising observations and the fact that TolC is almost ubiquitously expressed in gram-negative bacteria (128), this plasmid encoded delivery system for heterologous antigens can be adapted to display a multitude of different epitopes also in other live bacterial vaccines. One of these carriers, *Salmonella enterica* serovar Typhi Ty21a that is licensed for human use (79), was engineered the same way as described for Stm. TolC fusion proteins were also highly expressed and functional in a *tolC*-deficient Ty21a strain (Figure IV.5 and Figure IV.9) implying that this system can be easily transferred to human use.

In order to characterize the cellular basis of the adaptive response mediated by vaccination with *Salmonella*, presenting epitopes on the bacterial surface more generally, adoptive transfer models utilizing the model antigen ovalbumin were established. Unfortunately, no specific and convincing $CD8^+$ or $CD4^+$ T-cell responses were measurable. In one adoptive transfer experiment a slight but significant increase in specific $CD8^+$ T-cell frequencies after immunization with an OVA displaying *Salmonella* strain was detectable. However, this was not reproducible and cells were not activated as analysed by CD62L abundance. In activated (CD62L low) Ova specific T-cells no such increase was observed (Figure IV.12). Additionally, in a well established *in vitro* presentation assay StmTC displaying Ova failed to activate specific $CD8^+$ T-cells irrespective of the type of antigen presenting cell (DCs or macrophages) used (not shown, conducted in cooperation with G. Geginat, RUMMS, Mannheim).

It was demonstrated in earlier studies that frequencies of Ova specific T-cells after vaccination with Ova-expressing *salmonellae* are low, ranging in similar dimensions (approx 1 %) like presented in this work (141,217). The positive signal may therefore been overwhelmed by the “background noise”, as non-immunized mice showed similar frequencies of specific activated T-cells. However even if not detectable but present, these cells were not protective against challenge with B16-OVA xenografts (Figure IV.17). *Salmonella* evolved a multitude of mechanisms for escaping T-cell

recognition explaining those low frequencies. T-cell immune evasion is achieved by interfering with APC functions leading to decreased MHC class I and II presentation of peptides (for review see (218)) or directly by inhibiting T-cells due to secretion of inhibiting virulence factors or downregulation of the specific TCR (219,220). Nevertheless, T-cell immunity after *Salmonella* encounter was demonstrated several times (see II.2.3 and (217,221-223)) and also in the context of TolC-mediated surface display, protective immune responses were observed most likely facilitated, at least in parts, by T-cells recognizing their cognate epitope (127) and (Figure IV.18). These findings argue against a general inability of TolC-displayed antigens to prime specific T-cells moving the focus of possible explanations for the weak responses more to the nature of the individual epitope(s). Similar to TolC-OVA, TolC-BRAF also failed to mediate priming of specific T-cells as determined by ELISPOT (Figure IV.14).

In principle, two pathways exist that result in MHC class I presentation of antigens for pathogens restricted to vacuoles like *Salmonella* (i) the vacuolar alternative pathway facilitated by professional APCs (DCs and macrophages), involving vacuolar processing of antigens loaded on pre-existing pools of MHC I molecules (106,224) and (ii) cross-presentation of antigen-containing particles, e.g. apoptotic blebs of *Salmonella*-infected macrophages, mediated through bystander DCs relying on classical antigen processing via the endoplasmatic reticulum or the proteasome and loading of peptides onto nascent MHC I molecules (225-227). $CD4^+$ T-cell priming via MHC class II presentation of *Salmonella*-derived peptides is facilitated via the classical vacuolar pathway for exogenous antigens. Access to these presentation pathways may be impeded due to individual structural features of TolC fusion proteins.

In the case of TolC-OVA and TolC-BRAF, the antigen might be buried in the pore of its membrane anchor or display other conformational constraints rendering the antigen hardly accessible for degradation which is crucial for presentation via MHC molecules. To estimate structural features, loops of the different fusion proteins were analysed *in silico* for their hydrophobicity and tendency to build transmembrane regions. Comparing these factors might predict if loop structures are buried within the protein or membranes which could hinder accessibility for protein degradation and presentation by APCs. However, none of the “non-inducer” fusion proteins displayed significant higher hydrophobicity or tendency to build transmembrane regions within

their antigen-fused loop structure when compared to the “inducer” TolC-PSA (Figure V.1). Only the loop of TolC-LisTB shows lower tendency to form transmembrane segments and displays less hydrophobicity than the others. Taken together, loops of the “inducer” fusion proteins (TolC-PSA and TolC-LisTB) do not share common structural features that are distinct from the non-inducers (TolC-OVA and TolC-BRAF). Quite the contrary, TolC-PSA, TolC-BRAF and TolC-PSA seem to be very similar in the investigated structural characteristics with TolC-LisTB being the “odd one out”, thus rendering hydrophobicity and membrane affinity of the recombinant TolC loop structure unlikely to be the reason for low immunogenicity. Interestingly, TolC-PSA and TolC-LisTB exhibit shorter loop insertion fragments. Furthermore, TolC-PSA harbours more linker alanines (Figure IV.1) than TolC-OVA and TolC-BRAF. Those traits might influence immunogenicity, albeit being highly speculative.

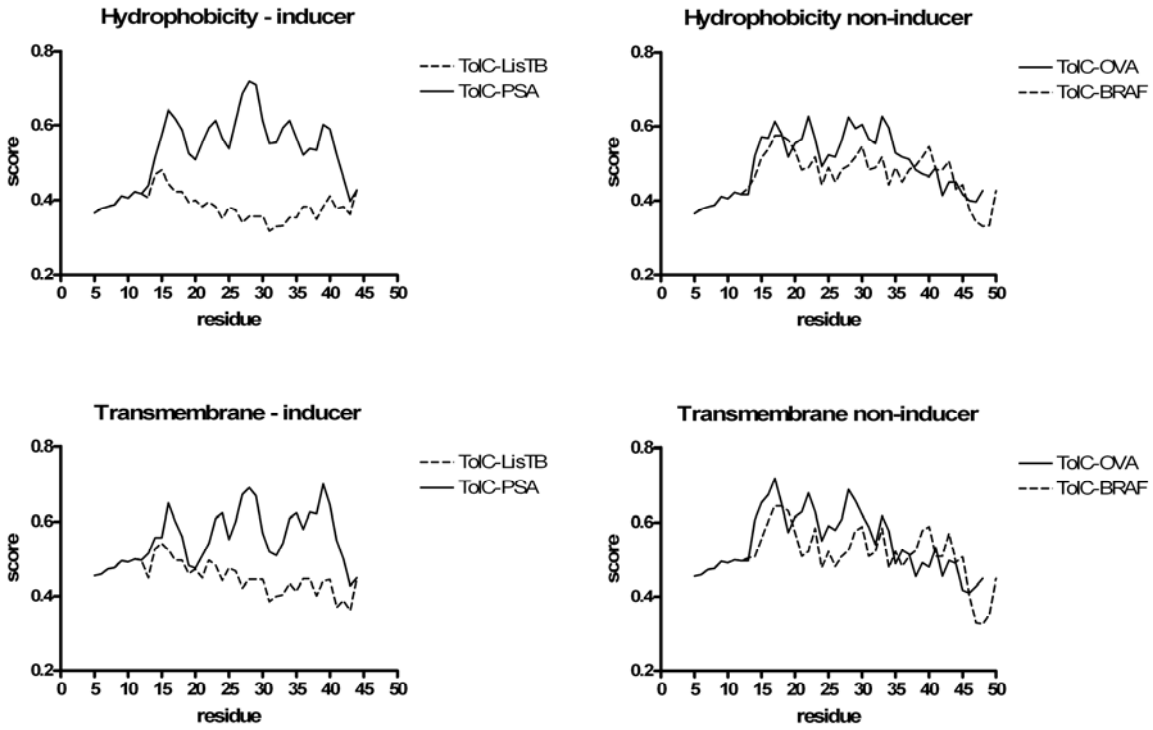

Figure V.1 Hydrophobicity and tendency to form transmembrane regions of loop structures derived from different TolC fusion proteins
The respective loops (encompassing aa 257-279 of the non-recombinant TolC protein containing the fused antigen) were analysed with ExPASy ProtScale (228) utilizing algorithms for estimating hydrophobicity from Rose *et al*. (229) (upper panel) and the tendency to form transmembrane segments (230) (lower panel). The scales were normalized from 0 to 1.

More generally, to my knowledge the question why surface displayed antigens are superior in eliciting T-cell responses still remains open. It was speculated earlier that surface bound expression (i) diminishes the metabolic burden opposed by cytoplasmic expression (127), (ii) increases the stability and accessibility of the antigen as it is not packaged into inclusion bodies and therefore not degraded in the bacteria like cytosolic expressed antigens (231), (iii) enhances immunogenicity as surface-exposed antigens co-localize to LPS which possesses intrinsic adjuvant properties (232) and (iv) allows easier processing of antigens by the described MHC presentation pathways (233).

However, the latter hypothesis has not been experimentally addressed. A possibility for such enhanced accessibility of outer membrane contents for processing prior to MHC presentation could be the occurrence of outer membrane vesicles (OMVs). Those vesicles are naturally discharged as discrete, closed outer membrane blebs from growing gram-negative bacteria (234,235). They encompass contents of the outer membrane and periplasm such as porins, receptors, pores and LPS, excluding products of the cytoplasm or inner membrane (236). OMVs are central to export and toxicity of different virulence factors, inter-bacterial material transfer and communication and play a role for pathogenicity of certain gram-negative species (for review see (237)). OMVs derived from *Salmonella* were shown to elicit protective B and $CD4^+$ T-cell responses against proteins of the outer membrane and display strong pro-inflammatory properties due to potent stimulation of the innate immune system (238,239). As TolC is found in those vesicles (Gentschev, I and Mayer, R. S., unpublished), shedding of those structures might represent an alternative way for the host immune system to gain access to TolC or other membrane proteins fused to antigens for MHC presentation besides vacuolar degradation of the whole carrier. On the other hand LPS shedding is suggested to inhibit T-cell activation in vivo (240) and excessive budding of OMVs could result in a more immune-dampening effect due to their LPS content.

Further studies in this respect may shed light into processing and superior presentation of outer membrane-anchored antigens. These observations pose possible options for elevating the T-cell stimulatory capacity. Increased shedding of these vesicles mediated by interfering with the *tol-pal* regulatory system (241) may form a wanted process to enhance membrane-bound antigen in the vacuolar compartment possibly leading to more efficient MHC class II presentation. However,

those mutants are fragile and enhanced shedding of OMVs correlates with virulence (242-244). An already established way to enhance $CD8^+$ responses is to allow excess of *Salmonella*-derived antigens into the class I presentation compartment, the cytosol. Listerolysin, a pore forming protein mediates escape of *Listeria monocytogenes* into the host cytosol (245). Applying this factor for export via the hemolysin secretion system allowed *Salmonella* to open the phagosome to some extent, providing access to the cytosolic compartment and thereby inducing potent CTL responses (246). Furthermore, a combination of Ova antigen and Listerolysin expressed in *E. coli* elicited potent protection from Ova-positive tumour cell challenge in mice compared to poor protection when immunizing with *E. coli* expressing Ova alone. This superior effect was not only achieved by changing the compartmentalization of the heterologous antigen but also by unexpectedly inhibiting $CD4^+$ $CD25^+$ regulatory T-cells (247) implying a very promising role for those modifying enzymes in cancer immune therapy.

As mentioned earlier vaccination with *Salmonella* expressing the BRAFV600E epitope on its surface failed to mediate priming of specific $CD8^+$ T-cells. Despite the discussed conformational constraints other observations could explain this low immunogenicity. Interestingly, the HLA-B27 allele and infection with pathogens like *Salmonella* are linked to reactive arthritis (248,249). It is hypothesized that HLA-B27-restricted CTLs activated against *Salmonella*-derived peptides might cross-react through molecular mimicry with endogenous peptides presented by HLA-B27, leading to autoimmunity (250). Furthermore, *Salmonella* interfere with HLA-B27 functions by inducing an alternative splicing of the pre-mRNA of this allele in transfected cell lines (251) and by downregulation of HLA-B27 molecules in infected patients (252). These described alterations of HLA expression could preclude efficient class I presentation of the BRAF epitope following vaccination with *Salmonella*.

Up to now, we were not able to observe convincing T-cell priming with *Salmonella* vaccines in this setting, earlier vaccination experiments employing secreted antigen delivery also failed to elicit specific $CD8^+$ T-cell responses (Yone, C, unpublished) and we are currently trying to assess the role of *Salmonella* itself on HLA-B27 presentation. By using DNA vaccines against mutated BRAF we want to exclude a general incompetence of HLA-B27 transgenic mice to present the epitope of mutated BRAF (Polzien, L., Hotz, C., and Fensterle, J.). In addition, colleagues are currently

working on the generation of BRAFV600E-positive and BRAFV600E-driven tumour xenograft models (Polzien, L.) as well as the generation of transgenic mice with BRAFV600E expression targeted to the lung (Luetkenhaus, K, Zanucco, E, Rapp, U.R.). These systems might be used in the future for immunization studies in tumour xenografts and spontaneous lung tumour settings, respectively.

In principle, targeting of oncogenes like BRAFV600E in their function as tumour-associated antigens poses an attractive and promising approach since it is believed that oncogene expression is not lost by immune evasion mechanisms due to their transforming and therefore indispensable character. This phenomenon was termed “oncogene addiction” (253). However, the BRAFV600E genotype was lost during transition from primary to metastatic disease in melanoma patients positive for HLA-B27 and specific T-cells (146), thus indicating the protective role of HLA-B27 but also representing a major limitation due to restriction of this epitope for vaccination against early stage melanoma. Our further experiments will elucidate the feasibility of *Salmonella*-based vaccination against the HLA-B27 restricted epitope as therapy against BRAFV600E-driven cancers.

V.2. Part II: Improvement of hemolysin secretion in Ty21a for vaccination purposes

Ty21a, the active compound of Vivotif®, represents the only licensed live typhoid vaccine in humans (83) and due to its well studied safety (for review see (254)) it represents an attractive carrier for delivery of heterologous antigens. Recently, two clinical trials assessed Ty21a as carrier for heterologous *Helicobacter pylori* antigens expressed in the cytoplasm of this live vaccine. The vaccine was safe but showed low immunogenicity (86,87). As mentioned several times in this work, the fashion of expression of heterologous antigens within live bacterial vaccines is crucial for immunogenicity and secreted or surface displayed antigens are superior in eliciting immune responses over cytoplasm-located antigens (110,118,127,255). One of the promising systems for such a delivery is the use of the hemolysin secretion system of *E. coli* which was used in numerous preclinical studies for delivery of pathogen derived (112) or tumour associated antigens (114,115) with *Salmonella* vaccines. Our group already demonstrated that HlyA secretion was functional in Ty21a and potent

humoral immune responses were elicited against the heterologous HlyA antigen in mice (159).

In this part of the work, the improvement of *S. typhi* Ty21a for hemolysin expression and secretion was described. In a first trial, it was shown that Ty21a is less efficient in expression and secretion of HlyA from plasmid pANN202-812 compared to other *Salmonella* vaccines (Figure IV.19). Different mutations account for attenuation of Ty21a of which *galE* and *rpoS* were supposed to have effects on hemolysin secretion. In contrast to *galE*, it was demonstrated that *rpoS* was involved in this process and complementation with pRpoS harbouring the Stm homolog of *rpoS* led to increase in expression and secretion of HlyA (Figure IV.21). It was speculated earlier in this work that RpoS exerts its action on HlyA via *rfaH*, a factor downstream of *rpoS* which regulates length of the O-side chain of LPS in *S. typhi* (192). RfaH also enhances elongation of *Escherichia coli hlyCABD* mRNA most likely via antitermination (193). For this reason the effect of *rpoS* on *rfaH*, *hlyA* and *hlyD* mRNAs was investigated. However, transcript levels of *hlyD* and *rfaH* were highly variable in Ty21a pANN202-812 pRpoS when comparing different experiments (Figure IV.22). The reason for this is not known but *rpoS* regulates various genes with in parts unknown functions in *Salmonella* (256) that could also influence expression and secretion via T1SS.

Complementation with pRfaH in the hemolysin-expressing strain Ty21a pANN202-812 strain showed a highly increased expression and secretion of hemolysin compared to the same strain without *rfaH* plasmid (Figure IV.23 A). Furthermore, *hlyA* transcript levels were doubled when pRfaH was present. This is not surprising as the RfaH protein not only acts as antiterminator but also enhances transcription initiation at the *hly* promoter (193,257). This 2-fold increase of *hlyA* was also observed when *rpoS* was reconstituted via plasmid pRpoS (Figure IV.22). Interestingly, pRfaH exhibited a rather slight effect on *hlyD* transcript which was only visible in the logarithmic growth phase (Figure IV.23 B). This is explainable as the effect of plasmid pRfaH on *rfaH* mRNA level weakened at the end of bacterial growth (Figure IV.23 B). Furthermore, Ty21a possesses a functional *rfaH* copy which may be sufficient for effective antitermination in the stationary phase, even though it could not be regulated by *rpoS* due to the *rpoS*-negative phenotype of Ty21a.

Finally, Ty21a pANN202-812 pRfaH induced significantly higher antibody titres against HlyA than control strains and even Ty21 pANN202-812 pRpoS in C57Bl/6

mice immunized intranasally. (Figure IV.25). This was unexpected as the latter strain also showed increased expression and secretion of HlyA (Figure IV.21). One possible reason for this could be improved survival inside macrophages as explained in the following.

Ty21a complemented with *rfaH* exhibited a higher titre of intracellular bacteria within the first 2 h of infection of RAW cells compared to Ty21a alone. This benefit is lost after 4 h because rfaHTy21a does not seem to replicate or the balance between killing and replication is shifted towards killing of the bacteria (Figure IV.24 B). The reason for this is not known, maybe *rfaH* mediates increased uptake by macrophages and/or increased susceptibility against killing by macrophages. Diametrically opposed to this hypothesis is a study by Nagy *et al.* which demonstrated that an *rfaH* deletion mutant of *Salmonella typhimurium* displayed increased invasion ability and decreased survival inside macrophages (258). However, maybe serovar-specific characteristics accounted for this contradictory observation.

Ty21a complemented with *rpoS* showed increased intracellular survival in RAW macrophages compared to the respective wildtype at 2 and 4 h p.i. (Figure IV.24 A). These data correspond to a study by Alam *et al.* in which a *S. typhi rpoS*-negative strain was more susceptible to intracellular killing by RAW macrophages. This killing was dependent on the action of nitric oxide synthetase (195). In contrast to that, a *S. typhi rpoS* deletion mutant showed no such susceptibility to intracellular killing in resting THP-I cells, a human acute monocytic leukemia cell line. However, this mutant was less cytotoxic than the respective wildtype (259) which indicates that RpoS could play a role in the virulence of serovar Typhi strains.

Furthermore, the observed reduced susceptibility to killing might result in less efficient MHC presentation of cytoplasmic or non-secreted antigens, which in turn could lead to reduced immunogenicity of the RpoS-positive strain. It was postulated earlier that decreased bacterial survival inside macrophages would be expected in increased availability of antigenic epitopes within the phagolysosome and consequently increased presentation of epitope/MHCII complexes (197). Resistance against intracellular killing might explain the lower antibody titres of Ty21a pANN202-812 pRpoS against the examined antigens (Figure IV.25) since the cytoplasmic fraction of hemolysin and LPS of the outer membrane are presumably of limited access for recognition when the carrier is not efficiently degraded. Of note, nearly all mice with one exception exhibited low antibody titres against the major antigen LPS.

This was anticipated due to culture conditions used to prepare Ty21a immunization aliquots. Since galactose was lacking in the medium, cells were devoid of full length O-antigen which in turn limited immunogenicity of LPS (79,189).
To rule out that instability of pRpoS accounted for low HlyA antibody titres, RAW cells were infected 24 h with strain Ty21a pRpoS and Ty21a pACYC184 as control. All colonies retained the Cm^R phenotype conferred by pRpoS whereas pACYC184 was readily lost after passage within macrophages (Table IV.5). A functional *rpoS* copy seems to underlie selective pressure and is therefore stabilized during intracellular replication presumably by counteracting nitric oxide. In addition plasmids pRfaH and pRpoS harbour the same origin of replication which would argue for at least similar stability rates. Taken together, it is unlikely that pRpoS is less stable than pRfaH *in vivo* which would lead to a loss of the beneficial effect on HlyA expression. Highly stable replication of pANN202-812 was demonstrated earlier (159).

Interestingly, the *rpoS* null mutation was inherited from the respective wildtype of Ty21a, *Salmonella typhi* Ty2 (260) and congruent to the presumed role of *rpoS* in virulence, the *rpoS*-positive Typhi ISP1820 serotype is more virulent in humans than Ty2 (261). Additionally, both genes, *rpoS* and *rfaH*, contribute to virulence in *Salmonella typhimurium* strains. Mutants of these factors were evaluated as live vaccine vectors in several studies (80,262-265). Introduction of these genes into the attenuated Ty21a strain might for those reasons increase virulence and affect attenuation. However, the exact contribution of *rpoS* in *S. typhi* virulence remains unclear except for a clear role in fine-tuning of the Vi capsule, balancing immunogenicity and adhesion and invasion into target cells (266). Nevertheless, Ty21a also lacks Vi antigen expression. Furthermore, the attenuating effect of the *rfaH* mutation in *S*. *typhimurium* is mainly due to downregulation of other virulence factors by silencing of LPS synthesis genes (258). In contrast, Ty21a represents a LPS defective strain resulting in a rough phenotype (78,267). It is therefore tempting to assume that overexpression of *rfaH i*n Ty21a will not interfere with safety concerns as full LPS synthesis is abrogated downstream of this regulator.
The present data clearly show that Ty21a pRfaH, secreting heterologous antigens via the hemolysin secretion system, allows vaccination against the carrier antigen. The feasibility of this system for vaccination against cancer in the murine model was demonstrated earlier (114,115). Currently, our group is preparing a Ty21a vaccine

based on secretion of a CtxB-PSA fusion protein via the hemolysin system for clinical trials (Bergmann, B., Gentschev, I., Kirchgraber, G., Weidmann, V., Meyer, R.S., Fischer, B., Fensterle, J., and Rapp, U.R.) and its success will determine the fate of cancer vaccines of this type. In future projects, this respective carrier could be "upgraded" for cancer immune therapy by *rfaH* complementation. Indeed, a two plasmid-based system is undesirable for a clinical vaccine. Hence, *rfaH* coupled to an active promoter can be incorporated on the antigen-determining plasmid, pMKhly1. Or as an alternative, promoters with high transcriptional activity can be integrated upstream of the *rfaH* genomic locus to uncouple this antiterminator from (putative) *rpoS* signalling or other regulation, leading to higher transcription rates *in vivo*. This can be achieved by strong promoters like P_{tac}, successfully used for antigen expression in live vaccines (268) or *in vivo* activated promoters like *nirB* (269) and *dmsA* (270) which allow strong transcriptional induction when the carriers localize within host cells while decreasing the metabolic burden opposed through growth *in vitro*.

Reflecting all announced facts, recombinant Ty21a strains, complemented with *rpoS*-independent *rfaH* loci, may form the basis of a novel generation of combination vaccines for human use which can be administered orally.

VI. References

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VII. Appendix

VII.1. Units

%	per cent
Å	Angstroms
bp, kb	base pairs, kilo base pairs
°C	degree Celsius
F	farad
g	gram
h	hour(s)
kDa	kilo Dalton
l	liter
M	molar [mol l^{-1}]
m	meter
min	minute(s)
rpm	revolutions per minute
s	second(s)
V	Volt
× *g*	factor of gravity

VII.2. Prefixes

f	femto	10^{-15}
p	pico	10^{-12}
n	nano	10^{-9}
µ	micro	10^{-6}
m	milli	10^{-3}
c	centi	10^{-2}
d	deci	10^{-1}
h	hecto	10^{2}
k	kilo	10^{3}
M	mega	10^{6}
G	giga	10^{9}

VII.3. Abbreviations

aa	amino acid(s)
approx.	approximately
AKP	alkaline phosphatase
Amp	Ampicillin
Amp^R	Ampicillin resistance
APC	antigen presenting cell
BCG	*Mycobacterium bovis* Bacille Calmette-Guerin
BHI	brain heart infusion
BSA	bovine serum albumin
CFU	colony forming units
CIAP	calf intestine alkaline phosphatase
Cm	Chloramphenicol
Cm^R	Chloramphenicol resistance
CTL	cytotoxic T-cell
CtxB	cholera toxin subunit B
DC	dendritic cell
dH_2O	de-ionized water
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
Ery	Erythromycin
Ery^R	Erythromycin resistance
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
Flp	flipase recombinase
FRT	flipase recombinase target
HLA	human leukocyte antigen
$HlyA_s$	HlyA secretion signal
HRP	horseradish peroxidase
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
Kan	Kanamycin
Kan^R	Kanamycin resistance
LB	Luria Bertani
LBV	live bacterial vaccine
LisTB	T and B cell epitope of the p60 protein from *Listeria monocytogenes*
Lm	*Listeria monocytogenes* EGDe *ΔtrpS*

LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
MSZ	Institute for Medical Radiation and Cell Research
NK	natural killer cell
NKT	natural killer T-cell
Novo	Novobiocin
OMVs	outer membrane vesicles
o/n	over night
ops	operon polarity suppressor
Ova	chicken ovalbumin
OVA	ovalbumin CD4 and CD8 epitope
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PSA	prostate specific antigen
RTX	repeats in toxin
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
Stm	*Salmonella enterica* serovar Typhimurium *aroA* SL7207
StmTC	*Salmonella enterica* serovar Typhimurium *aroA* Δ*tolC* SL7207
TAA	tumour associated antigen
TC	*tolC* deleted
TCA	trichloric acid
TCR	T-cell receptor
Tet^R	tetracycline resistance
Th	T-helper (cell, response)
TNF	tumour necrosis factor
Treg	regulatory T-cell
Tris	tris (hydroxy methyl) aminomethane
TS	temperature sensitive replication
Ty21a	*Salmonella enterica* serovar Typhi Ty21a
Ty21aTC	*Salmonella enterica* serovar Typhi Ty21a Δ*tolC*
UDP	uridine diphosphate
w/o	without
×	times

VII.4. Publications

VII.4.1. Articles

Stegmeier, J. F., Polleichtner, G., Brandes, N., Hotz, C., and Andersen, C. (2006) Importance of the adaptor (membrane fusion) protein hairpin domain for the functionality of multidrug efflux pumps. *Biochemistry* 45 (34), 10303-10312

Fensterle, J., Bergmann, B., Yone, C. L., Hotz, C., Meyer, S. R., Spreng, S., Goebel, W., Rapp, U. R., and Gentschev, I. (2008) Cancer immunotherapy based on recombinant Salmonella enterica serovar Typhimurium aroA strains secreting prostate-specific antigen and cholera toxin subunit B. *Cancer Gene Ther* 15 (2), 85-93

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Hotz, C., Fensterle, J., Goebel, W., Meyer, S. R., Kirchgraber, G., Heisig, M., Fuerer, A., Dietrich, G., Rapp, U. R., and Gentschev, I. (2008) Improvement of the live vaccine strain Salmonella enterica serovar Typhi Ty21a for antigen delivery via the hemolysin secretion system of Escherichia coli. *Int J Med Microbiol* accepted

Galmbacher, K., Hotz, C., Heisig, M., Wischhusen, J., Galmiche, A., Bergmann, B., Gentschev, I., Goebel, W., Rapp, U. R., and Fensterle, J. Block of tumor growth in an experimental breast cancer model by elimination of macrophages with a novel attenuated variant of Shigella flexneri. submitted

Hotz, C., Wagner, S., Fensterle, J., Goebel, W., Rapp, U. R., and Gentschev, I. Cancer Immunotherapy based on a novel recombinant *Salmonella enterica* serovar Typhimurium strain expressing epitopes of prostate-specific antigen (PSA) in surface exposed form. in preparation

VII.4.2. Patents

Recombinant bacteria with *E. coli* hemolysin secretion system and increased expression and/or secretion of HlyA, process of manufacturing and uses thereof. Patent pending (Z08-02), Æterna Zentaris (Hotz, C, inventor share 35 %)

Attenuated bacteria capable of inducing apoptosis in macrophages, process of manufacturing and uses thereof. Patent Pending (Z08-01), Æterna Zentaris (Hotz, C., inventor share 5 %)

VII.5. Curriculum vitae

PERSONAL DATA

Name	Christian Hotz
Date of birth	24.01.1979
Place of birth	Erbach/Odenwald, Germany
Nationality	German

ACADEMICAL CAREER

Since Feb. 2005	PhD at the Institute of Medical Radiation and Cell Research, University Würzburg, PD Dr. Ivaylo Gentschev. Title of thesis: "Improvement of *Salmonella* vaccine strains for cancer immune therapy based on secretion or surface display of antigens"
Feb. 2005- Jan. 2008	Student speaker of the DFG Research training group 1141/1 "Signal transduction: Where cancer and infection converge" Würzburg-Nice (GCWN)

Oct. 1999 – Dec. 2004	Study of biology at the University Würzburg Major subject: Biotechnology, minor subjects: Microbiology and Genetics Diploma thesis: „Investigation of the protein-protein crosstalk of periplasmic adapterproteins with the outer membrane channel-tunnel TolC of *Escherichia coli*.”
Dec. 2004	Diploma, Grade: “sehr gut” (1)
Aug. 2002-Jan. 2003	Semester abroad in Umeå, Sweden

SCHOOLING

Aug. 1985-June 1998	Primary school, Grammar school, Gymnasium
June 1998	Abitur, Grade: 1,9

Date Signature

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