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Target identification for prevention and therapy of *Salmonella* infections

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auf Antrag von

- Prof. Dr. Dirk Bumann
- Prof. Dr. Christoph Dehio

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Prof. Dr. Martin Spiess

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Summary

Summary

In this thesis we investigated maintenance requirements of *Salmonella* during chronic infections, and determined the relevant antigen properties that might facilitate the development of *Salmonella* vaccines. Overall, we wanted to achieve two goals:

a) To identify **suitable targets to eradicate persisting *Salmonella***.

b) Identify **antigen properties for developing enteric fever vaccine**.

To achieve the first goal we established a practical *Salmonella* mouse infection model for identifying bacterial maintenance functions essential for persistency.Using this model, we evaluated twelve *Salmonella* defects. Our data revealed extremely relaxed environment of *Salmonella* during persistency compared to the acute infection. On the other hand, we identified that unsatuarated/ cyclopropane fatty acid synthesis pathway might contain suitable targets for antimicrobial chemotherapy of chronic infections.

To achieve the second goal, we tested thirty seven *in vivo* expressed antigens for immunogenicity and protectivity in a mouse typhoid fever model. We identified novel *Salmonella* antigens that conferred partial protection against virulent *Salmonella* in a typhoid fever model. The identified antigens had high sequence conservation among several *Salmonella* serovars suggesting that these antigens might be suitable as vaccine candidates against systemic *Salmonella* infection caused by diverse serovars. Using model antigens expressed in *Salmonella* and autologous antigens, our data also revealed that surface associated antigens might be promising for inducing both humoral and cellular immunity to *Salmonella*, as recognition of such antigens might enable uniquely detection and destruction of live *Salmonella*. This may provide a strategy to discover additional protective antigens for *Salmonella* and other intracellular pathogens.

Introduction

1. Introduction

The introduction of the thesis is divided into three parts (Part A, Part B, and Part C).

Part A: General introduction to the *Salmonella* diseases (refer to section 1.1).

Part B: Mostly focuses on chronic *Salmonella* infections (refer to section 1.2).

Part C: Prevention and effective control of *Salmonella* diseases through vaccination with more focus on the urgent need for a new enteric fever vaccine
(refer to section 1.3)

Introduction

Part A – *Salmonella* diseases

1.1 *Salmonella* diseases

Enteric fever, an inclusive term for typhoid and paratyphoid fever is a human - restricted systemic infection caused by *Salmonella enterica* serovar Typhi (*S. typhi*) and serovar paratyphi (*S. paratyphi A, B,* and *C*). Unlike enteric fever, there are also a range of other clinical syndromes, including diarrhoeal disease caused by non - typhoidal *Salmonella* (NTS) that have a broad vertebrate host range. These organisms are the important causes of febrile illness where overcrowding, poor sanitation, and untreated water are the norm [1-4].

1.1.1 Global disease burden of *Salmonella* infections

It was estimated in the year 2000 that typhoid fever caused 21.7 million illnesses and 217,000 deaths and paratyphoid fever 5.4 million illnesses worldwide [2, 5]. In the United States and Europe, typhoid fever was the major cause of morbidity and mortality in the 19th century [6]. With the provision of good sewage systems and clean water, there has been a dramatic decrease in the incidence of typhoid fever in these regions. But, enteric fever still remains a public health problem in developing countries [7]. In Asia, recently a large population- based prospective study confirmed the high incidence rate of typhoid fever in China, India, Indonesia, Pakistan, and Vietnam amongst children and adolescents (Fig 1) [8].

Outbreaks of typhoid fever are frequently reported from sub –Saharan Africa with large number of patients presenting with intestinal perforations [9].

Currently, serovars of *Salmonella* other than *S. typhi* have been emerging as causes of the disease and mortality in various sub continents of the world [10, 11]. In a number of Asian countries, *S. paratyphi A* appeared to be responsible for a growing proportion of enteric fever, sometimes accounting for 50% of *Salmonella* bloodstream

isolates from enteric fever patients. In addition, NTS that had previously been associated mostly with diarrhoea increasingly causes bacteraemia and focal infections especially in sub-Saharan African countries [12, 13]. Distinct strains of serovar *Typhimurium* are associated with this emerging disease. In a current study, two main *Salmonella enterica* serovars, *S. typhimurium* and *S. enteritidis* were isolated from patients of NTS bacteraemia and gastroenteritis [13, 14]. Overall, this trend raises serious concerns about the impact of enteric fever and failures of control strategies [15, 16].

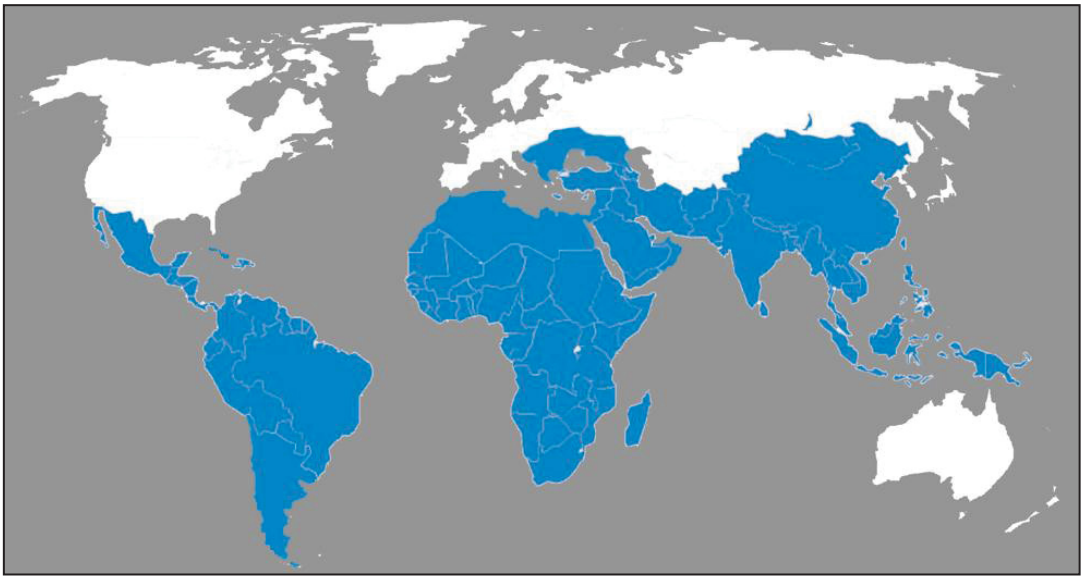

Figure 1. World map representing countries endemic for typhoid (WHO, 2009).

1.1.2 Symptoms, transmission and pathogenesis

The clinical symptom of typhoid fever includes diarrhoea, sustained fever, headache, malaise, and anorexia [17]. Approximately, 20% - 30% of patients develop rose spots on abdomen and chests [18]. Finally, gastrointestinal bleeding or perforations are usually observed during the third week of illness, but they may occur at any time. Other complications include abdominal pain, bloody stools, cholecystitis, and rarely pancreatitis [16, 19, 20]. The severity of the illness mainly depends on

immunocompetence, age, virulence of the *S. typhi* strain, and nutritional status of the patient [17]. Approximately, 1% to 4% of the patients become chronic carriers and shed *Salmonella* for years [21]. The chronic carriers of *S. typhi* although are highly contagious, but remain typically asymptomatic, making the identification of carriers difficult [22, 23].

The only source of *S. typhi* infections are humans, there are no animal reservoirs. The organisms are mostly shed in the stool and urine. The infection is mainly spread via the faecal – oral route through water or food that has been contaminated by a person with acute disease or, an asymptomatic chronic carrier [17, 24]. The chronic carriers form a reservoir for further spread of the disease through bacterial shedding in urine and faeces [21, 25].

Salmonella are introduced into the gastrointestinal tract by ingestion of contaminated food or water (Fig 2). The infection dose has been estimated to be 10^5 to 10^9 organisms [21, 26]. A certain percentage of the bacteria survive the acidic barrier in the stomach, and are internalized by the M cells (microfold epithelial cells) overlying the Peyer's patches in the small intestine. The organisms are then transported to the lymphoid tissue including the mesenteric lymph node. *Salmonella* may gain entrance into the bloodstream directly, or via the thoracic duct [21, 26, 27]. The incubation period is approximately 7 – 14 days [21]. The bacteria then disseminates through the bloodstream and lymphatics systematically to colonize liver, spleen, gall bladder, and bone marrow. Infection of the gall bladder followed by biofilm formation on the gallstones contributes to the asymptomatic carrier state. The carriers continue to periodically discharge *Salmonella* from the gall bladder and infect new hosts.

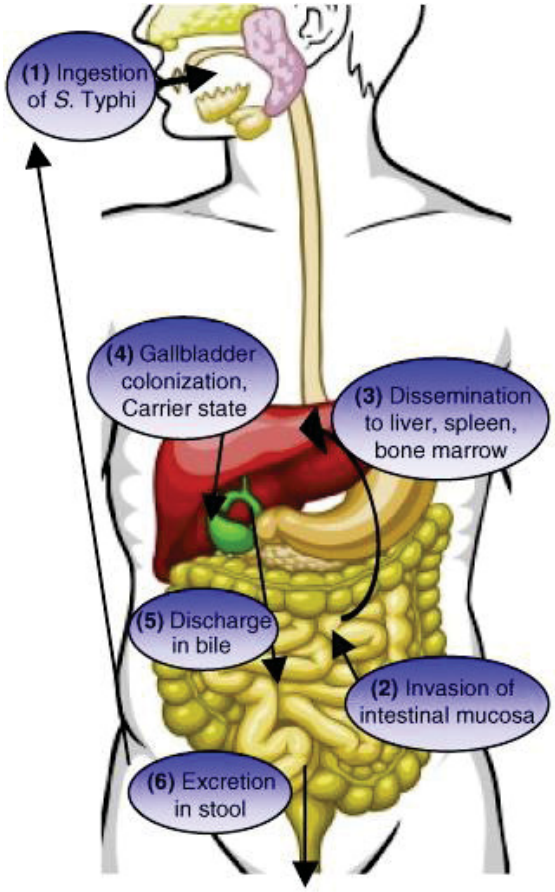

Figure 2. *Salmonella* infection cycle and persistence mechanism [28].

1.1.3 History of the disease burden

From the late 1800s to 1949, typhoid fever caused by *S. typhi* was one of the most serious health problems in the United States. Between 1895 and 1910, Philadelphia experienced three typhoid fever epidemics due to unfiltered water supplies [29]. European countries have also experienced endemic outbreaks of typhoid fever. In 1964, there were 507 cases of typhoid fever in Scotland from canned Argentenian corned beef that was cooled in contaminated river water. A 1998 Danish outbreak and 1997 French outbreak of multiresistant *S. typhi* were linked to the ingestion of contaminated pork [30, 31]. Over the past centuries, improvements in water treatment, sanitation, and food handling practices has led to the dramatic fall in the incidence of typhoid fever in the developed countries [21, 32].

Despite the availability of clean water, still in 1900 typhoid fever claimed the lives of 20,000 Americans in locations where the drinking water was known to be pure[33]. The reason were chronic carriers such as a woman named Mary Mallon, better known as "Typhoid Mary", the first healthy *S. typhi* carrier to be identified and charted in North America. An Irish born cook, who didn't have any signs of the disease, continued to infect the wealthy New York citizens to whom she provided her culinary services[34].

1.1.4 Current problems with the disease control

Although there are indications of an overall downward trend in the global incidence of typhoid, enteric fever continues to remain a major public health challenge in the developing countries [35].

One of the public health concerns in these countries is the overcrowded slums that have poor waste disposal and drainage facilities. Due, to poor sanitation conditions, contaminated food and water is common in these communities resulting in typhoid fever [36]. Secondly, knowledge about typhoid fever is still limited in the economically disadvantaged countries. Often half–measures are taken by self-medications to avoid the unaffordable costs of modern healthcare leading to misdiagnosis of the disease[37]. Thirdly, Multidrug-resistant (MDR) strains of *S. typhi* has also become a challenge in the management of typhoid fever in these endemic locations. Resistance has been noticed to all suitable drugs including trimethoprim, ampicillin, quinolones, and cephalosporins with increasing rates[35]. Upto 60% of *Salmonella* strains isolated exhibit multidrug resistance to almost every available first – line antibiotics.

Finally, despite the availability of several licensed vaccines, the use of these vaccines in endemic areas has not been as extensive as it should be resulting in the mismanagement of the disease burden [35].

1.1.5 Diagnosis and treatment of *Salmonella* infections

Diagnosing typhoid fever is difficult because the symptoms and signs overlap with other common febrile illness. After the first week of illness, stool and urine cultures may be positive however these cultures cannot distinguish acute disease from a carrier state [17, 38]. The antibiotic of choice for treating active typhoid fever is the fluoroquinolone ciprofloxacin. The drug regimen is for 7 – 10 days and has a high cure rate. Other quinolones including ofloxacin, norfloxacin, or levofloxacin and third generation cephalosporins can also be used [39-41].

Fortunately, with adequate treatment, most of the patients recover from the acute phase of typhoid but the treatment of a chronic carrier is very difficult [42, 43]. The impact of high incidence rate of typhoid fever in many parts of the world highlights the importance of understanding the mechanisms of persistent *Salmonella* infection [21]. The *S. typhi* carrier state must be completely eradicated as it is the main reason for continuous transmission of the pathogen.

Introduction

Part B – Chronic *Salmonella* infections

1.2 Antimicrobial chemotherapy of chronic *Salmonella* infections

1.2.1 *Salmonella* persistence as a problem

Bacterial persistence is the phenomenon in which a small subpopulation of the cells survives antibiotic treatment ("tolerance") [44]. In contrast to antibiotic resistance, persistent bacteria do not grow in presence of antibiotics and their tolerance arises from physiological processes rather than genetic mutations[45]. As a result, the antibiotics might not eradicate infections completely and the persistent bacteria could later cause relapse of the disease [46].

Epidemiological studies indicated that approximately 90% of chronically infected carriers have gall stones [47, 48]. The typhoid carrier state, both with and without gall stones, is implicated as a predisposing factor for the development of gall bladder cancer [21]. *S. typhi* forms biofilm on gall stones and antibiotic treatment has not proved to be effective in the resolution of chronic *S. typhi* colonization of gall bladder. The persisters are contained in the biofilm and can partially survive the antibiotic treatment. When the antibiotic therapy is discontinued, the persistent bacteria can repopulate the biofilm, producing relapse of the infection (Fig 3) [44, 49].

For patients with gall stones, cholecystectomy (complete removal of the gall bladder) increases the cure rate, but does not guarantee the eradication of the carrier state[44]. In addition, chronic *Salmonella* carriers should be treated by a four week course of a quinolone such as ciprofloxacin[50]. The long term chemotherapy results in poor patient compliance, and inability to eradicate the carrier state. Persisters, likely to be responsible for chronic infections, can be suppressed but cannot be always completely eliminated with the existing antimicrobials[44].

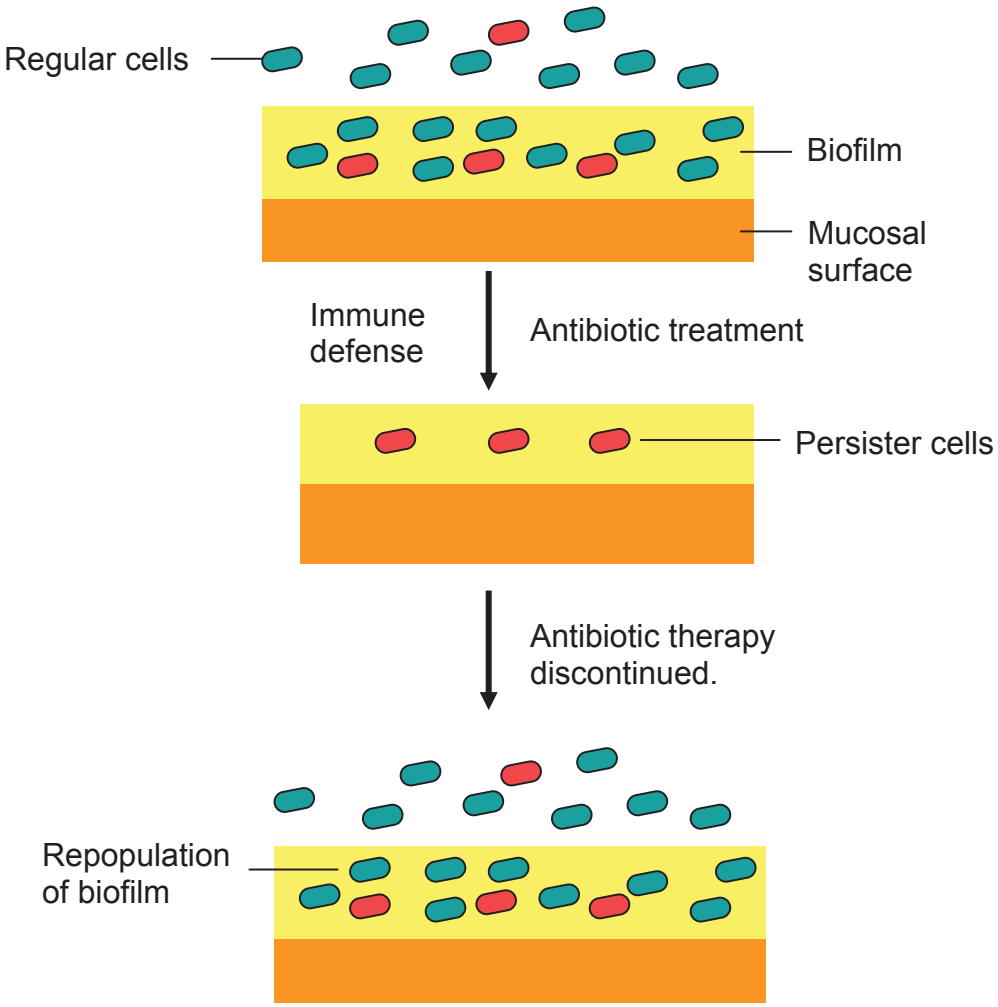

Figure 3. Treatment of biofilm with antibiotics. Initial antibiotic treatment kills most cells (green) in both the biofilm and planktonic populations. The immune system eradicates planktonic persisters but the biofilm persisters (pink) continue to stay protected within the exopolymer matrix and escape clearance by the host defense systems. After the discontinuation of the antibiotic therapy, the persisters repopulate the biofilm and the infection relapses. Adapted from [51].

1.2.1.1 Mechanisms of tolerance versus resistance to bactericidal antibiotics

Persisters often represent small subpopulation of cells that spontaneously enter a dormant, non-dividing state that is tolerant to most antibiotics [44]. Antibiotic tolerance might function by preventing target corruption by an antibiotic through blocking its target. If the persisters are dormant and do not have topoisomerase or translation activity, or have no or little cell wall synthesis, then the antibiotics will bind to, but will not be able to corrupt, the function of respective target molecule (Fig 4) [51].

In contrast to antibiotic tolerance, resistance represents the ability of cells to grow in presence of antibiotics [44]. Different types of resistance mechanisms include antibiotic modification/destruction by specialized enzymatic reactions, target modification by mutation, restricted permeability to antibiotics, antibiotic efflux, and target substitution [52, 53] . All of these mechanisms limit antibiotic efficacy and allow bacteria to grow even at an elevated concentration of antibiotics [51].

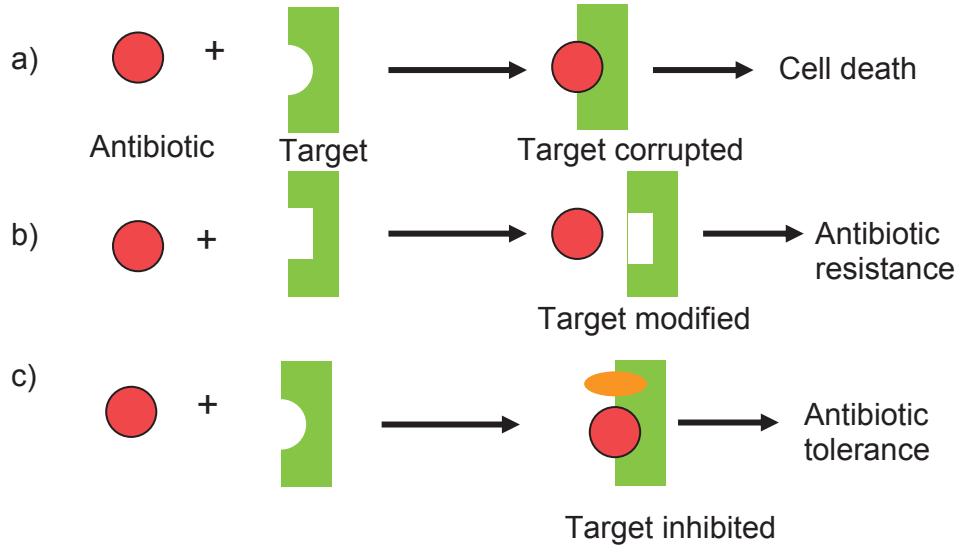

Figure 4. Tolerance versus resistance to antibiotics. **a)** The antibiotic (pink) binds to the target (green) altering its function, which causes cell death. **b)** The target of the antibiotic has been modified so that it's unable to bind the antibiotic and the cells become resistant to treatment with the drug.

c) The antibiotic target is inhibited by a different molecule (orange). This prevents the antibiotic from disrupting its function, resulting in tolerance. Adapted from [51].

1.2.2 General mechanisms of persistency

Persistency refers to the clinical observation of the infection without apparent disease.

It is very difficult to study persistency, because the bacteria are inaccessible and problematic to visualize in humans. Instead much research has been focused on a bacterial phenotype – dormancy.

The term dormancy has been much debated. Generally, dormancy is defined as a stable but reversible, non-replicating state. However, this definition covers a broad range of phenotypes that has been observed in bacteria as explained below (a,b,c):

a) In case of some bacteria, there are *in vitro* conditions that restrict replication, but allow the bacteria to remain culturable under standard conditions[54].

b) Some species can form spores, making them metabolically inactive and extremely resistant to environmental stresses [54].

c) In some cases, prolonged cultures induce a state where bacteria remain viable but non culturable (VBNC) unless they are resuscitated with the addition of exogenous factors [54].

Since, some of these phenotypes were observed in the persistent bacteria several researchers consider persisters as the dormant variants of regular cells [44].

However, another persistence strategy was proposed by Munoz Elias *et al* in *M. tuberculosis.* In chronically infected mice, persistent bacteria might represent a static equilibrium in which the bacterial cell division is very slow or non-existent [55].

This widespread notion that chronic tuberculosis is in static equilibrium, was completely contradicted by another study. The data showed that persistent *M. tuberculsosis* represents a dynamic equilibrium where the bacterial replication is

balanced by an equally rapid host mediated killing, thus maintaining steady tissue loads [56].

It is difficult to understand which of these states is relevant in human disease. Predominantly, for simplicity it has been mostly assumed that persisters might be dormant cells and due to nutrient and other limitations, do not metabolize and replicate sufficiently for the antimicrobials to function effectively.

Different mechanisms have been proposed for persister cell formation. A combination of deterministic and stochastic events is thought to give rise to persisters in a bacterial population [57-59]. This can happen through redundant independent mechanisms such as induction of TA (toxin antitoxin) modules [60, 61] and/or SOS stress responses [62, 63] .

Whatever the mechanism of persister formation, to eradicate the persistent bacteria it would be necessary to identify the "Achilles heel" of persisters – the essential maintenance functions required to survive within the host [44]. It is possible that there is actually no such "achilles heel", as completely dormant bacteria might lack any maintenance requirements but just survive without any activity.

1.2.3 Models for studying persisting pathogens

Critical maintenance requirements during persistency can be identified using gene deletion mutants in a well validated model. Various *in vitro* and *in vivo* models have been established to study the chronic infection of different pathogens. The important criteria for such models are their practicality, and their ability to closely mimic relevant *in vivo* conditions. The hypoxia [64] and nutrient starvation [65] models of latent tuberculosis are well studied and elucidated. However, these models are probably still far from the human granuloma conditions. The hypoxia induced model captures the hypoxic nature of the granuloma, but lacks the effect of macrophage

phagocytosis, immune response, and eventual release to the extracellular milieu. The nutrient starvation models are not hypoxic and it is unlikely that the host environment in which *M. tuberculosis* persists will be limiting of all nutrients [66].

Another model, the *in vitro* drug - tolerant phenotype that occurs in static 100–day old stationary phase *M. tuberculosis* cultures is worth mentioning. It has been used for screening new drugs that might be active against drug–tolerant microorganisms [67, 68]. However, the relationship between the bacteria in human TB lesions and the metabolic state of bacteria in the model has not been validated. So, none of the above mentioned *in vitro* models fully capture the complex heterogeneous of granuloma with a gradient of oxygenation and nutrients [69].

In vivo models might be more useful for analyzing persister maintenance requirements. Two mouse models have been studied in *M. tuberculosis*. In the first model, the termination of the growth of the bacilli coincides with the onset of host immune response (The Untreated Mouse model) [70, 71]. In the second one, chronic infection is established by administering antimicrobial drug combination (The Drug – Induced Cornell mouse model) [70, 72]. However, the granulomas that develop in mice are not well formed structures like human lesions [66, 73].

Several rodent models have been established to study *E. faecalis* persistency. But, the experimental conditions defined by these models are inadequate for investigation of persistency, since the bacteria are readily cleared and fail to establish chronic cystitis. These models fail to provide changes in bladder homeostasis following urinary catheterization to achieve successful infection of the urinary tract [74-77].

To study S. *typhimurium* persistence, a Nramp1^{r} mouse model was established. Nramp 1 is a natural resistance associated macrophage protein that localizes to the membrane of *Salmonella* containing vacuole, removes cations from the vacuole and in

turn controls the replication of intracellular bacteria [78]. In these mice, an initial acute disease (approximately 2 weeks) is followed by chronic infection. A microarray – based negative screen was performed to identify *S. typhimurium* genes that contribute to persistency in Nramp1^{r} mouse. A transposon - mutagenized library was used to infect mice intraperitoneally, and the disappearance of the mutants was monitored after 7, 14, 21, and 28 days post infection.

Most of the SPI 1 and SPI 2 (*Salmonella* pathogenecity island 1 and 2, which encodes for type III secretion, system) genes were required by *Salmonella* for systemic persistence. However, the contribution of many genes during persistent infection could not be analyzed as corresponding mutants already dropped out during the initial acute infection. Moreover, the final time point (day 49) that is the actual persistent stage, had to be removed from further analysis because the bacterial loads in spleen and liver were too small [79].

Taken together, despite considerable effort practical *in vivo* models are still largely lacking. There is thus a need to establish persistency *in vivo* models for identifying genes crucial for chronic phase survival that could be targeted for therapy.

1.2.4 Potential mechanisms of persistency in other pathogens

Although a well validated model is largely lacking yet, several individual maintenance function requirements during persistency have been proposed in various pathogens. It has been suggested that during persistent state, *Mycobacterium tuberculosis* performs limited protein synthesis, but may need to spend its available resources on maintaining cell wall, genome integrity, and membrane potential as well as resisting host defense. Studies indicate that *de novo* ATP synthesis is required for the viability of hypoxic non replicating mycobacteria [80]. *In vitro* data also suggested that expression of ribonucleotide reductase (encoded by *nrdZ*) was

upregulated indicating that a pool of deoxyribonucleotides is required either for the process of DNA repair or maintenance of chromosomal integrity during *M. tuberculosis* persistency [73, 81]. However, in infected mice this mutant had no apparent virulence defect [81]. Even polyphosphate metabolism seemed to be crucial in metabolic adaptation for survival in the *in vitro* models of *Mtb* persistency [82].

In *E. coli,* degradation of membrane phospholipids is important for persistent bacteria. The *FadR* regulon involved in fatty acid β – oxidation pathway seemed to provide the persistent bacteria with endogenous carbon and energy from the membrane derived fatty acids [44].

Recent data in *E. coli* also suggests that ATP generation through TCA cycle is crucial for persistency [83]. On the other hand, this result was in apparent contradiction to another study showing that decrease in ATP levels in *E. coli* sends cells into dormancy [44]. Hence, this reflects that data obtained using different model system shows differential perturbation effects.

Most of these pathways discussed above were identified in *in vitro* models and it is unclear if relevant host microenvironments in which the pathogen persists will be actually limiting for nutrients [81]. So, all these data needs to be validated *in vivo* to understand the actual maintenance requirements.

In vivo data showed that nitrate reductase (encoded by *narGHJI* genes) activity is required for maintaining redox balance in *M. bovis* during persistency. But, it is not clear why nitrate reductase activity is required for virulence in lung, liver, kidney, and not in spleen in SCID (severe combined immunodeficiency) mice [84].

Taken together, all these different studies reflect that maintenance function requirements to survive during persistency might be different for each pathogen.

Identifying such basal maintenance activities could represent attractive targets for therapy of chronic infections.

1.2.5 Goal and aims of the first part of thesis

Goal

The overall goal for this part of the thesis was to identify suitable targets to eradicate persisting *Salmonella*.

Aims

To achieve this goal I followed several specific aims:

a) Generate a relevant *Salmonella* persistency model that is practical, and mimic the *in vivo* conditions (refer to section 2.1).
b) Experimental testing of candidate target genes against persistent *Salmonella* infection (refer to section 2.1).
c) Deduce general properties of *Salmonella* persistency (refer to section 2.1).

Introduction

Part C – Prevention and effective control of *Salmonella* diseases

1.3 Prevention and effective control of *Salmonella* diseases

Public awareness regarding proper food preparation, hand washing, safe water supply, and control of flies is essential for preventing typhoid fever. Also typhoid carriers must be excluded from handling food and caring for patients as an additional measure of the prevention strategy [17].

To further reduce the impact of typhoid fever efficacious vaccines are needed (Fig 5). Vaccination is considered as the most cost–effective tool for the prevention of infectious diseases, and could offer long term solution against infection.

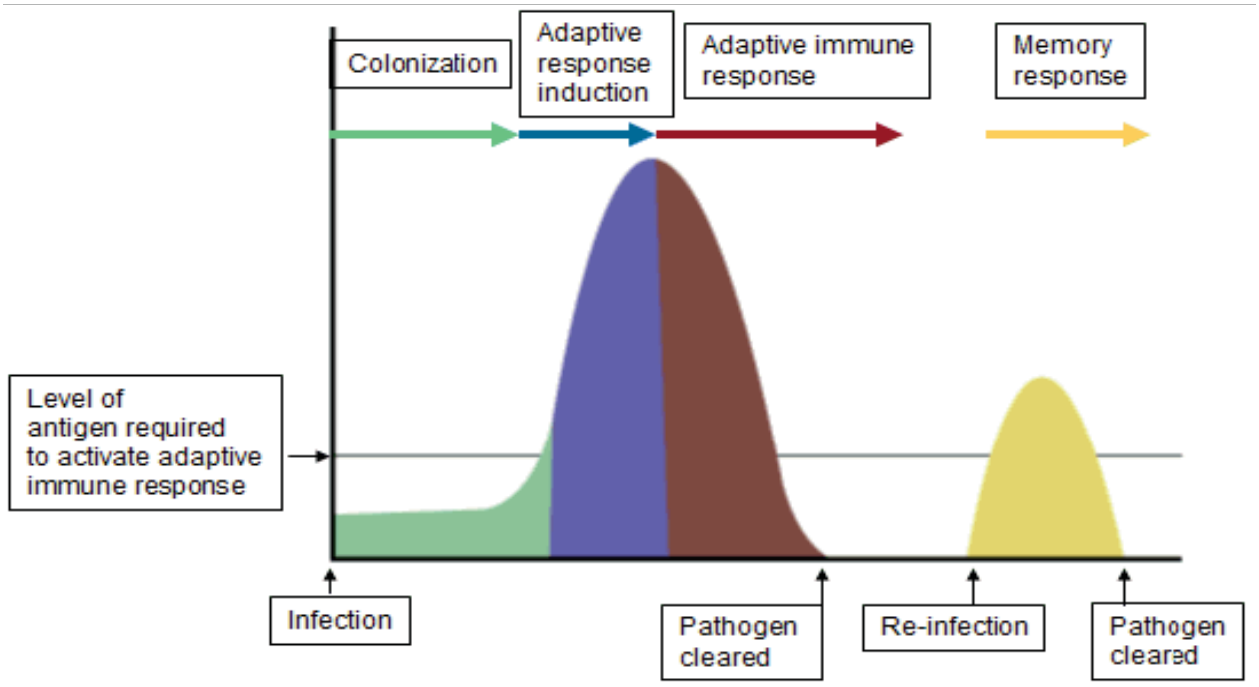

Figure 5. Time course of immune response after vaccination that leads to the formation and maintenance of active immunological memory.

1.3.1 Currently available vaccines and their disadvantages

Different approaches have been pursued to develop vaccines against typhoid fever. This includes inactivated whole cell vaccines, live attenuated vaccines or subunit vaccines (Table 1)

	Ty21a vaccine	Vi polysaccharide vaccine	Vi conjugate vaccine
Type	Live	Subunit	Subunit
Route of Administration	Oral	IM/SC	IM/SC
Doses/regimen	3	1	1-3
Revaccination	5-7 years	3 years	Min 4y-max life
Efficacy	50-80%	50-80%	Upto 90%
Duration of Efficacy	62% at 7 years	55% at 3 years	90%
Cross protection against paratyphoid, NTS	Yes	No	No

Table 1. Characteristics of current typhoid fever vaccine. Adapted from [5].

1.3.1.1 Inactivated whole cell vaccines

The parenteral whole cell typhoid vaccines were obtained by inactivating virulent microorganisms with heat or chemicals. The first typhoid vaccine mainly consisted of inactivated *S. typhi* by heat and phenol or by acetone [85]. Its protection efficacy ranged from 51% to 88% in children and young adults and the effect lasted up to 7 years [86-88]. These parenteral vaccines containing mixture of different serovars were able to confer broad protection against typhoid fever. However, their global use for

routine vaccination was discontinued due to high reactogenicity. It caused severe local pain (upto 35% of the recipients), headache (10%) and fever (6 – 30%) after immunization [28, 89]. Hence, the whole cell vaccine was replaced by oral live attenuated, and Vi –based parenteral vaccines.

1.3.1.2 *S. typhi* Ty21a live oral vaccine

Ty21a is an attenuated mutant strain of *S. typhi* Ty2 that has a GalE-and Vi- negative phenotype [90]. Galactose residues are an important component of the smooth LPS O-antigen in *S. typhi*. When grown in absence of galactose, Ty21a expresses rough O-antigen and is non-immunogenic [91]. *galE* gene encodes for uridine diphosphate (UDP)–galactose–4–epimerase which is responsible for the conversion of UDP–glucose to UDP–galactose and vice versa. Due to deficiency of this enzyme, UDP galactose cannot be metabolized and accumulates in the cytoplasm causing cell lysis and attenuation [85]. Further spontaneous mutations in the *via* and *ilvd* genes resulted in the loss of Vi capsular polysaccharide, and an auxotrophic phenotype for isoleucine and valine repectively [92]. An additional mutation in the *rpoS* gene, inherited from the wild type parental Ty2 also contributed to the avirulence of the Ty21a strain [93]. Partly from the *rpoS* mutation, Ty21a has poor capacity to survive starvation conditions, and various environmental stresses [94].

Ty21a vaccine induces strong $CD4^+$ T helper type 1 and $CD8^+$ T cell immune response. Ty21a vaccine has excellent tolerability and protective efficacy of 67% - 80% (applying three doses of enteric–coated capsule or liquid formulation).

Ty21a has the drawbacks of requiring multiple doses and being cold–chain dependent, both factors that impairs its widespread use in endemic areas [85, 95]. Moreover, this vaccine cannot be used in children under 2 years of age, an age group that is significantly affected by the disease. It also has only moderate protection

against *S. paratyphi* A, B, and NTS strains that were recently found to be associated with multidrug resistance [96].

1.3.1.3 Novel attenuated *S. typhi* strains

The need to administer multiple doses to elicit a protective immune response is viewed as a drawback of the Ty21a vaccine. So, novel attenuated *S. typhi* strains that may serve as a single dose, oral typhoid vaccines, were developed as an alternative to the Ty21a strain.

The most extensively evaluated metabolically attenuated strains were mutants deficient in the biosynthesis of aromatic amino acids (*aroA*, or *aroC*, and *aroD*), and purines (*purA*, and *purE*). In the CVD 908 strain (Ty2 *aroC aroD*) mutation in the *aro* genes lead to an auxotrophy for aromatic amino acid causing strong attenuation [85, 97]. Although this vaccine was well tolerated, and highly immunogenic, CVD 908 resulted in silent vaccinemia (viable organisms in blood) in a proportion of subjects. Due to this reason, development of CVD 908 was discontinued [85, 98]. Other attenuated strains like aroC *aroD* derivative of the serovar typhi isolate ISP1820 (strain CVD 906), and *aroA aroD* (strain PBCC211) or *aroA aroD htrA* (strain PBCC222) derivatives of CDC10-80 were also found to cause vaccinemia, fever, and other adverse reactions [98]. The *cya crp* mutant also caused vaccinemia and adverse reactions [99]. More heavily attenuated strains were also tested but found to induce poor immune response in vaccinees (e.g *pur* and *gua* mutations) [100]. The general problem associated with the attenuated live bacterial vaccine is the frequent rate of shedding, and the risk of horizontal gene transfer [101]. Live attenuated *Salmonella* vaccines like *aroA* mutants or *htrA* mutants caused lethal infections in mice deficient in T cells, IL-12, or IFN-γ. Therefore, live attenuated vaccines could be dangerous for immunocompromised individuals (HIV) [102].

The recently developed M01ZH09 (also referred to as ZH9) lacks the *aroC* and *ssaV* (a key component of the SPI 2 type III secretion system machinery) genes. This strain was found to be safe and highly immunogenic in phase I clinical trial and is considered to be a future vaccine [103].

1.3.1.4 Vi – based subunit vaccine

Compared to live attenuated vaccines, subunit vaccines contain only few, broadly conserved *Salmonella* components and cannot cause infection even in heavily immunocompromised individuals.

The only currently used subunit vaccines contain purified Vi capsular polysaccharide of *S. typhi*. It consists of (α–1-4),2–deoxy–2–N–acetyl galacturonic acid, which is partially O–acetylated at carbon 3 and forms a capsule that protects the bacteria against lysis and phagocytosis [104, 105] . The protective efficacy of these vaccines is approximately 64% - 72% and lasts up to 17–21 months [85]. The most important drawback of simple Vi polysaccharide vaccines is its poor efficacy in infants and toddlers. And immune responses against polysaccharide donot involve T cells, so immunological memory was not established [106]. These limitations were recently overcome by linking T cell independent Vi polysaccharide antigen to a T–dependent protein carrier molecule that resulted in T–dependent conjugate vaccine. For *S. typhi* conjugate vaccine, covalent binding of Vi polysaccharide to recombinant *Pseudomonas aeruginosa* exotoxin A elucidated higher and more sustained IgG antibody response than pure Vi polysaccharide [106]. It also showed high seroconversion in vaccinees in endemic and non-endemic areas (a fourfold rise in anti – vi antibodies is defined as seroconversion) [107].

However, *S. paratyphi* A and B, as well as NTS all lack the capsular Vi antigen. Therefore, the Vi polysaccharide vaccines is ineffective against these bacterial strains with rapidly increasing clinical importance.

The limitations and drawbacks of all the available vaccines emphasize the urgent need of effective and affordable enteric fever vaccines with broad serovar coverage [108, 85].

1.3.2 Immune response against *Salmonella* infection

In order to design specific vaccination strategies against *Salmonella*, it is important to have an increasing knowledge on the functionality of the immune system and the cells involved in generation of an immune response.

A schematic representation of infection with *S. typhi* in mice is shown in Fig 6. The innate immune response plays an essential role in the early response to *Salmonella* infections. The initial stages of *Salmonella* infection are characterized by recruitment and activation of phagocytic macrophages and neutrophils [109]. This is followed by production of large amounts of proinflammatory cytokines (TNF–α, IL–1, IL– 6, IL–8, IL–12, IFN–γ) as well as macrophage migration inhibitory factor, and iNOS [110, 111] by a variety of cells as a reaction to the toll receptors (flagellin, LPS, lipoproteins) and bacterial effectors of the type III secretion system [112, 113]. Due to this primary line of defense, many but not all *Salmonella* are eliminated [114].

For effective control and eradication of the bacteria from tissues, activation of acquired immunity through generation of *Salmonella* specific lymphocytes is essential [109].

1.3.2.1 Importance of T cells for immunity to *Salmonella*

Evidence for a crucial role of T cells is provided by the high susceptibility of mice deficient in αβ T cells [115] and HIV infected individuals with low $CD4^+$ T cell counts [116]. In most experiments, $CD4^+$ T cells appeared more important than $CD8^+$ T cells [115, 117]. Depletion of $CD4^+$ T cells in mice had more pronounced effect on the control of *Salmonella* infection, and also on protection induced by vaccination of attenuated *S. typhimurium* strain. Transfer of $CD4^+$ T cells from vaccinated mice to naive recipients resulted in better protection compared to transfer of $CD8^+$ T cells [115, 117, 118]. Additionally, mice deficient in MHC class II that are $CD4^+$ T cells deficient showed increased susceptibility to *Salmonella*, reflecting the crucial role of $CD4^+$ T cell**s** [119].

$CD4^+T_{H1}$ cells produce IFN–γ and TNF–α and activate cellular immunity, whereas TH2 cells produce IL-4, IL–5, IL–13 that activate and differentiate B cells [114]. A number of studies indicated that *Salmonella* infection induces predominantly T_{H1} responses [118, 120]. Importantly, mice deficient for IFN–γ receptors are highly susceptible to *Salmonella* infection [119]. This supports the crucial role of $CD4^+$ T_{H1} derived IFN–γ for immunity to *Salmonella*.

1.3.2.2 Role of antibodies and B cells for immunity to *Salmonella*

During different stages of infection, antibodies can perform several functions of protection [109]. Evidence from humans and animal studies suggest that infection caused by non-typhoidal *Salmonella* causes severe bacteremia when specific antibodies from different B cell subsets are lacking [121]. *Salmonella* infection also results in potent antibody response particularly against LPS [122] but these are actually detrimental for protection. A recent study indicated that excess inhibitory

antibodies against *Salmonella* LPS resulted in impaired immunity against NTS bacteremia in HIV patients [123].

In the intestinal lumen, IgA and IgM antibodies could block penetration of *Salmonella* into deeper tissues. Injection of B cell hybridoma producing *Salmonella* specific IgA prevents against *Salmonella* oral infection in mice [124].

The contribution of B cells to *Salmonella* immunity has been always controversial providing conflicting results after induction of passive immunity by transfer of serum to naive mice [114]. But, a recent review stated that B cells and antibodies contribute to protection against secondary infection in mice and also in humans. For example, in vaccinated mice antibodies contribute to control in the very early stages of infection [125].

Experiment with mice mutated in *Igμ* gene (Igh–6 $^{-/-}$) which are deficient in B cells, showed increased susceptibility to *Salmonella* infection and were unable to mount strong immune response when infected with *S. typhimurium aroA* vaccine [126]. This study indicated B cells are required for acquired resistance[114]. But, other than just producing *Salmonella* specific antibodies, B cells are also involved in the engenderment and expansion of anti–*Salmonella* Th1 cells. Further, work in chimeric mice also revealed the early (innate) and late (cognate) contributions of B cells to Th1 programming [125, 127].

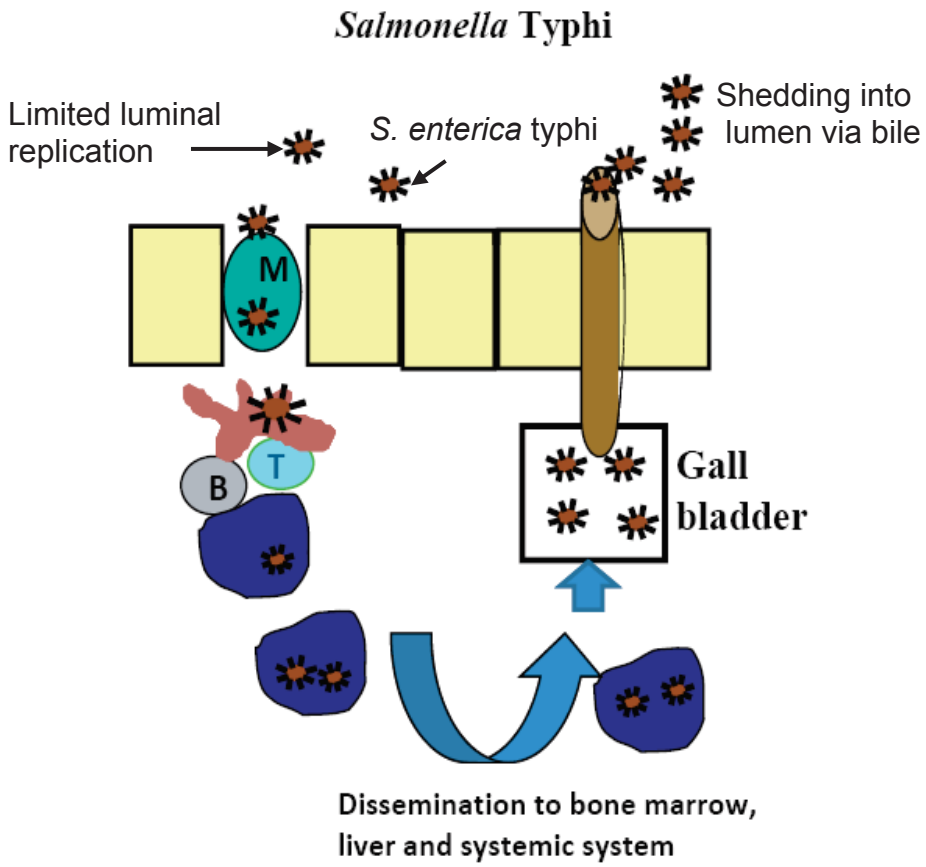

Figure 6. Schematic representation of infection with *S. typhi*. Bacteria enter by invading M cells. This is followed by inflammation and phagocytosis of the bacteria by neutrophils, macrophages, and recruitment of T and B cells. *Salmonella* targets specific type of host cells like dendritic cells and/or macrophages that favors dissemination into deeper tissues (spleen, bone marrow, liver, gall bladder). Periodic reseeding of the mucosal surface via the bile duct takes place and shedding can occur from the mucosal surface. Adapted from [125].

1.3.3 Antigen parameters important for generating protective immune responses

It is clear that both humoral and cellular immune responses are important for protection against *Salmonella* infection [125]. In particular, $CD4^+$T cells are crucial for immunity to *Salmonella* [128]. Unfortunately, only few antigens can be recognized by protective $CD4^+$T cells, and their identification thus requires major resources. Identifying relevant antigen properties could help to narrow down the

search to only the most promising candidates. Indeed, several criteria like sequence conservation across *Salmonella* serovars, *in vivo* expression, immunogenicity, and surface localization of the antigens have been put forward to prioritize candidates for novel antigen identification.

High sequence conservation across different serovars of *Salmonella* could represent a potentially relevant antigen property especially to identify vaccine candidates with broad serovar coverage. The availability of complete genome sequences of microorganisms in combination with comparative genomics has helped to compare related bacterial pathogens and identify conserved genes amongst them. Indeed, this property was utilized for identifying protective antigens against *Neisseria meningitides* serogroup B [129].

Expression of antigens during an infection could be another antigen property that can affect $CD4^{+}$T cell responses. *In vivo* expression is essential for antigen recognition and protective immunity. The small minority of highly expressed antigens might be preferentially recognized by the host's immune system, and testing of this small subset of attractive antigens could allow rapid identification of protective antigens [128]. *In vivo* expression can be deduced from several approaches like *in vivo* expression technology (IVET), signature–tagged mutagenesis (STM), differential fluorescence induction (DFI), *in vivo* induced antigen technology (IVIAT), and transcriptional and proteomic profiling [130, 131] .

Other than antigen expression, **antigen immunogenicity** can be another prerequisite for an antigen to stimulate antibody and $CD4^{+}$ T cells response. Sera from covalescent individuals who have survived infection can demonstrate the antigens that were expressed *in vivo* and recognized by the immune system. This can be deduced from approaches like Serological proteome analysis (SERPA). Interestingly, this approach

has been utilized to identify T and B cell antigens of *Chlamydia trachomatis*. However, many immunodominant antigens in covalescent individuals lacks protective efficacy [132].

Antigen localization is another antigen property that can affect CD4+T cell responses. Secreted or surface associated antigens might induce strong cellular immune response because of efficient antigen processing, and/or their association with classical inflammatory molecule lipopolysaccharide that provokes strong humoral and cellular immune response. Indeed, partially protective antigens FliC (flagella) and SseB (needle complex of type III secretion system) [128] are part of surface structures. Another surface protein, the outer membrane porin OmpD, might represent candidate vaccine target against non-typhoidal salmonellosis [121]. Recent reports have also indicated that antibodies against *S. typhimurium* other outer membrane proteins (OmpF, OmpC) might protect against *Salmonella* in the mouse model [123].

However, evidence supporting the relevance of all these antigen properties is still rather weak because antigens with different properties have not yet been systematically compared in immunization experiments.

If relevant antigen properties would be known, then reverse vaccinology could be employed as a fast way of identifying protective antigens (Fig 7). This genome based approach uses *in silico* tools to predict novel antigens based on criteria such as predicted localization from the genome sequence of the bacteria, virus, or pathogen of interest and subsequently confirming their activity by experimental biology [133, 134].

Areas in reverse vaccinology such as functional (transcriptomics and proteomics) genomics, structural genomics have the potential to identify promising vaccine

candidates based on antigen properties like *in vivo* expression level, and/or localization from the transcription, and expression profiles of the selected antigens/proteins.

Pan–genomic reverse vaccinology, comparative genomics, immunoinformatics can predict novel candidates based on criteria like high sequence conservation from the genome sequence of the pathogens [133].

But till date, reverse vaccinology has been useful for identifying vaccine candidates that induces serum antibodies and B cells. Identification of T cell antigens has been technically challenging, and has not been readily feasible with this approach because relevant antigen properties for candidate selection are unclear [133].

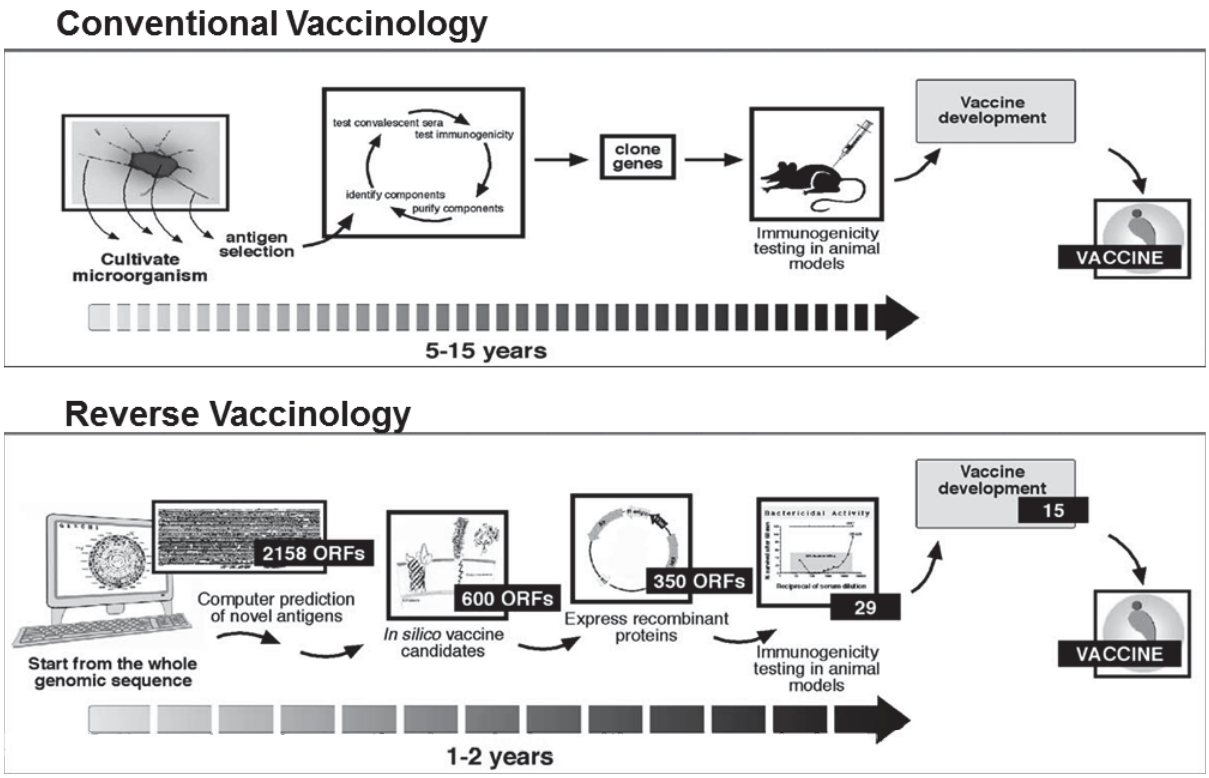

Figure 7. Comparison of conventional vaccinology with reverse vaccinology. Schematic representation showing different steps from *in silico* analysis to vaccine development in reverse vaccinology[135].

1.3.4 Goal and aims of the second part of thesis

Goal

None of the currently available licensed vaccines protect against all important serovars of *Salmonella*, and all of these vaccines also have other limitations. For developing novel vaccines, identifying protective antigens is an important bottleneck. Unraveling antigen properties that are important for generating protective immune response might help to more efficiently identify the rare suitable antigens. So, the goal of this part of the thesis was to identify predictive antigen properties that could help to develop a safe, enteric fever vaccine with broad serovar coverage.

Aims

To achieve this goal, two specific aims were followed:

a) Comparison of diverse *Salmonella* antigens in immunization/challenge experiments (refer to section 2.2).
b) Identification of relevant antigen properties that might be predictive for protectivity (refer to section 2.2).

Results

2. Results

The results section is divided into two parts (Part A and Part B).

Part A: Research Publication I – Extensive in vivo resilience of persisting *Salmonella* (refer to section 2.1).

Part B: Research Publication II – Immunity to intracellular *Salmonella* depends on surface associated antigens (refer to section 2.2).

Results

Part A – Extensive in vivo resilience of persisting *Salmonella*

2.1 Extensive in vivo resilience of persistent *Salmonella*

PloS one, 2012, 7(7), e42007

Somedutta Barat, Benjamin Steeb,Alain Maźe, Dirk Bumann.

2.1.1 Abstract

Chronic infections caused by persisting pathogens represent a tremendous health problem throughout the world. Such infections are often difficult to treat because it is largely unknown whether the persisters have any maintenance requirements for survival. We have established a simple, practical, and stringent mouse model to identify *Salmonella* maintenance functions that are essential for persistency. Our data revealed extremely relaxed requirements for *Salmonella* during persistency compared to acute infection. Only inactive biosynthesis of unsaturated/cyclopropane fatty acid resulted in clearance to non-detectable tissue loads within few days. This clearance kinetics was faster compared to the currently recommended antimicrobial drug enrofloxacin for chronic salmonellosis suggesting that unsaturated/cyclopropane fatty acid pathway might contain suitable targets to treat chronic *Salmonella* infections.

Statement of my work

I contributed to this work by generating all the data in the figures except the *in silico* analysis.

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PLoS one

Extensive In Vivo Resilience of Persistent *Salmonella*

Somedutta Barat, Benjamin Steeb, Alain Mazé, Dirk Bumann*

Focal Area Infection Biology, Biozentrum, University of Basel, Basel, Switzerland

Abstract

Chronic infections caused by persistent pathogens represent an important health problem. Here, we establish a simple practical mouse *Salmonella* infection model for identifying bacterial maintenance functions that are essential for persistency. In this model, a substantial fraction of *Salmonella* survived even several days of treatment with a potent fluoroquinolone antibiotic indicating stringency of the model. Evaluation of twelve metabolic defects revealed dramatically different requirements for *Salmonella* during persistency as compared to acute infections. Disrupted synthesis of unsaturated/cyclopropane fatty acids was the only defect that resulted in rapid *Salmonella* clearance suggesting that this pathway might contain suitable targets for antimicrobial chemotherapy of chronic infection.

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* E-mail: Dirk.Bumann@unibas.ch

Introduction

Persistent pathogens represent a major problem for control of infectious diseases [1]. Extensive drug tolerance of persisters to all available antimicrobials often leads to treatment failures and relapse. Persistent pathogens may adopt a non-replicating dormant stage with no requirement for macromolecular synthesis comprising most current antimicrobial targets [2]. As a consequence, such dormant stages are tolerant to most antibiotics. In addition, low metabolic activity during dormancy might minimize vulnerability to perturbation. In fact, it remains unclear if dormant persisters have any essential maintenance requirements for survival that could provide opportunities for eradication through antimicrobial chemotherapy.

Various in vitro models have been used as an approximation of chronic infection with dormant persisters. Data obtained with these models revealed differential perturbation effects depending on the particular model and the respective pathogen [3,4,5,6,7,8]. As an example, proton motive force-driven ATP synthesis has been shown to be essential for *Mycobacterium tuberculosis* survival in a hypoxia in vitro model [9]. Indeed, inhibition of ATP synthase accelerates mycobacterial eradication in patients [10]. On the other hand, diminishing ATP levels can actually promote *E. coli* in vitro persister formation [1]. Additional in vivo models could help to compare persister maintenance requirements under relevant conditions. However, except for *Mycobacterium tuberculosis*, practical in vivo persistency models are largely lacking.

Salmonella enterica can cause diarrhea or systemic disease called typhoid/paratyphoid fever. A substantial fraction of systemically infected individuals develops asymptomatic chronic infection [11,12]. In many cases, *Salmonella* persists in biofilms on gallstones but persisting *Salmonella* have also been detected in liver [13] and lymph nodes [14]. Surgical removal of gallstones is required for successful treatment of *Salmonella* in gallstones biofilms, while extended treatment with potent fluoroquinolone antibiotics is recommended for treatment of chronic *Salmonella* tissue colonization [15].

In genetically resistant mice, *Salmonella* cause an acute infection with exponential *Salmonella* proliferation. However, after *Salmonella* peak colonization and partial clearance *Salmonella* persist at low levels and this can cause relapses [14]. Genetic screens have identified some factors that might support chronic *Salmonella* survival in this model [12]. However, *Salmonella* mutants defective for genes relevant during the initial acute phase would be lost early on without reaching persistency. This problem could be circumvented using inducible gene cassettes but this is impractical for testing many candidate genes.

In this study, we used a simple chronic mouse *Salmonella* infection model in which a substantial *Salmonella* subpopulation survived without previous exponential proliferation. Interestingly, *Salmonella* survived even prolonged treatment with a fluoroquinolone antibiotic thus mimicking treatment failures. In this stringent in vivo model, almost all tested *Salmonella* activities were dispensable confirming extensive resilience of persistent pathogens against perturbation. On the other hand, the data also revealed a few novel candidate targets that could be explored for their suitability to control chronic infections.

Results

Persistency Model using *Salmonella purA ssaGH*

Wildytpe *Salmonella* SL1344 grew exponentially in spleen of infected genetically susceptible BALB/c mice (Figure S1A). To generate a practical *Salmonella* persistency model, we constructed a *Salmonella* SL1344 derivative that survived but largely failed to proliferate in systemically infected mice. Specifically, we combined two mutations that had previously been shown to impair *Salmonella* in vivo growth: *purA* which blocks adenosine biosynthesis [16], and *ssaGH* which inactivates the SPI-2 (*Salmonella* pathogenicity island 2)-associated type three secretion system required for intracellular

Salmonella growth and virulence [17]. Both *purA* and SPI-2 mutations have previously been shown to result in long-term persistence with minimal acute virulence, but our initial characterization revealed some in vivo proliferation of the individual mutants after i.v. administration (Figure S1B). In contrast, the double mutant *Salmonella purA ssaGH* was initially largely cleared from spleen and liver (Figure S1C) consistent with early killing during acute salmonellosis [18], but maintained largely constant bacterial tissue loads thereafter (Fig. 1A, B) suggesting limited net growth.

To determine the suitability of this model for evaluating antimicrobial targets during persistency, we treated infected mice with the antibiotic enrofloxacin. This antibiotic belongs to the fluoroquinolone class, which is uniquely effective against non-growing bacteria in vitro [19], and the treatment of choice for human persistent salmonellosis although effective therapy might require several weeks of treatment [15]. Enrofloxacin has been shown to be well absorbed after oral administration, with penetration into all tissues [20]. Indeed, enrofloxacin is the most effective drug in the mouse typhoid fever where it diminishes wildtype *Salmonella* loads in spleen and liver to levels below the detection threshold within one to two days of treatment although relapses occur unless treatment is continued for several days indicating some residual *Salmonella* persistence [21,22]. In our persistency model, the same enrofloxacin treatment initially diminished spleen loads of *Salmonella purA ssaGH*, but in contrast to previous findings for wildtype *Salmonella*, a substantial surviving subpopulation of *Salmonella purA ssaGH* stabilized within two days and remained clearly detectable during at least four days of treatment (Fig. 1A). Liver loads continuously decreased during prolonged treatment suggesting somewhat different *Salmonella* physiological states and/or differential pharmacokinetics in the two host tissues. We determined MIC (minimal inhibitory concentration) values of the inocculum and ten clones recovered from spleen and liver of two different mice after four days of enrofloxacin treatment. All clones were enrofloxacin sensitive with the same MIC value of 0.06 mg l^{-1} indicating that *Salmonella* persisted because of partial tolerance or limited antibiotic availability, but not emergence of resistant mutants. The substantially increased persistence of *Salmonella purA ssaGH* during enrofloxacin treatment indicated that our model offered a practical approach to study treatment failures during persistency. Enrofloxacin efficacy also provided a suitable benchmark for potential new *Salmonella* persistency targets.

Salmonella Defects with Minor Persistency Phenotypes

Only a small number of *Salmonella* genes are absolutely essential for *Salmonella* survival and growth in host tissues during acute salmonellosis [23]. Some of these genes might also be relevant for *Salmonella* persistency. To test this hypothesis we transduced 12 mutations into the parental *Salmonella purA ssaGH* strain, and determined persistence capabilities of the resulting strains in competitive infections with mixtures with the parental strain. At day 7 post infections, most strains had small colonization defects compared to the parental strain as indicated by competitive indices that were close to 1. These data suggested that most tested genes had only minor impact on *Salmonella* persistency in our model despite their crucial importance during acute infections (Fig. 2).

As an example, *ubiC* encoding chorismate lyase is required for ubiquinone biosynthesis. During acute infection, *Salmonella ubiC* is completely cleared from infected mice within one day indicating absolute essentiality [23]. In striking contrast, *Salmonella purA ssaGH ubiC* survived at high levels indicating dispensability of ubiquinone-mediated oxidative respiration during persistency. Similarly, functional ATPase is essential for acute *Salmonella* virulence [24], but we found it to be fully dispensable during persistency. This was in striking contrast to various *Mycobacterium tuberculosis* models that suggest ATPase to be a particularly attractive target for this pathogen [9,10]. Another case with strikingly different relevance in acute [25] vs. persistent *Salmonella* infections was *recA* involved in DNA repair. More expectedly, *trxA* encoding a thioredoxin essential for SPI-2 function [26], had no detectable role in *Salmonella purA ssaGH* presumably because SPI-2 was already inactive in this strain.

Polyphosphate biosynthesis or fatty acid degradation were known to be largely dispensable during acute infection but had

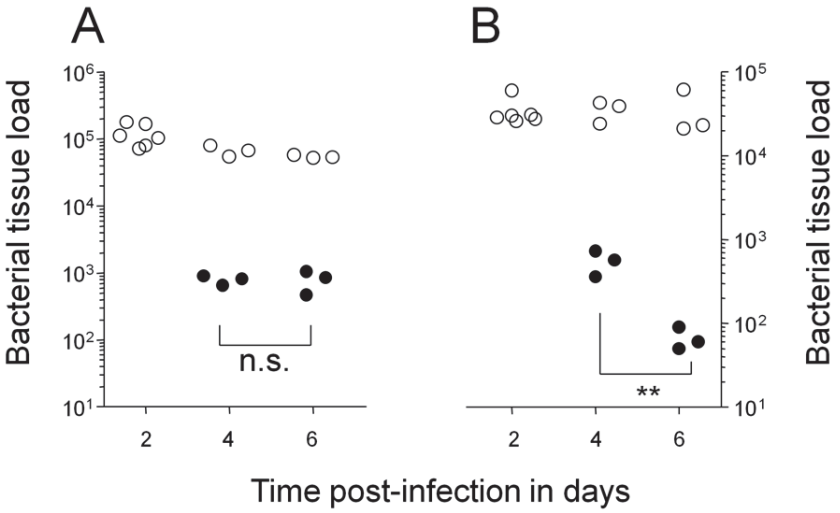

Figure 1. Colonization kinetics of *Salmonella enterica* serovar Typhimurium *purA ssaGH* in systemically infected BALB/c mice. Data are shown for spleen (**A**) and liver (**B**) of individual untreated mice (open circles), and mice that were treated from day two post infection with enrofloxacin (filled circles). Statistical significance of clearance at day 6 compared to day 4 were determined by t-test of log-transformed data (**, $P<0.01$; n.s., not significant).
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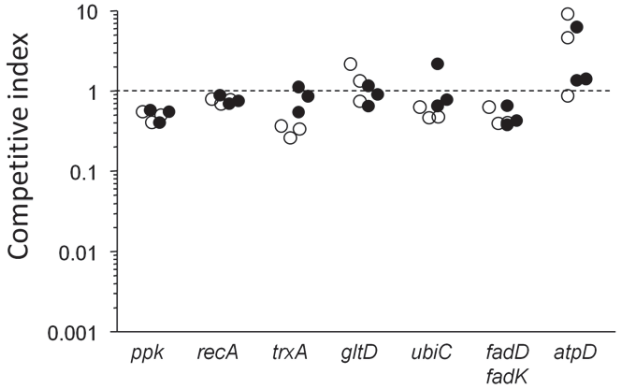

Figure 2. Competitive indices (CI) of various *Salmonella* mutants vs. the parental *Salmonella purA ssaGH* strain in infected spleen (open circles) and liver (filled circles). Data are shown for individual mice at day seven post infection. A competitive index of 1 indicates equal colonization capabilities of mutant and parental strains. Statistical significance was determined by t-test of log-transformed data. Spleen colonization of mutants *ppk, recA, trxA, ubiC*, and *fadD fadK* was significantly lower compared to the parental strain ($P<0.05$). Liver colonization of mutants *ppk, recA,* and *fadD fadK* was significantly lower compared to the parental strain ($P<0.05$).
doi:10.1371/journal.pone.0042007.g002

some role in other chronic *Salmonella* infection models [27,28]. However, in our stringent model both activities had weak effects indicating their dispensability for persistency. All these negative results suggested a severely limited number of suitable targets for control of persistent *Salmonella* infections.

Salmonella Defects with Moderate Persistency Phenotypes

In contrast to all these cases, two mutations, *asd* and *gutQ yrbH*, showed moderate phenotypes in our model (Fig. 3). *asd* encoding aspartate semialdehyde dehydrogenase is required for biosynthesis of the cell-wall peptidoglycan component diaminopimelic acid. A *Salmonella asd* strain spontaneously lyses in vitro and is completely cleared within one day from systemically infected mice [23]. However, *Salmonella purA ssaGH asd* was only partially cleared during the first day post infection which might reflect residual proliferation of some *Salmonella* and/or difficulties in establishing a suitable systemic niche [18]. Thereafter, this strain persisted at slowly declining levels in spleen. This could reflect non-essentiality of cell-wall synthesis for non-growing bacteria [19]. In contrast, liver loads rapidly declined suggesting a substantial fraction of *Salmonella purA ssaGH* with active cell-wall turnover/growth in liver. Similarly, *Salmonella purA ssaGH gutQ yrbH* that required supplementation with the lipopolysacharide precursor arabinose-5-phosphate to grow in vitro [29] and was highly attenuated during acute infections (our unpublished data), maintained high levels in spleen but was cleared from liver suggesting limited lipopolysaccharide demands during *Salmonella* persistency. Both genes thus were unsuitable as targets.

Two additional mutants had very severe colonization defects but still maintained stable small loads indicating non-essentiality for seven day persistence. *Salmonella purA ssaGH ribB* defective for 3,4-dihydroxy-2-butanone 4-phosphate synthase which is involved in riboflavin biosynthesis, was cleared within one day post infection to very low levels in both spleen and liver, but stabilized thereafter particularly in liver. This might reflect differential availability of host riboflavin supplementation in these two tissues. Importantly, these data showed that *Salmonella* with defective riboflavin biosynthesis can survive in vivo for extended periods. Another strain that was rapidly cleared from spleen had a defect in *iscS* encoding cysteine desulfurase involved in repair of iron-sulfur clusters and tRNA modification [30]. This mutant also dropped to very low loads in liver but still maintained detectable loads at seven days post infection.

β-ketoacyl-ACP Synthase I Essentiality for *Salmonella* Persistency

Finally, there was a single mutant with a more promising phenotype (Fig. 4A). *Salmonella purA ssaGH fabB* defective for β-ketoacyl-ACP synthase I required for biosynthesis of unsaturated fatty acids and cyclopropane fatty acids, was progressively cleared from both liver and spleen. During clearance, residual *Salmonella purA ssaGH fabB* were recovered from mice mostly as small-colony variants. Withdrawal of fatty acid supplementation in vitro similarly enriched small-colony variants of this strain (Fig. 4B), suggesting that reduced growth and metabolism might enhance survival of this mutant when external fatty acids are unavailable. However, even small-colony variants were rapidly cleared from mouse tissues. Small colony variants usually reflect decreased growth rate which can be caused by diverse *Salmonella* defects such as dimished respiratory activity [31]. Elucidation of the actual mechanisms that caused our SCV's was difficult because small-colony variants of *Salmonella fabB* generated in vivo or in vitro quickly reverted to fast growth upon sub-culturing in presence of oleic acid supplementation.

To test the suitability of this target for antimicrobial chemotherapy, we administered thiolactomycin [32], a slow onset inhibitor of β-ketoacyl-ACP synthase I that is effective in mouse infection models with extracellular pathogens [33]. However, safe doses did not diminish *Salmonella purA ssaGH* loads in spleen (data not shown). This could reflect the low target affinity of this inhibitor and/or poor delivery to *Salmonella* that persist intracellularly in infected macrophages [14,21].

Discussion

Chronic infections represent a major health problem. Eradication often requires long-term treatment that causes compliance problems, facilitates resistance development, and often fails to prevent relapse. Many chronic infections are likely to be caused by persistent pathogens in a dormant state with minimal cellular and metabolic activities. In fact, it remains unclear if such dormancy has any basal maintenance requirements that could be targeted for therapy. Various in vitro and in vivo models have been established to determine requirements of persistent pathogens. However, results depend on the particular model and it remains unclear how well these models mimic relevant conditions during chronic infections. It is likely that even within one infected host tissue, various microenvironments exist that might induce distinct forms of persistency [34,35].

Here we established a simple *Salmonella* mouse infection model in which *Salmonella* with dual metabolic and virulence defects persisted at constant tissue loads without an initial acute infection that hampers functional analysis in a more natural *Salmonella* infection in genetically resistant mice [12]. Indeed, in this model a substantial fraction of such *Salmonella* reached a non-proliferating state with minimal cell wall turnover within one day post infection in spleen, and a substantial *Salmonella* subpopulation even survived chronic treatment with a fluoroquinolone. This was surprising since the same treatment diminishes wildtype *Salmonella* loads in spleen to non-detectable levels [21,22], and since fluoroquinolones are the most potent, but still only partially effective antibiotic to

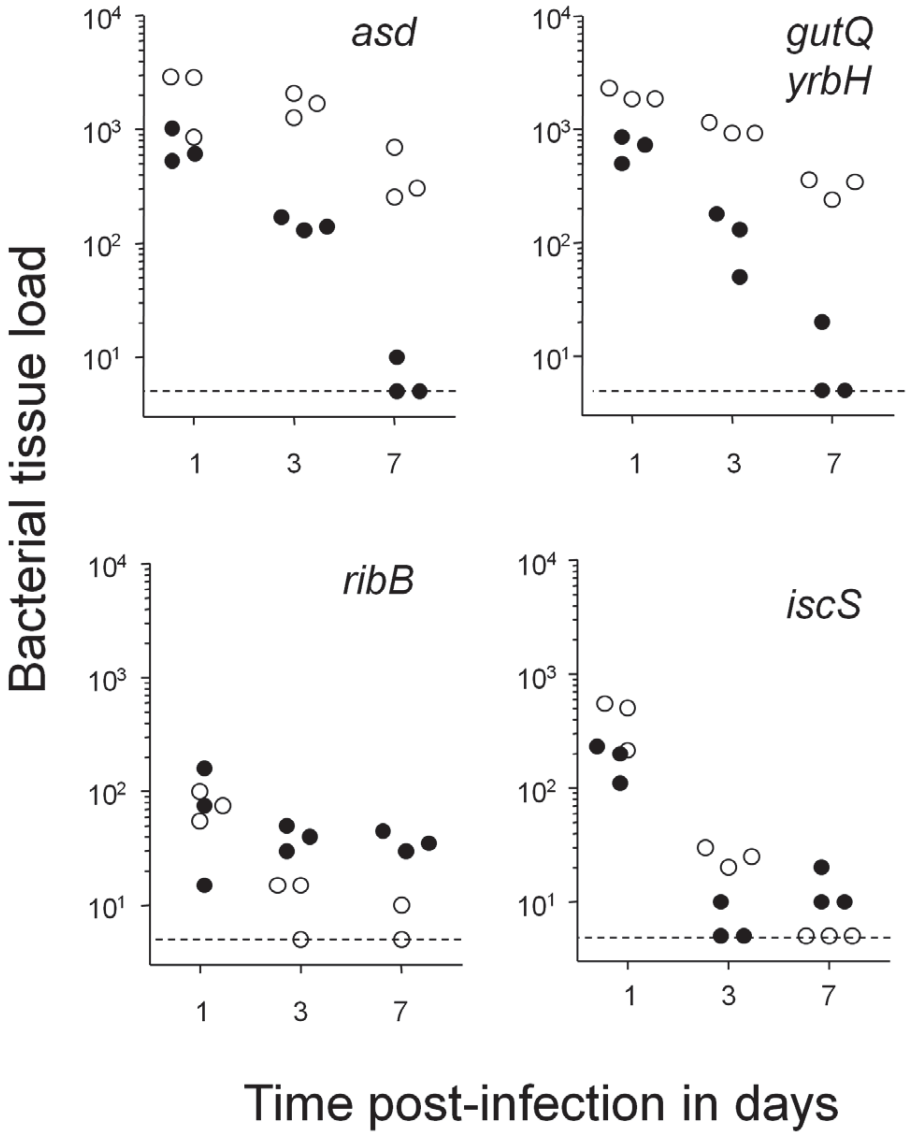

Figure 3. Colonization kinetics of four compromised mutants in spleen (open circles) and liver (filled circles). Small residual colonization levels after seven days of infection suggested that all shown genes contributed to *Salmonella* survival but were not absolutely essential. Statistical significance of clearance at day 7 compared to day 1 in spleen was determined by t-test of log-transformed data (***, $P<0.001$).
doi:10.1371/journal.pone.0042007.g003

eradicate persistent salmonellosis [15] and non-growing bacteria in general [2,19]. These data suggested that our model represented a stringent test for identifying targets that might be useful in clinically relevant settings. On the other hand, the emergence of small-colony variants of a *fabB* mutant suggested that in this model persistent *Salmonella* still had some metabolic activities that could be diminished to relax residual maintenance requirements.

Salmonella in liver remained sensitive to fluoroquinolone treatment and required continuous de novo cell wall synthesis. These data suggested that despite purine auxotrophy and inactivity of the SPI-2 type III secretion system, liver microenvironments might permit residual *Salmonella* proliferation in this tissue. Liver colonization was therefore less suitable as readout for *Salmonella* maintenance requirements during persistency. On the other hand, distinct *Salmonella* microenvironments in this organ provided complementary information for target evaluation. As an example, liver seemed to provide conditions that enable at least partial survival of *Salmonella* mutants defective for riboflavin biosynthesis or repair of iron-sulfur clusters, in contrast to conditions in spleen. Antimicrobial chemotherapy should eradicate *Salmonella* from all host organs including liver suggesting that the corresponding targets might be unsuitable.

The *Salmonella* metabolic network contains more than 1200 different enzymes that could all represent potential antimicrobial targets. However, only a very small number of these enzymes are sufficiently important for *Salmonella* physiology to qualify as potentially suitable targets to control acute infections [23]. Interestingly, the data from this study suggested that almost all of these targets might be unsuitable to treat persistent infections indicating strikingly different *Salmonella* requirements for survival as compared to growth in host tissues. Activities that were absolutely essential during acute infection, but dispensable during

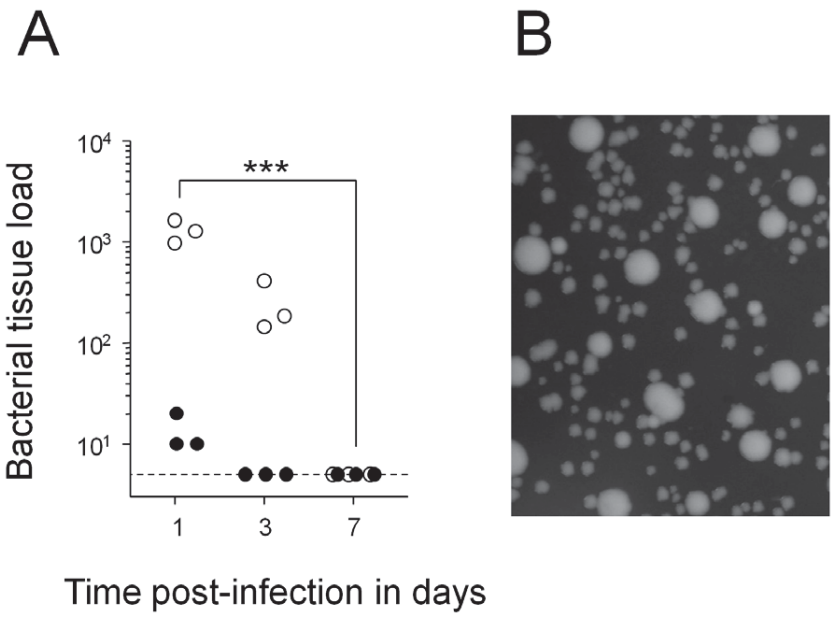

Figure 4. Clearance of *Salmonella purA ssaGH fabB* from infected mice. A) Colonization kinetics in spleen (open circles) and liver (filled circles). Similar results were obtained in three independent experiments. Statistical significance of clearance at day 7 compared to day 1 were determined by t-test of log-transformed data (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; n.s., not significant). B) Heterogeneity of colony size on agar plates. Similar data were obtained for two independent in vitro cultures and five independent ex vivo cultures.
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persistency, include cell wall synthesis, ubiquinone-dependent aerobic respiration, proton motive force-dependent ATP synthesis, translational accuracy, DNA repair, and thioredoxin-mediated redox balance. Dispensability of PMF-driven ATP synthesis highlighted the stringency of our model but might also reflect differences between *Salmonella* and other pathogens. In addition, activities that play important roles in other persistency models including fatty acid degradation and polyphosphate storage were also dispensable in our model. These data indicate that *Salmonella purA ssaGH* had limited requirements for extended in vivo survival.

We found only a single defect, inactive biosynthesis of unsaturated fatty acids and cyclopropane fatty acids that resulted in clearance to non-detectable tissue loads within a few days. Interestingly, clearance kinetics for defective mutants were faster compared to the best current antimicrobial drug enrofloxacin for chronic salmonellosis suggesting that the corresponding targets could potentially help to improve treatment of such disease. It is possible that defective fatty acid biosynthesis could result in accumulation of toxic intermediates although such toxic intermediates have not yet been described in the respective pathway and the mutant grows normally in vitro if supplemented with oleic acid. Flux-Balance Analysis [36] of a genome-scale metabolic model [37] predicted additional expected essential genes in the fatty acid biosynthesis pathway (*accA, accB, accC, accD, acpP, fabA, fabD, fabG, fabI*) but no other pathways reflecting redundancy in providing required precursors such as malonyl-CoA, NADPH, and NADH.

Unsaturated fatty acids and their derivatives cyclopropane fatty acids together comprise about one-half the *Salmonella* fatty acid content [38]. Essentiality of de novo synthesis could suggest continuous internal turnover, damage, and or loss to the environment. Damage/loss of membranes has previously been proposed as a potential strategy to control persisters [39]. Reactive oxygen species can readily damage mammalian polyunsaturated fatty acids, but bacterial unsaturated fatty acids that usually contain only a single double bond are refractory to oxidative damage [40]. Alternatively, membranes could also be lost by shedding outer membrane vesicles [41]. On the other hand, continuous synthesis of another outer membrane component, lipopolysaccharide may not be needed for *Salmonella* persistence based on the slow clearance of *Salmonella purA ssaGH gutQ yrbH* from infected spleen (Fig. 3). Further studies are needed to clarify the function of de novo fatty acid synthesis and the impact of the host immune response on fatty acid requirements during *Salmonella* persistency. It is also important to note that host fatty acids (both saturated and unsaturated) might be sufficiently available in other infectious disease models, especially in case of extracellular pathogens [42].

In conclusion, we established a practical, highly stringent in vivo persistency model. Data obtained with this model revealed that key metabolic activities that are essential during acute salmonellosis might be dispensable during persistent *Salmonella* infections. On the other hand, at least some *Salmonella* metabolic activities might be crucial for persistency and the model could help to identify additional requirements in subsequent studies.

Materials and Methods

Bacterial Genetics

We used strain *Salmonella enterica* serovar typhimurium SL1344 *hisG xyl* [43] as parental wild type strain. *Salmonella* mutants were constructed by lamda red- recombinase mediated allelic replacement [44] followed by general transduction using phage P22 *int* [45]. Resistance cassettes were flanked with FRT sites for removal using FLP recombinase [44]. All strains were cultivated at 37°C in Lennox LB medium containing 90 μg/ml streptomycin and 50 μg/ml kanamycin, 20 μg/ml chloramphenicol, and/or 100 μg/ml ampicillin, as appropriate. Auxotrophs were supplemented with 40 μg/ml riboflavin (*ribB*), 0.1% oleate (*fabB*), 50 μg/ml diamino pimelic acid (*asd*), 15 μM D-arabinose-5-phosphate/

10 μM glucose-6-phosphate (*gutQ_yrbH*). Agar plates containing oleate were always freshly prepared and maintained at 37°C to keep oleate homogeneously dispersed. Minimal inhibitory concentrations (MIC) for enrofloxacin were determined as described [46].

Mouse Infections

All animals were handled in strict accordance with good animal practice and all animal work was approved by local animal care and use committee (license 2239, Kantonales Veterinäramt BS). Eight to 12 weeks old female BALB/c mice were infected intravenously with 10^6 CFU *Salmonella* from late exponential LB cultures. For some experiments, we administered enrofloxacin (2 mg/ml) in the drinking water beginning two days post infection [21], or thiolactomycin (two doses of 2 mg per mouse). For competitive infections, mutant *Salmonella* carrying different antibiotic resistance cassettes were mixed before administration. The actual bacterial dose was confirmed by plating. At various time intervals post infection, mice were sacrificed, spleen and liver collected aseptically in 1 ml of 0.1% Triton Tx-100, and number of viable bacteria per organ was determined by plating tissue homogenates on appropriate selective media. Competitive indices (CI = output ratio/input ratio) were calculated based on plate counts for inoculum and tissue homogenates collected at seven days post infection.

In Silico Modeling

To predict additional targets, we used a genome-scale computational *Salmonella in vivo* metabolism model STMv1.1, an updated version of the consensus genome-scale metabolism reconstruction STMv1 [37] (manuscript in preparation). We used production of unsaturated fatty acids as objective function and determined all genes that were predicted to be essential for this function with Flux-Balance Analysis [36] using MatLab and the COBRA toolbox [47].

Supporting Information

Figure S1 Colonization kinetics of various *Salmonella* mutants in spleen (open circles) and liver (closed circles) of systemically infected BABL/c mice. A) Colonization of wildtype *Salmonella* SL1344 after systemic infection with 350 CFU. B) Colonization of SL1344 *purA* after infection with 1.85×10^6 CFU and SL1344 *ssaGH* after infection with 1.2×10^6 CFU. C) Initial colonization of SL1344 *purA ssaGH* after infection with 8.5×10^5 CFU. Statistical significance of colonization level differences at day 2 and 4 (for data in A), clearance at day 7 compared to day 1 (for data in B), or colonization levels at 24 h compared to 2 h (for data in C) were determined by t-test of log-transformed data (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$).
(TIF)

Author Contributions

Conceived and designed the experiments: SB BS AM DB. Performed the experiments: SB BS AM. Analyzed the data: SB BS AM DB. Wrote the paper: SB BS DB.

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2.1.3 Additional Results

Supplementary Figure 1 (Fig S1)

Colonization kinetics of various *Salmonella* mutants in spleen (open circles) and liver (closed circles) of systemically infected BABL/c mice.

A) Colonization of wildtype *Salmonella* SL1344 after systemic infection with 350 CFU. B) Colonization of SL1344 *purA* after infection with 1.85×10^6 CFU and SL1344 *ssaGH* after infection with 1.2×10^6 CFU. C) Initial colonization of SL1344 *purA ssaGH* after infection with 8.5×10^5 CFU. Statistical significance of colonization level differences at day 2 and 4 (for data in A), clearance at day 7 compared to day 1 (for data in B), or colonization levels at 24 h compared to 2 h (for data in C) were determined by t-test of log-transformed data (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$).

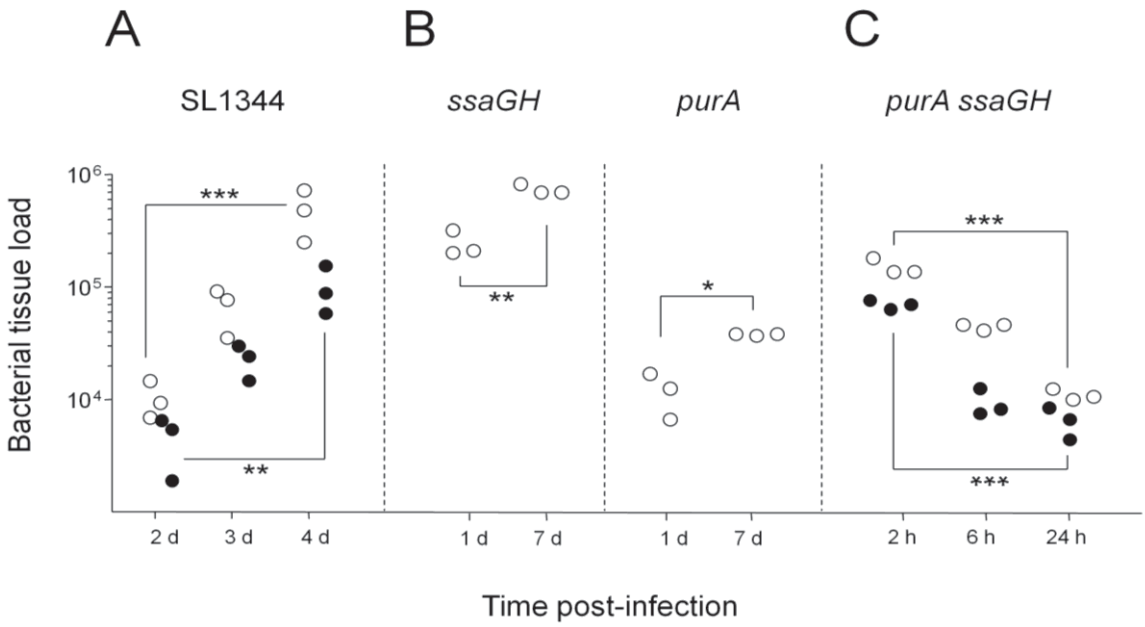

Figure S1

Results

Part B – Immunity to intracellular *Salmonella* depends on surface associated antigens

2.2 Immunity to intracellular *Salmonella* depends on surface associated antigens.

PLoS pathogens, 2012, 8(10), e1002966.

Somedutta Barat[†], Yvonne Willer[†], Konstantin Rizos[†], Beatrice Claudi, Alain Maze´,Anne K. Schemmer, Dennis Kirchhoff, Alexander Schmidt, Neil Burton, Dirk Bumann.

[†]equal contribution

2.2.1 Abstract

Enteric fever causes tremendous morbidity and moratality worldwide. Current control strategies are becoming inefficient because of the increasing antibiotic resistance, and emergence of new *Salmonella* serovars that are not covered by the currently licensed vaccines. Hence, there is an urgent need for novel *Salmonella* vaccine with broad serovar coverage. In this study we did experiments to determine the relevant antigen properties in a mouse *Salmonella* typhoid fever model that might be relevant for developing a new *Salmonella* vaccine. Our data revealed novel protective *Salmonella* antigens that conferred partial protection against virulent *Salmonella*. Moreover, we also found that the protective antigens were all surface associated suggesting a crucial role of antigen localization. Results from model antigens expressed in *Salmonella* suggested that surface localization might enhance antigen immunogenicity. However, data from protective surface autologous *Salmonella* antigens did not induce superior immune responses during infection compared to the internal antigens. We also observed that live *Salmonella* segregated away from dead *Salmonella* that released internal antigens. Detection and destruction of live *Salmonella* possibly required

recognition of surface exposed antigens. In conclusion, these data suggested prioritization of *Salmonella* antigen candidates based on surface association.

Statement of my work

I contributed to this work by generating sixteen *Salmonella* outer membrane associated antigens, and one inner membrane antigens, and tested their protectivity in mouse typhoid fever model with immunization/challenge experiments. I also predicted the T cell epitopes of all the thirty seven *Salmonella* antigens using bioinformatic tools, and contributed in the strucutural modeling for the selected *Salmonella* antigens.

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Immunity to Intracellular *Salmonella* Depends on Surface-associated Antigens

Somedutta Barat[1☯], Yvonne Willer[2☯¤a], Konstantin Rizos[3☯¤b], Beatrice Claudi[1], Alain Mazé[1], Anne K. Schemmer[1], Dennis Kirchhoff[4¤c], Alexander Schmidt[5], Neil Burton[1], Dirk Bumann[1,2,3]*

1 Focal Area Infection Biology, Biozentrum, University of Basel, Basel, Switzerland, **2** Junior Group "Mucosal Infections", Hannover Medical School, Hannover, Germany, **3** Department of Molecular Biology, Max-Planck-Institute for Infection Biology, Berlin, Germany, **4** Immunomodulation Group, Deutsches Rheuma-Forschungszentrum Berlin, Berlin, Germany, **5** Proteomics Core Facility, Biozentrum, University of Basel, Basel, Switzerland

Abstract

Invasive *Salmonella* infection is an important health problem that is worsening because of rising antimicrobial resistance and changing *Salmonella* serovar spectrum. Novel vaccines with broad serovar coverage are needed, but suitable protective antigens remain largely unknown. Here, we tested 37 broadly conserved *Salmonella* antigens in a mouse typhoid fever model, and identified antigen candidates that conferred partial protection against lethal disease. Antigen properties such as high in vivo abundance or immunodominance in convalescent individuals were not required for protectivity, but all promising antigen candidates were associated with the *Salmonella* surface. Surprisingly, this was not due to superior immunogenicity of surface antigens compared to internal antigens as had been suggested by previous studies and novel findings for CD4 T cell responses to model antigens. Confocal microscopy of infected tissues revealed that many live *Salmonella* resided alone in infected host macrophages with no damaged *Salmonella* releasing internal antigens in their vicinity. In the absence of accessible internal antigens, detection of these infected cells might require CD4 T cell recognition of *Salmonella* surface-associated antigens that could be processed and presented even from intact *Salmonella*. In conclusion, our findings might pave the way for development of an efficacious *Salmonella* vaccine with broad serovar coverage, and suggest a similar crucial role of surface antigens for immunity to both extracellular and intracellular pathogens.

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* E-mail: dirk.bumann@unibas.ch

☯ These authors contributed equally to this work.

¤a Current address: Department of Radiation Oncology, University Hospital Erlangen, Erlangen, Germany.
¤b Current address: Genetic ID AG, Augsburg, Germany.
¤c Current address: Global Drug Discovery, Bayer HealthCare, Berlin, Germany.

Introduction

Enteric fever caused by systemic *Salmonella* infection causes tremendous morbidity and mortality worldwide [1]. Current control strategies become increasingly inefficient as a result of increasing antimicrobial resistance [2,3] and emergence of *Salmonella* serovars that are not covered by currently available safe vaccines [4,5]. This situation generates an urgent medical need for novel *Salmonella* vaccines with broad serovar coverage.

Early killed whole-cell vaccines containing mixtures of different serovars provide broad protection, but cause unacceptable adverse reactions [1]. As an alternative to whole-cell vaccines, subunit vaccines containing a few defined *Salmonella* components could minimize adverse reactions. Indeed, vaccines containing the capsular polysaccharide Vi antigen provide moderate protection and excellent safety [1]. On the other hand, serovars Paratyphi A and non-typhoidal *Salmonella* (NTS) that cause an increasing number of invasive salmonelloses [6], lack the Vi antigen and are therefore not covered by Vi vaccines [5]. Apart from Vi, few *Salmonella* antigens have been identified, and all of these provide at best moderate levels of protection against challenge infection with virulent *Salmonella* strains in the commonly used mouse typhoid fever model. Moreover, antigens such as flagellin [7] and OmpD [8] are poorly conserved among relevant serovars.

For extracellular pathogens with antibody-mediated immunity, protective antigens must be surface-exposed [9], and this enables an effective strategy for priorization of antigen candidates [9]. Humoral response to surface antigens can also contribute to immunity to intracellular pathogens such as invasive *Salmonella* [10]. Indeed, Vi which induces protective antibody responses in human vaccinees, forms an extracellular capsule around *Salmonella* Typhi [11]. Two additional antigens that confer partial immunity in the mouse typhoid fever model, flagellin [7] and SseB [12], are also part of *Salmonella* surface structures (flagella, translocon complex of a type III secretion system). Furthermore, outer membrane preparations (but not the outer membrane component lipopolysaccharide) have been suggested to mediate protective humoral immune responses against extracellular *Salmonella* bac-

Author Summary

Salmonella infections cause extensive morbidity and mortality worldwide. A vaccine that prevents systemic *Salmonella* infections is urgently needed but suitable antigens remain largely unknown. In this study we identified several antigen candidates that mediated protective immunity to *Salmonella* in a mouse typhoid fever model. Interestingly, all these antigens were associated with the *Salmonella* surface. This suggested that similar antigen properties might be relevant for CD4 T cell dependent immunity to intracellular pathogens like *Salmonella*, as for antibody-dependent immunity to extracellular pathogens. Detailed analysis revealed that *Salmonella* surface antigens were not generally more immunogenic compared to internal antigens. However, internal antigens were inaccessible for CD4 T cell recognition of a substantial number of infected host cells that contained exclusively live intact *Salmonella*. Together, these results might pave the way for development of an efficacious *Salmonella* vaccine, and provide a basis to facilitate antigen identification for *Salmonella* and possibly other intracellular pathogens.

teremia [13] and attenuated *Salmonella* strains in the mouse model [8,14]. A number of porins such as OmpC, OmpD, and OmpF are highly abundant in such outer membrane preparations suggesting that they might represent the actual protective antigens [8,14,15].

However, immunity to *Salmonella* critically depends also on CD4 T cells [10]. Unfortunately, protective T cell antigens seem to be rare, and priorization of candidates is difficult since relevant antigen properties for CD4 T cell responses remain unclear [9,16,17]. One key precondition for protective responses is expression of the respective *Salmonella* antigen during infection [18], and some data suggest that highly abundant antigens might be particularly well recognized by CD4 T cells [12,19]. Antigen in vivo expression can be deduced from various complementary approaches including screening of promoter trap libraries [20,21], proteomics [22], serum antibody response [23–26], as well as mutant virulence phenotypes.

In addition to antigen expression, antigen immunogenicity could play a major role. Antigen detection by cognate CD4 T cells requires antigen processing and presentation of the resulting small peptides by major histocompatibility (MHC) class II molecules. Peptide sequence properties that are characteristic for well recognized epitopes, can be used for genome-wide prediction of promising antigens [27]. However, a large number of non-protective antigens contain putative high-score epitopes [16,18,28] which could compromise the discriminatory power of this approach.

Experimental detection of immune responses to an antigen in convalescent individuals that have survived infection, demonstrates that this antigen was expressed in vivo and could be recognized by the immune system [23–25]. Indeed, this approach has been recently shown to facilitate identification of protective *Chlamydia* antigens [29]. On the other hand, many immunodominant antigens in convalescent individuals lack protective efficacy, while a number of protective antigens may induce immune responses below the detection threshold during natural infection [17].

Another antigen property that can affect CD4 T cell responses is antigen localization. In particular, secreted or surface-associated antigens might induce particularly strong cellular immune responses because of superior processing, kinetic advantages compared to internal antigens, and/or physical association with pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide that provide potent stimuli for innate and adaptive immunity [14,30–36]. Indeed, secretion/surface localization has been widely used to prioritize candidates for antigen identification. However, antigens with likely internal localization can also induce specific CD4 T cell responses that mediate protection against various intracellular pathogens [37,38].

Taken together, relevant antigen properties for CD4 T cell mediated immunity to intracellular pathogens remain poorly characterized, and this impairs antigen priorization for vaccine development. To address this issue, we compared here 37 diverse *Salmonella* antigens in a mouse model that closely mimics human typhoid fever [39]. The results suggested that recognition of surface-associated antigens might be necessary to detect and combat live intracellular *Salmonella*, whereas recognition of internal antigens would mediate futile non-protective attack of already dead *Salmonella*. In conclusion, we propose a similar crucial role of surface-associated antigens for immunity to both extracellular and intracellular pathogens.

Results

Immune responses to *Salmonella* antigens in convalescent individuals

To determine immune responses to *Salmonella* antigens, we selected 21 broadly conserved *Salmonella* proteins. We selected several subunits of the SPI-2 type III secretion system since the putative translocon subunit SseB of this system showed promising protectivity in previous studies [12,26]. We also included several porins since a previous study had shown that OmpD conferred protection against an attenuated *Salmonella* mutant [8]. To explore the role of antigen localization we selected additional proteins localized in *Salmonella* cytosol, inner membrane, periplasm, and outer membrane/surface. To explore the role of antigen abundance, we determined absolute quantities of more than 1100 *Salmonella* in infected mouse spleen. Specifically, we purified *Salmonella* from infected mouse spleen using flow cytometry as described [22]. We determined absolute protein quantities in these ex vivo purified *Salmonella* using shot-gun proteomics with 30 isotope labeled reference peptides and the iBAQ quantification method [40] (for detailed description see Materials and Methods section). From these data, we selected additional antigens with a large range of abundances (Tab. 1).

To determine potential cross-protection between different serovars, we cloned the corresponding genes from *Salmonella enterica* serovar Typhi (except for OmpD which was obtained from serovar Typhimurium since it is absent in serovar Typhi). We expressed the proteins as C-terminal His_6-fusions in *E. coli* followed by Ni-affinity chromatography purification. We purified the control antigen GFP-His_6 using the same protocol.

We determined immune responses to these antigens in genetically resistant, convalescent mice that had survived infection with virulent *Salmonella enterica* serovar Typhimurium. We detected antigen-specific CD4 T cells in spleen using a sensitive CD154 assay [41] and measured serum IgG antibody responses using ELISA. All tested antigens were recognized by CD4 T cells (Fig. 1A; Tab. 1), many of which secreted IFNγ or IL-17 upon stimulation. Both cytokines play crucial roles in immunity to *Salmonella* [10]. Frequencies of responsive CD4 T cells were in the same range as for flagellin, which has been considered an immunodominant antigen [42]. These data suggested that *Salmonella* infection elicited a broad cellular immune response

Table 1. Properties of *Salmonella* antigens.

STY antigen	STm orthologue	Name	Compartment	In vivo abundance in copies per cell ± SEM	CD154+ cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	IFNg-secreting cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	IL17-secreting cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	Serum IgG in ng ml^{-1} ± SEM	*P*-value[2]	Survival time extension in days	*P*-value[3]
T2461	STM0402		Cytosol	21725 ± 2837	1949 ± 733	0.008	591 ± 251	0.014	268 ± 82	0.005	3406 ± 1852	<0.001	−3	0.924
T1689	STM1231	*phoP*	Cytosol	24060 ± 1410	938 ± 295	0.009	221 ± 45	0.001	140 ± 53	0.012	85 ± 20	0.045	−1	0.702
T1265	STM1397	*sseA*	Cytosol	2163 ± 877	703 ± 245	0.030	137 ± 17	0.001	84 ± 20	0.002	36 ± 7	0.310	−3	0.639
T0782	STM2090	*rfbH*	Cytosol	4260 ± 392	1035 ± 481	0.019	270 ± 140	0.030	170 ± 56	0.008	79 ± 19	<0.001	−3	0.555
T0524	STM2340		Cytosol	922 ± 434	923 ± 520	0.038	276 ± 119	0.020	103 ± 30	0.009	36 ± 0	<0.001	−1	0.782
T3053	STM3132		Cytosol	7504 ± 1374	1083 ± 618	0.030	322 ± 98	0.009	198 ± 71	0.016	52 ± 10	<0.001	−2	0.676
T3595	STM4026	*yihX*	Cytosol	5101 ± 902	1014 ± 621	0.119	206 ± 85	0.044	100 ± 42	0.045	42 ± 9	0.287	0	0.146
T1506	STM1597	*ydcW*	Cytosol	2328 ± 462	952 ± 652	0.378	139 ± 66	0.030	106 ± 65	0.022	46 ± 9	0.006	3	0.268
T2763	STM2861	*sitA*	Inner Membrane	644 ± 136	873 ± 399	0.024	87 ± 27	0.126	76 ± 45	0.043	52 ± 29	0.011	−4	0.938
T3872	STM3647	*yiaF*	Inner Membrane	829 ± 354	n.d.		n.d.		n.d.		n.d.		0	0.880
T1508	STM1599	*pcgL*	Periplasm	14605 ± 2135	503 ± 235	0.077	75 ± 22	0.006	63 ± 66	0.110	1212 ± 940	<0.001	−3	0.868
T0300	STM2556	*hmpA*	Periplasm	7658 ± 1154	853 ± 484	0.043	302 ± 216	0.088	125 ± 74	0.045	38 ± 1	<0.001	−1	0.398
T4225	STM4319	*phoN*	Periplasm	37606 ± 2742	559 ± 263	0.021	155 ± 39	0.004	59 ± 30	0.038	2891 ± 760	<0.001	−1	0.761
T2415	STM0445	*yajG*	Lipoprotein (outer membrane)	1057 ± 360	n.d.		n.d.		n.d.		n.d.		−2	0.690
T1058	STM1819	*slp*	Lipoprotein (outer membrane)	599 ± 208	n.d.		n.d.		n.d.		n.d.		−1	0.835
T3199	STM3281	*nlpL*	Lipoprotein (outer membrane)	b.t	n.d.		n.d.		n.d.		n.d.		−1	0.418
T0371	STM2488	*nlpB*	Lipoprotein (outer membrane)	2593 ± 363	n.d.		n.d.		n.d.		n.d.		1	0.921
T0336	STM2520	*yfgL*	Lipoprotein (outer membrane)	2131 ± 286	n.d.		n.d.		n.d.		n.d.		2	0.386
T2619	STM2663	*yfiO*	Lipoprotein (outer membrane)	763 ± 289	n.d.		n.d.		n.d.		n.d.		4	0.441
T1459	STM1540		Lipoprotein (outer membrane)	5485 ± 694	n.d.		n.d.		n.d.		n.d.		6	0.150
T3874	STM3645	*yiaD*	Lipoprotein (outer membrane)	3856 ± 859	n.d.		n.d.		n.d.		n.d.		8	0.196
T0937	STM1940		Lipoprotein (outer membrane)	1864 ± 396	546 ± 650	0.128	67 ± 89	0.140	49 ± 25	0.013	37 ± 2	<0.001	9	0.023
T1313	STM1445	*slyB*	Lipoprotein (outer membrane)	7270 ± 719	n.d.		n.d.		n.d.		n.d.		12	0.228
T1262	STM1394	*ssaC*	Outer membrane	b.t	618 ± 378	0.073	114 ± 36	0.007	74 ± 47	0.062	35 ± 1	<0.001	−3	0.240
T3107	STM3186	*tolC*	Outer membrane	b.t	898 ± 550	0.050	24 ± 32	0.273	79 ± 44	0.042	527 ± 446	<0.001	−3	0.370
T0463	STM2395	*pgtE*	Outer membrane	8466 ± 1032	1924 ± 583	0.044	768 ± 208	0.016	245 ± 109	0.057	80 ± 23	<0.001	−1	0.214
T1119	STM1246	*pagC*	Outer membrane	11993 ± 3667	851 ± 122	0.009	273 ± 79	0.002	110 ± 13	0.001	36 ± 1	<0.001	−1	0.478

Table 1. Cont.

STY antigen	STm orthologue	Name	Compartment	In vivo abundance in copies per cell ± SEM	CD154+ cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	IFNg-secreting cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	IL17-secreting cells per 10^6 CD4 T cells ± SEM	*P*-value[1]	Serum IgG in ng ml^{-1} ± SEM	*P*-value[2]	Survival time extension in days	*P*-value[3]
–	STM1572	*ompD*	Outer membrane	b.t	n.d.		n.d.		n.d.		n.d.		−1	0.958
T2450	STM0413	*tsx*	Outer membrane	6835 ± 510	n.d.		n.d.		n.d.		n.d.		0	0.910
T0597	STM2267	*ompC*	Outer membrane	146 ± 81	n.d.		n.d.		n.d.		n.d.		0	0.540
T0225	STM0224	*yaeT*	Outer membrane	1835 ± 233	n.d.		n.d.		n.d.		n.d.		1	0.479
T1935	STM0999	*ompF*	Outer membrane	b.t	n.d.		n.d.		n.d.		n.d.		1	0.231
T2283	STM0585	*fepA*	Outer membrane	b.t	n.d.		n.d.		n.d.		n.d.		7	0.344
T2672	STM2777	*iroN*	Outer membrane	b.t	1038 ± 569	0.037	353 ± 166	0.023	155 ± 58	0.013	41 ± 6	0.180	16	0.012
T0656	STM2199	*cirA*	Outer membrane	b.t	n.d.		n.d.		n.d.		n.d.		16	0.139
T1266	STM1398	*sseB*	Secretion apparatus needle	n.a.	1048 ± 304	0.016	329 ± 143	0.012	104 ± 28	0.006	410 ± 170	<0.001	10	0.104
T1269	STM1401	*sseD*	Secretion apparatus translocon	n.a.	2177 ± 410	0.003	823 ± 357	0.050	381 ± 125	0.031	202 ± 85	<0.001	3	0.162

[1], t-test vs. non-infected control mice;
[2], t-test of log-transformed values vs. non-infected control mice;
[3], log-rank test vs. control mice immunized with GFP;
b.t.; below quantification threshold; n.d., not determined; n.a., not applicable for secreted proteins SseB and SseD.
doi:10.1371/journal.ppat.1002966.t001

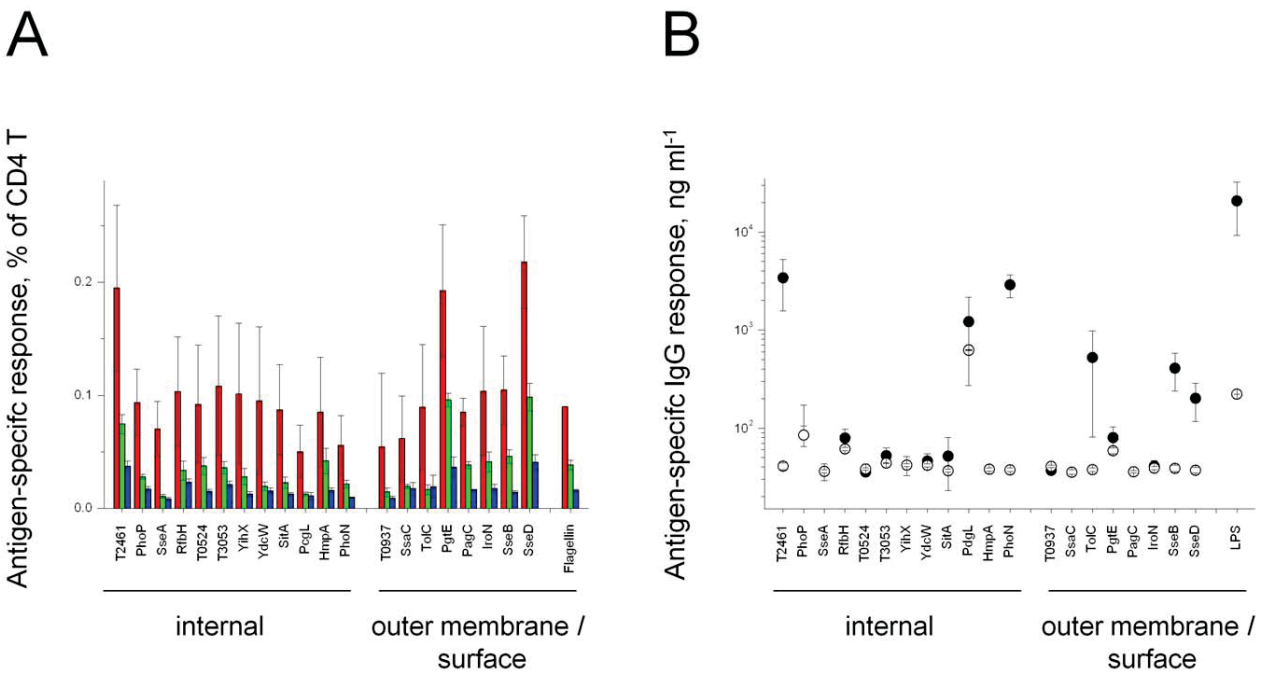

Figure 1. Cellular and humoral immune responses of convalescent *Salmonella*-infected mice to recombinant *Salmonella* antigens. A) Antigen-specific CD4 T cell frequencies as detected by CD154 upregulation (red) and IFNγ (green) or IL-17 (blue) secretion. The data represent means $\pm$ SE of three mice. Responses to *Salmonella* antigens in non-infected control mice were subtracted (see also Fig. S1). **B**) Serum antibody responses to *Salmonella* antigens. The data represent means $\pm$ SE of 11 convalescent mice (filled circles) and means $\pm$ SE for ten non-infected control mice (open circles).
doi:10.1371/journal.ppat.1002966.g001

against a large number of in vivo expressed antigens from all *Salmonella* compartments in agreement with data observed for *S.* Typhi infected human patients [43]. There was no correlation between in vivo antigen abundance as determined by proteome analysis of ex vivo purified *Salmonella*, and CD4 T cell frequency or cytokine profile (Tab. 1).

Serum antibody responses revealed similar broad recognition of antigens from several *Salmonella* compartments (Fig. 1B) in agreement with previous data for human typhoid fever patients [24–26,44]. Interestingly, the three immunodominant humoral antigens T2461, PhoN, and PcgL were all highly expressed in vivo (Tab. 1) suggesting a potential impact of antigen dose on antibody responses to *Salmonella*, although responses to minor antigens did not correlate with antigen abundance. PhoN has been previously recognized as an immunodominant antigen [26].

Immunization and challenge infection

Many of the tested *Salmonella* antigens were capable to induce cellular and humoral immune responses. To test if these responses could confer protective immunity, we tested the 21 recombinant *Salmonella* antigens in immunization/challenge infection experiments in genetically susceptible BALB/c mice. Based on the results, we selected 16 additional *Salmonella* antigens primarily from the outer membrane, and tested them using the same experimental immunization/challenge approach (however, we did not measure their immunogenicity in convalescent mice). For simplicity, we discuss results for both antigen sets together. Out of 37 tested antigens, only few antigens enabled prolonged survival after oral challenge infection with virulent *Salmonella* compared to control immunization with the unrelated antigen GFP (Fig. 2; Tab. 1; poor survival of PhoN-vaccinated animals confirmed recently published data [26]). In fact, only two antigens (T0937 and T2672) mediated protective immune responses with *P*-values below 0.05 in our small experimental groups of only five mice per antigen. Replicate experiments with larger group sizes might yield statistical significant results for additional candidates such as SseB that has already been shown to be protective in two independent previous studies. Such experiments will be required to select individual antigens for vaccine development in future studies. On the other hand, the primary focus of this study was to identify antigen properties that correlate with protectivity. For this purpose, the somewhat noisy survival times detected with small animal groups were still helpful.

As an example, survival times did not correlate with CD4 T cell responses (Fig. 3A) or serum antibody levels (Fig. 3B) during natural infection of resistant mice. This could partially reflect differences in MHC class II haplotypes ($H2^d$ in BALB/c vs. $H2^b$ in 129/Sv), courses of infection, and potential differences in *Salmonella* biology in susceptible vs. resistant mice. However, a recent large-scale study reports comprehensive immunogenicity data for BALB/c mice and other mouse strains that had been immunized with attenuated *Salmonella*, as well as for human patients [26]. Several antigens that prolonged survival of immunized BALB/c mice after *Salmonella* challenge infection in our experiments, elicit detectable antibody responses in various mouse strains including BALB/c. However, none of these antigens was found to be immunodominant [26] and antibodies to antigens IroN and CirA with the longest survival times were not detected in this and previous studies. This could reflect differential antigen expression in virulent vs. attenuated *Salmonella*, different routes of administration, and/or differential expression at various stages of disease progression. Together, these data provide no evidence for immunodominance in convalescent or immune individuals as a prerequisite for protectivity.

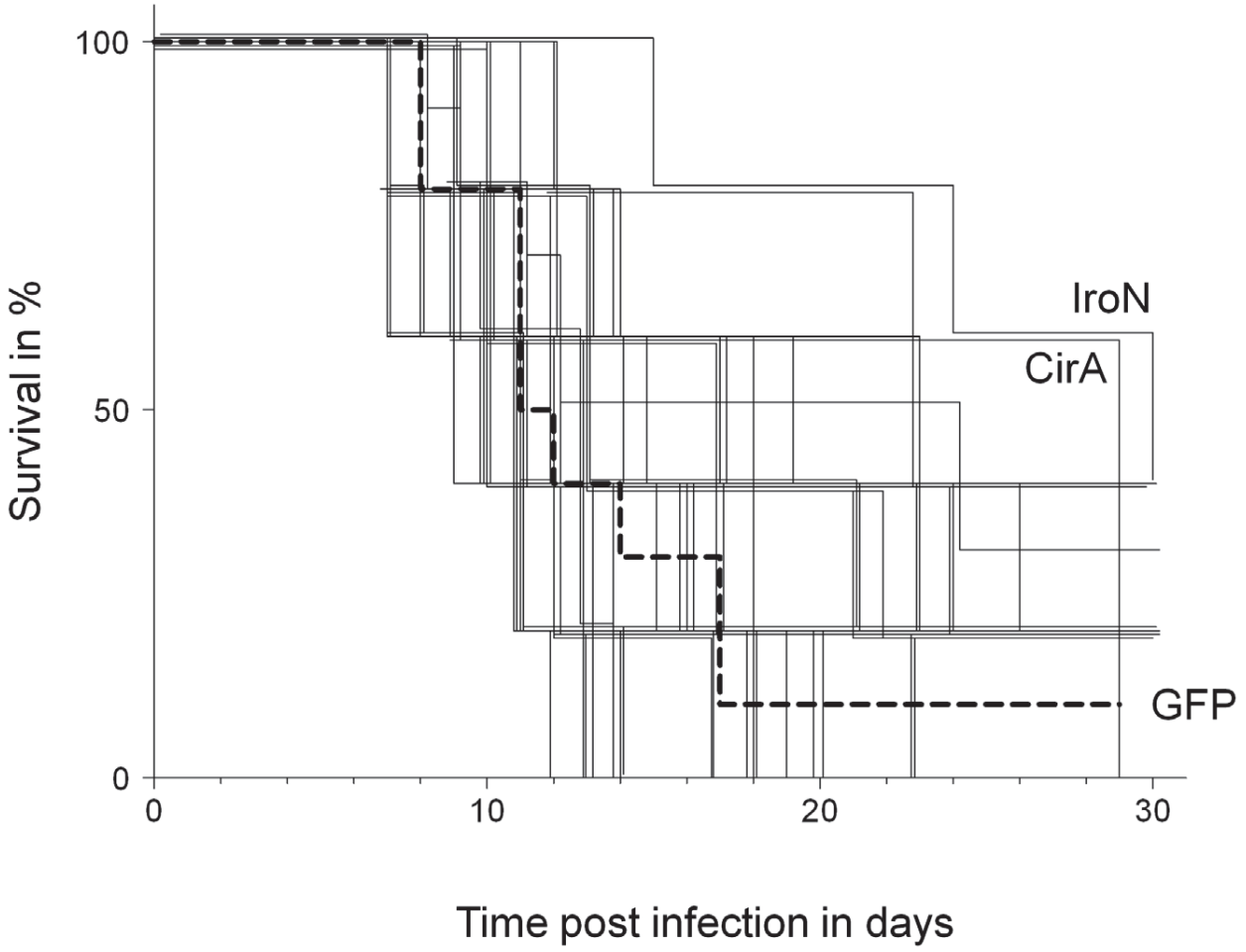

Figure 2. Survival curves of mouse groups immunized with 37 different *Salmonella* antigens (thin lines) or the control antigen GFP (thick dashed line). For better visualization, curves were slightly shifted. The longest survival was observed for antigens IroN and CirA. For statistical analysis by log-rank test see Table 1.
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Interestingly, in vivo expression levels also did not correlate with survival times (Fig. 4A). In fact, the two antigens that enabled the longest survival, IroN and CirA, had in vivo expression levels that were below our detection threshold. By comparison, antigens T2461 and PhoN were highly expressed in vivo and induced potent CD4 T cell and humoral responses in convalescent individuals, yet failed to prolong survival (in agreement with previous observations [26]).

In contrast to immunogenicity and in vivo abundance, antigen localization seemed to be crucial (Fig. 4B). In fact, antigens enabling prolonged survival times were exclusively associated with the *Salmonella* surface, either as experimentally validated outer membrane-associated lipoproteins [45], as outer membrane proteins, or as the translocon complex of the type III secretion system encoded by *Salmonella* pathogenicity island two (SPI-2) (Tab. 1). These data suggested distinct immune responses to *Salmonella* outer membrane/surface antigens that fundamentally differ from those to internal antigens.

On the other hand, surface localization alone was not sufficient for protectivity. As examples, membrane proteins PgtE, PagC, and Tsx were highly expressed in vivo and PgtE and PagC elicited potent CD4 T cell responses in convalescent individuals (Fig. 1A). PagC is also well recognized by antibodies and CD4 T cells of human typhoid fever patients [24]. However, PagC, PgtE, and Tsx failed to prolong survival. Interestingly, structural models revealed that these proteins were largely buried in the outer membrane bilayer (Fig. 5), and their extracellular loops contained at most one predicted CD4 T cell epitope each, and only up to two linear antibody epitopes, respectively. Importantly, key amino acids in exposed T cell epitopes differed among *Salmonella* serovars which might have impaired cross-protectivity of serovar Typhi antigens against serovar Typhimurium challenge infection. Similar observations were also made for non-protective TolC, OmpC, OmpD, and OmpF. By contrast, antigens IroN, CirA, and FepA that enabled extended survival after challenge infection, had extracellular loops with several highly conserved T and B cell epitopes (Fig. 5). Further studies with larger data sets will be required to validate the relevance of these structural properties for protectivity.

Impact of *Salmonella* antigen localization in an ovalbumin model

The strong bias for surface-associated *Salmonella* antigens might have been expected based on previous data for model antigens suggesting superior immunogenicity of surface antigens compared to internal antigens [30,46–50]. However, these model antigen

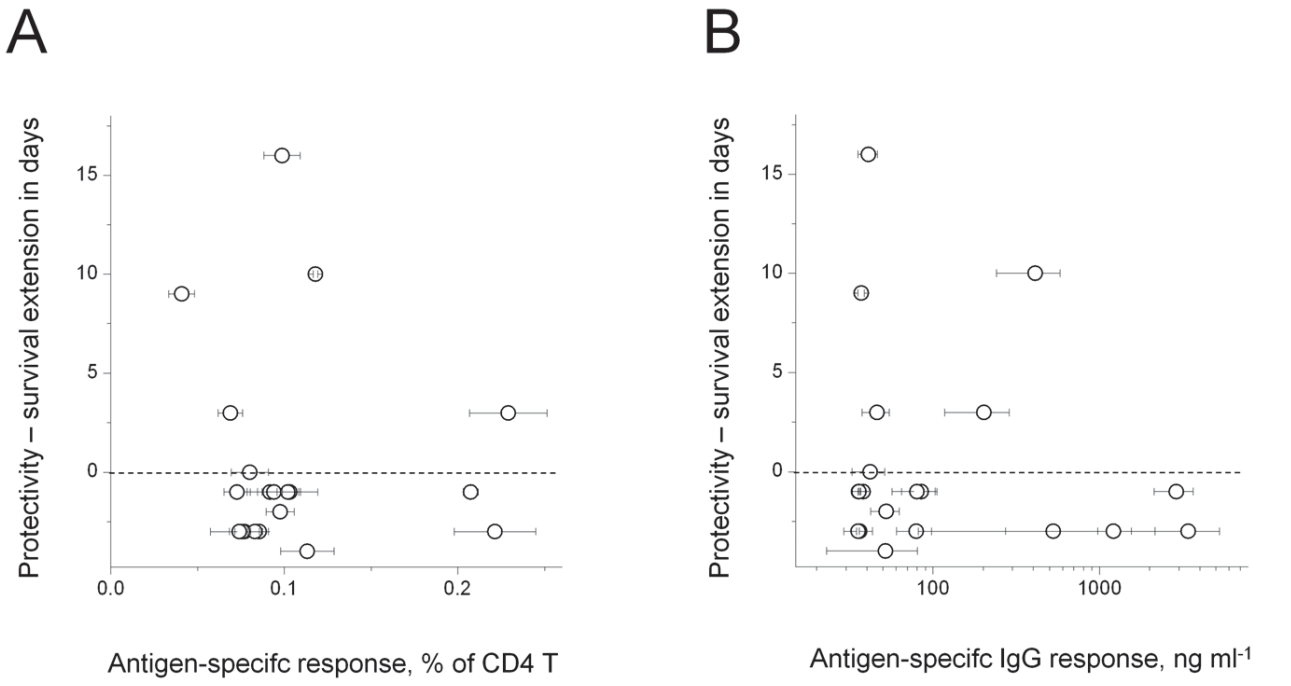

Figure 3. Comparison of *Salmonella* antigen protectivity against primary infection and immunogenicity in convalescent resistant mice. A) Relationship between antigen protectivity against primary infection and cognate CD4 T cell responses in convalescent mice (same data as Fig. 1A). Protectivity is expressed as ''survival time extension'', which is the difference in median survival time of a group of five immunized mice compared to a control group of five mice that were immunized with GFP. **B**) Relationship between antigen protectivity and serum antibody responses in convalescent mice (same data as Fig. 1B).
doi:10.1371/journal.ppat.1002966.g003

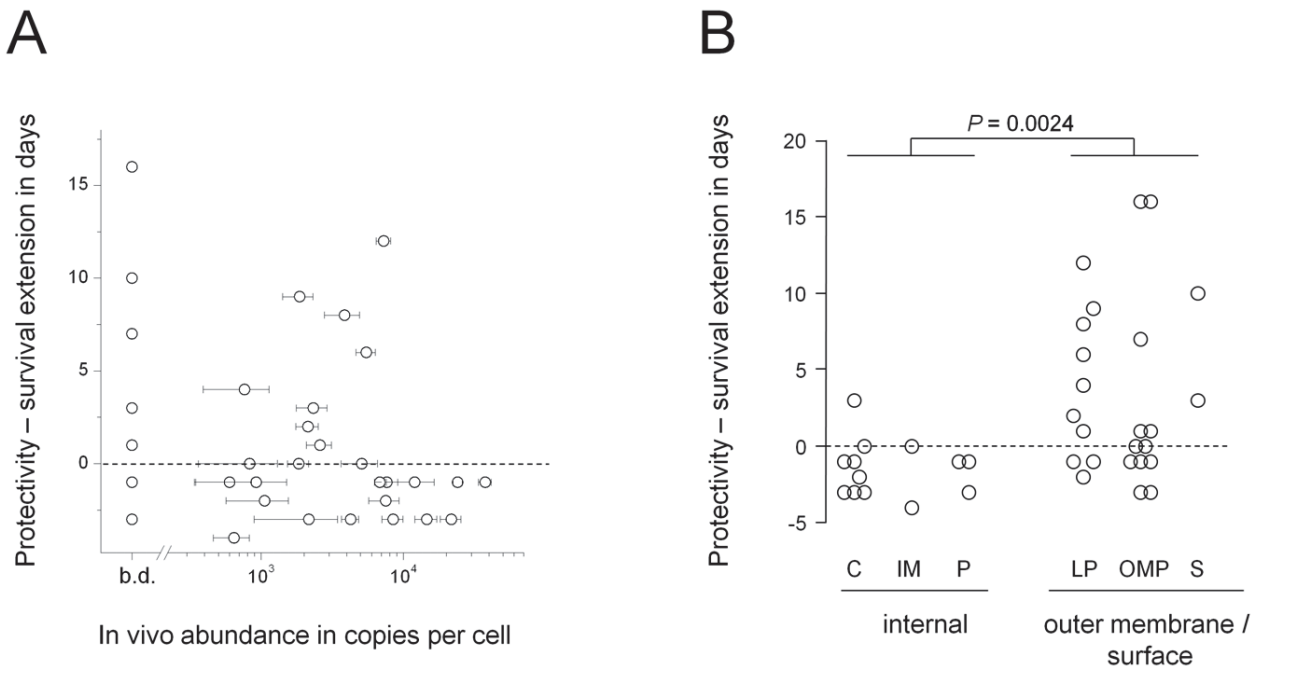

Figure 4. *Salmonella* antigen protectivity does not correlate with in vivo antigen abundance but depends on antigen localization within the *Salmonella* cell. A) Relationship between antigen protectivity and in vivo abundance as determined by quantitative proteome analysis of ex vivo purified *Salmonella* (means ± SD for three independently infected mice; b.d., below detection threshold). **B**) Relationship between antigen protectivity and antigen localization within *Salmonella* (C, cytosol; IM, inner membrane; P, periplasm; LP, outer-membrane associated lipoprotein; OMP, outer membrane protein; S, surface). Statistical significance of differences between internal and outer membrane/surface antigens was tested using the non-parametric Mann-Whitney U test.
doi:10.1371/journal.ppat.1002966.g004

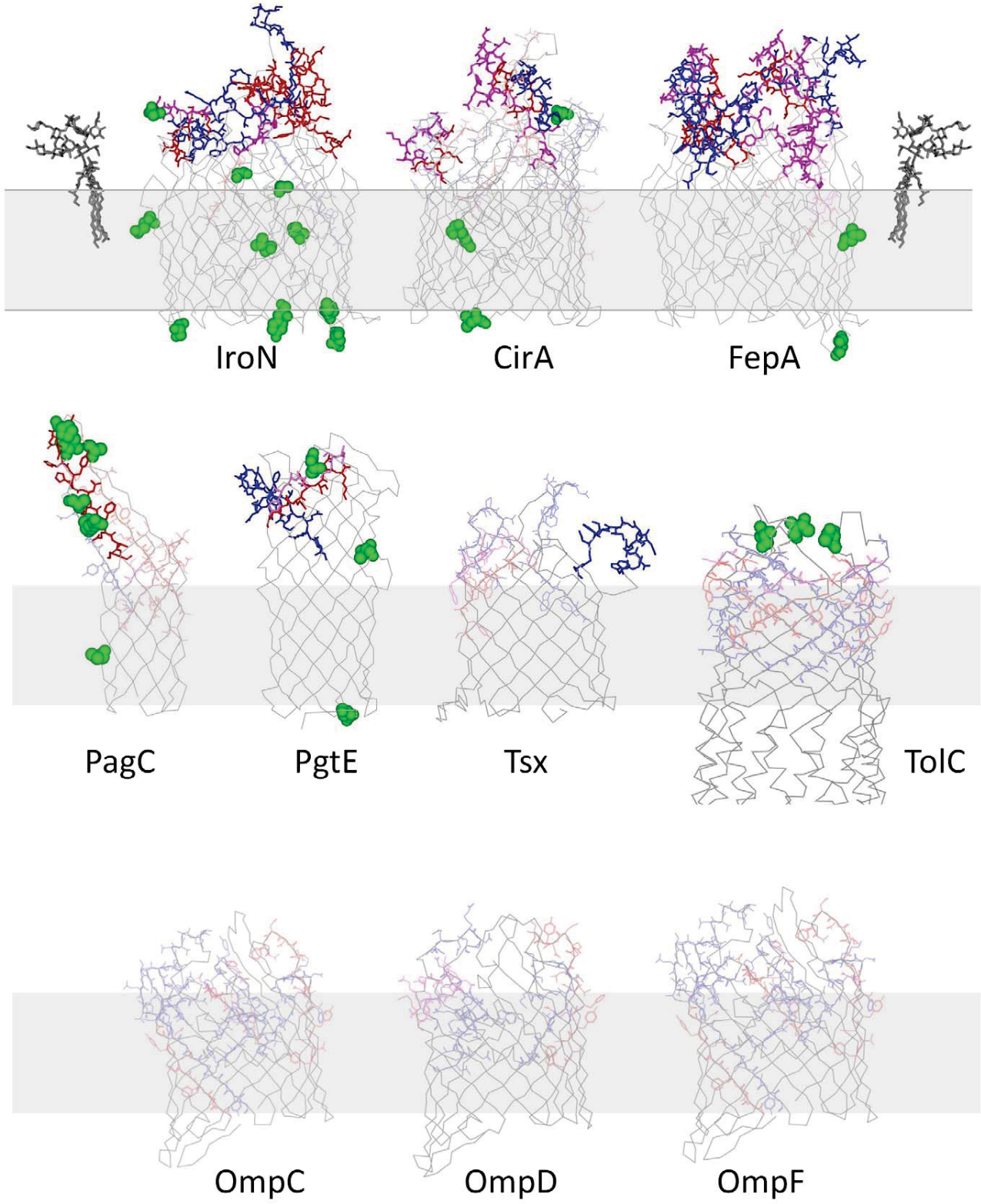

Figure 5. Structural models and exposed immune epitopes of various *Salmonella* outer membrane proteins. The outer membrane is shown as a grey area, predicted CD4 T cell epitopes in exposed loops are shown in red, potential antibody binding sites are shown in blue, and overlapping T and B cell epitopes are shown in magenta. Partially exposed epitopes are shown in pale colors. Amino acid residues that differ between *Salmonella enterica* serovars Typhimurium and Typhi are shown in green. For TolC only the outer membrane-associated part is shown. LPS structures as observed in FhuA-LPS crystals [93] are also shown.
doi:10.1371/journal.ppat.1002966.g005

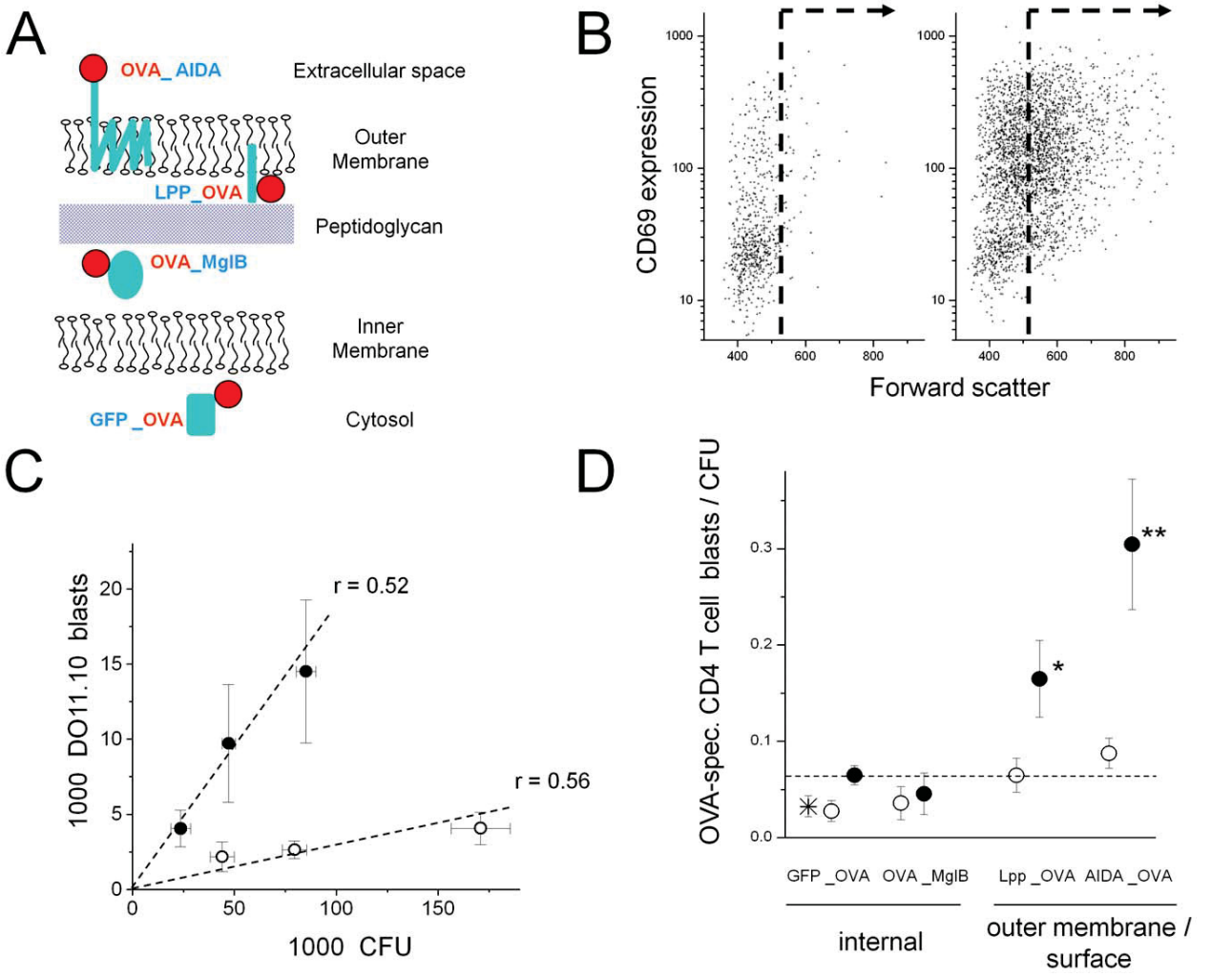

Figure 6. CD4 T cell responses to *Salmonella* expressing an ovalbumin model antigen in various compartments. A) Schematic overview of fusion proteins that target an immunodominant ovalbumin epitope (OVA) to various *Salmonella* cell compartments. **B**) Flow cytometric analysis of ovalbumin-specific CD4 T cell activation in a T cell receptor-transgenic adoptive transfer model. Mice were infected with control *Salmonella* expressing GFP (left) or *Salmonella* expressing LPP_OVA (right). Ovalbumin-specific transgenic CD4 T cells were detected with a clonotypic monoclonal antibody and analyzed for forward scatter and expression of the very early activation marker CD69. The dashed line was used to count CD4 T cell blasts. Similar observations were made for more than hundred mice in several independent experiments. **C**) Relationship between *Salmonella* Peyer's patches colonization and OVA-specific CD4 T cell induction in mice infected with *Salmonella* expressing high levels of LPP_OVA (filled circles) or low levels of GFP_OVA (open circles). Data represent means ± SEM's for groups of five to six animals from three independent experiments. CD4 T cell blasts correlated with *Salmonella* Peyer's patches colonization for both strains (Spearman test, $P<0.05$ in both cases). The slopes of the two curves differed (ANCOVA; $P<0.05$). **D**) OVA-specific CD4 T cell induction in mice infected with *Salmonella* expressing OVA at various levels (open circles, low abundance; filled circles, high abundance) in four different compartments. The dashed line represents CD4 T cell responses to saturating levels of cytosolic OVA. The star represents data for *Salmonella* expressing moderate levels of cytosolic OVA together with cholera toxin B and AIDA. Data represent means ± SEM's for groups of ten to twenty mice. Statistical significance of differences to *Salmonella* expressing saturating levels of cytosolic OVA were tested using Mann-Whitney U test (*, $P<0.05$; **, $P<0.01$).
doi:10.1371/journal.ppat.1002966.g006

data were in striking contrast to results from us and others demonstrating comparable immune responses to autologous *Salmonella* antigens from all *Salmonella* compartments (Fig. 1A,B). Furthermore, there was no obvious correlation between immunogenicity and survival times (Fig. 3A,B).

To better understand these discrepancies between model antigens and autologous *Salmonella* antigens, we re-visited the impact of antigen localization using a well-characterized, sensitive model system in which a MHC II-restricted T cell epitope from ovalbumin comprising amino acids 319 to 343 (OVA) is recognized by adoptively transferred cognate T cell receptor transgenic CD4 T cells [51,52]. We targeted the OVA epitope to different *Salmonella* compartments by fusing it to various proteins with known localization: GFP_OVA (cytosol [53]), OVA_MglB (periplasm [54]), Lpp_OVA (inner leaflet of the outer membrane [55]), and OVA_AIDA (outer leaflet of the outer membrane [56]) (Fig. 6A). To modulate expression levels, we used ribosome binding sites with differential translation initiation efficiency [12]. We expressed these fusion proteins from an in vivo inducible promoter [57] in an attenuated *Salmonella enterica* serovar Typhimurium *aroA* strain [58]. Antigen expression and localization was validated in in vitro cultures using cell fractionation followed

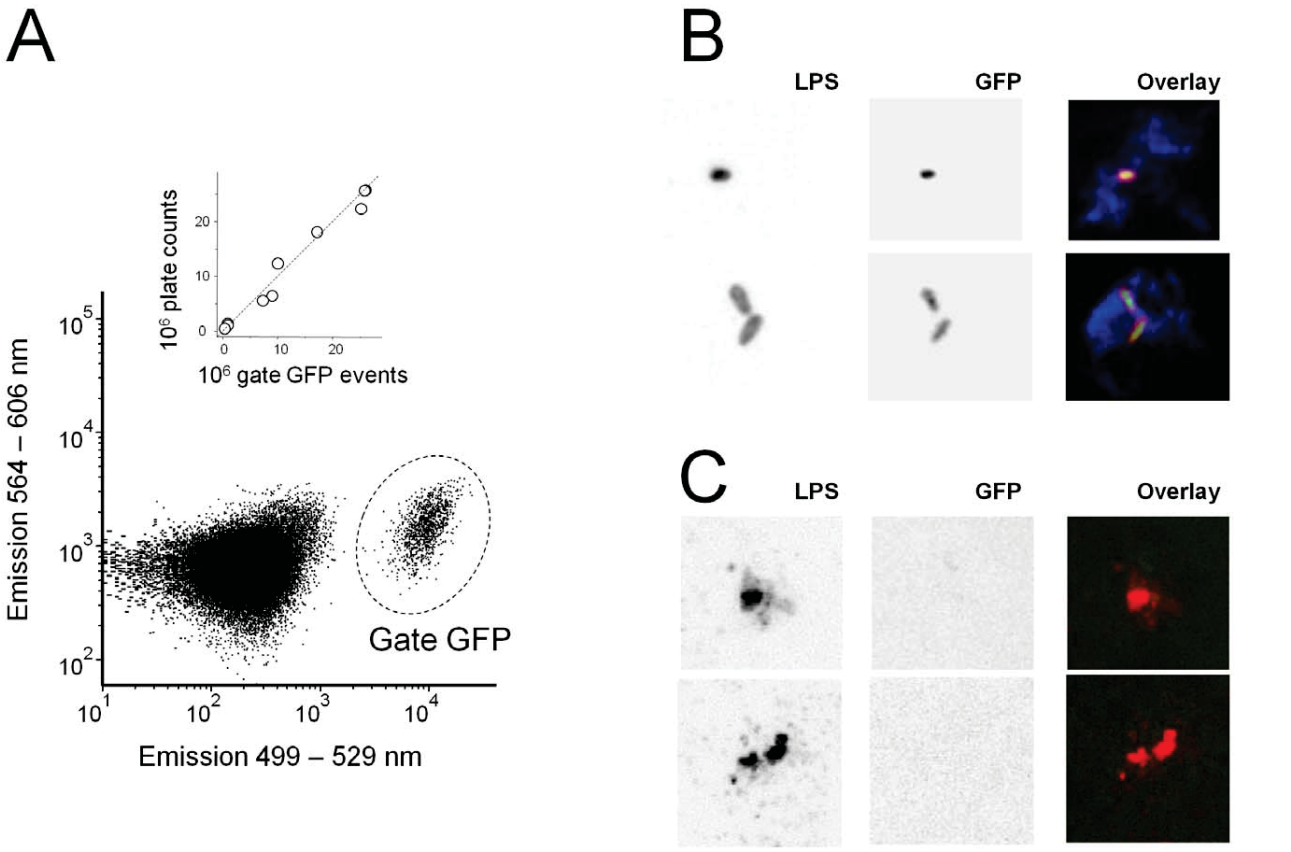

Figure 7. Detection of intact and damaged *Salmonella* cells in infected mouse tissues. A) Flow cytometry of a spleen homogenate infected with *Salmonella sifB::gfp* using 488 nm excitation. Gate 1 contains GFP-positive *Salmonella*. The inset shows the relationship between flow cytometry data and plate counts for individual mice, the dashed line represents a 1:1 ratio. **B**) Confocal micrographs of liver cryosections infected with *Salmonella sifB::gfp* that were stained with antibodies to *Salmonella* lipopolysaccharide (red) and macrophage marker CD68 (blue). Individual color channels are shown with inverted grey scale for better visualization of weak staining. Micrographs represent typical observations for four independently infected mice. **C**) Confocal micrographs of lipopolysaccharide-positive particles that lack detectable GFP (even when contrast was increased compared to B). Such particles were absent in non-infected control sections.
doi:10.1371/journal.ppat.1002966.g007

by western blotting, trypsin treatment, and antibody binding (Fig. S2). Interestingly, small fractions of both outer membrane antigens LPP_OVA and partially processed OVA_AIDA were released to the extracellular surroundings when expressed at high levels (Fig. S2C) in agreement with previous findings for similar proteins [59–61].

We infected BALB/c mice with *Salmonella* strains by intragastric gavage of 10^{10} CFU. All *Salmonella* strains colonized intestinal Peyer's patches with peak tissue loads of 3×10^4 to 1.5×10^5 CFU at day seven post infection as observed before for attenuated *Salmonella aroA* [62]. All constructs stably maintained their respective ovalbumin-expression plasmids (>80% at 7 days post infection). To determine antigen-specific CD4 T cell induction, we adoptively transferred OVA-specific TCR-transgenic CD4 T cells one day prior to *Salmonella* infection. OVA-specific T cells upregulated the early activation marker CD69 and formed blasts in mice infected with *Salmonella* expressing ovalbumin model antigens, but not in mice infected with control *Salmonella* (Fig. 6B) as observed previously [53]. CD4 T cell induction kinetics were similar for all constructs and consistent with our previous observations [53] suggesting a response to *Salmonella* in situ antigen expression, but not to the inoculum [57,63].

To compare T cell responses against the various *Salmonella* constructs, we measured T cell blast formation at peak *Salmonella* colonization at day seven post infection. *Salmonella* tissue loads varied somewhat between individual mice but for each construct, there was a linear relationship between the number of ovalbumin-specific DO11.10 blasts and *Salmonella* loads (Fig. 6C) in agreement with our earlier observations [57]. To determine the specific immunogenicity of each *Salmonella* strain, we calculated the average ratio of DO11.10 CD4 T cell blasts per viable *Salmonella* (i.e., the slopes in Fig. 6C) [57]. The data revealed comparable immunogenicity of model antigens GFP_OVA and OVA_MglB (Fig. 6D). In contrast, high-level expression of surface-associated LPP_OVA and OVA_AIDA induced superior responses that clearly surpassed responses even to saturating amounts [12] of internal GFP_OVA.

The OVA_AIDA fusion protein contained a fragment of the virulence factor AIDA from enteropathogenic *E. coli* and a cystein-deficient variant of the cholera toxin B subunit from *Vibrio cholerae* [64]. Both components might have stimulatory effects [65,66] that could potentiate ovalbumin immunogenicity. To test this potentially confounding factor, we compared *Salmonella* expressing a suboptimal level of cytosolic GFP_OVA [12] (some 54.000 copies per *Salmonella* cell) to *Salmonella* expressing the same amount of GFP_OVA together with AIDA and cholera toxin B. Both strains induced DO11.10 T cell blasts with similar efficacy (Fig. 6D)

suggesting that AIDA and cholera toxin B expression had no impact on the immunogenicity of *Salmonella*-encoded OVA.

Taken together, these findings suggested that antigens from all *Salmonella* compartments could induce specific CD4 T cell responses, but highly expressed outer membrane-associated antigens were clearly superior in agreement with previous observations in other model systems. However, these data were in striking contrast to responses to autologous *Salmonella* antigens (see discussion).

Distribution of intact and damaged *Salmonella* in infected tissues

The fundamentally superior protectivity of surface-associated *Salmonella* antigens might reflect their unique accessibility to antigen processing and presentation in infected host cells in contrast to internal *Salmonella* antigens that are shielded by the *Salmonella* envelope, and thus remain invisible for the host immune system until *Salmonella* is damaged and the bacterial cell breaks open. To detect intact and damaged *Salmonella* in infected tissues, we used cytosolic GFP as a marker for internal antigens.

Salmonella expressing GFP from the chromosomal in vivo induced locus *sifB* were readily detected in infected tissue homogenates using flow cytometry [12] (Fig. 7A). Flow cytometric counts for GFP^+ *Salmonella* closely correlated with viable counts as determined by plating (Fig. 7A, inset) suggesting that detectable GFP levels were present in all live *Salmonella*.

Confocal microscopy of infected spleen and liver sections revealed many particles that were stained by a polyclonal antibody to *Salmonella* lipopolysaccharide, had typical *Salmonella* size and morphology, and contained GFP (Fig. 7B) as previously observed [53] suggesting that these particles represented live intact *Salmonella*. In addition, we also detected numerous lipopolysaccharide-positive particles with distorted shapes that lacked detectable GFP (Fig. 7C), and likely represented killed and partially degraded *Salmonella*. Such particles were absent in non-infected control sections. Some *Salmonella* killing during acute infections had previously been proposed [67–69]. We observed some infected cells containing both intact and damaged *Salmonella*, but a large number of live *Salmonella* resided alone (or together with other live *Salmonella*) in infected cells with no detectable dead *Salmonella*. In such infected cells, internal *Salmonella* antigens were thus shielded and inaccessible for immune recognition.

Discussion

There is an urgent medical need for an efficacious *Salmonella* vaccine with broad coverage of invasive serovars. One important bottleneck in the development of such a vaccine is the identification of suitable protective antigens. In this study, we identified broadly conserved *S.* Typhi antigen candidates that prolonged survival after *S.* Typhimurium challenge infection in the mouse typhoid fever model. The protectivity of some of these candidates should be confirmed with larger experimental groups to select the best antigen candidates for vaccine development in future studies.

Two siderophore receptors (IroN, CirA) enabled the longest survival (Tab. 1) consistent with previous studies that revealed siderophore receptors including IroN as promising vaccine antigens in other models [70–72]. Interestingly, siderophore receptors are induced by iron starvation and/or activation of the PhoPQ two component sensory system [73]. IroN and CirA induction could thus contribute to increased protective efficacy of membrane preparations from iron-starved *Salmonella* [74], or live attenuated *Salmonella* $phoQ^{24}$ with constitutive hyperactivation of the PhoP response regulator [75].

On the other hand, all identified antigens still provided at most partial protection against challenge infection with virulent *Salmonella* suggesting a need for additional antigens. Unfortunately, protective *Salmonella* antigens might be rather rare as even among the 37 tested in vivo expressed antigens that were all highly immunogenic during infection, only a small minority enabled prolonged survival. OmpC, OmpD, and OmpF were previously proposed as potential protective antigens based on data obtained for enriched *Salmonella* membrane preparations. However, all three antigens failed to protect in our model. This could reflect higher stringency of our model (challenge infection with virulent *Salmonella* vs. highly attenuated mutant *Salmonella*), denatured three-dimensional structures of our recombinant antigen preparations vs. native antigens, and/or presence of undetected minor protective antigens (such as IroN and CirA) besides OmpC, OmpD, and OmpF in the previously used outer membrane antigen preparations.

Additional protective *Salmonella* antigens could be identified by comprehensive immunization/challenge experiments, but this would require extensive animal experimentation. Antigen priorization using relevant antigen properties could help to narrow down the number of antigen candidates to more practical numbers. Unfortunately, some previously proposed antigen properties seemed to have limited relevance for protectivity in our model. This included *Salmonella* in vivo expression levels, sequence-based antigenicity predictions, and immunodominance in convalescent individuals. Poor correlation of antigen immunodominance with protective efficacy has also been observed in tuberculosis [17]. On the other hand, immune recognition in convalescent individuals can still provide valuable information about antigen expression during at least some stage of infection that might be difficult to obtain otherwise [23,29]. Such data thus could greatly help to prioritize antigen candidates [26].

In contrast to antigen abundance and immunodominance, surface-association appeared to be an essential prerequisite. Surprisingly, some surface-associated proteins that enabled prolonged survival also included lipoproteins which were likely to reside in the inner leaflet of the outer membrane facing the internal periplasmic space with no exposure to the outside. It is possible that some lipoproteins might flip across the outer membrane as observed for other Gram-negative bacteria [76]. Moreover, some lipoprotein fraction is constantly released to the outside through outer membrane vesicle shedding [59,60].

Several mechanisms could contribute to the striking superiority of surface-associated antigens. Antibody responses are important for full protection against virulent *Salmonella* [10], and protective antibody responses must be directed against surface antigens [9]. On the other hand, CD4 T cells are even more important for immunity to *Salmonella* at least in the mouse typhoid fever model [10], and it is unclear why CD4 T cells should respond to surface-associated antigens in a fundamentally different way compared to the much larger number of internal antigens.

In fact, early cell culture experiments suggested no impact of *Salmonella* antigen localization on CD4 T cell recognition of infected cells [77]. However, in this study a large amount of antigen was already present in the inoculum, and rapid killing of the majority of phagocytosed *Salmonella* [78] would have released this antigen from all *Salmonella* compartments. Several subsequent in vivo studies suggested that surface-associated model antigens might have intrinsically higher immunogenicity compared to internal model antigens [30,46–50]. However, the various model antigen targeting constructs could have differed in antigen in vivo

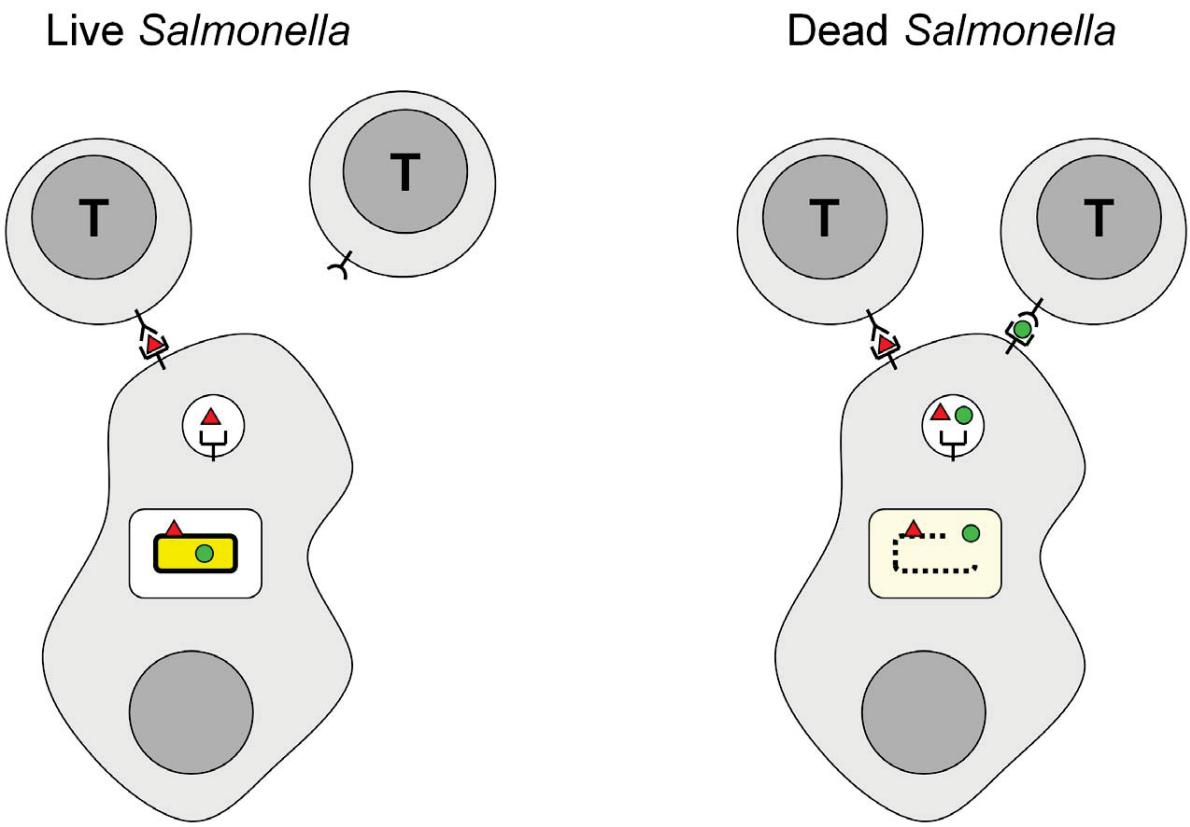

Figure 8. Schematic model for cellular immunity to *Salmonella*. *Salmonella* (yellow) reside in intracellular vacuoles in infected host cells. *Salmonella* possesses internal (green) and surface-associated (red) antigens. **Left**) Live *Salmonella* shield internal antigens, but some of their surface-associated antigens are accessible for processing and presentation. As a consequence, T cells specific for *Salmonella* surface antigens can recognize these infected cells and initiate antibacterial immune effector mechanisms. In contrast, T cells specific for internal *Salmonella* antigens fail to detect host cells that contain exclusively intact *Salmonella*. **Right**) Dead *Salmonella* release internal antigens. As a consequence, both surface-exposed and internal antigens can be processed, presented, and recognized by cognate T cells. However, this recognition is unproductive for infection control since it targets *Salmonella* that are already dead.
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expression levels, antigen stability, and epitope processing. Fusion partners could also have direct immunomodulatory effects. We therefore re-visited this issue and tried to control some of these factors. Our results clearly supported the previous finding of superior immunogenicity of highly expressed surface-associated model antigens in *Salmonella*.

In surprising contrast to these data from model antigens, however, humoral and cellular immune responses in *Salmonella*-infected convalescent mice did not show any bias for surface-associated autologous *Salmonella* antigens in this as well as in a recent large-scale study [26]. Broad recognition of antigens from all pathogens compartments has also been observed in *Salmonella* Typhi-infected or *Chlamydia*-infected human patients [24,25,29,43,44]. Model antigens and autologous antigens were also discordant with respect to the impact of antigen abundance. Specifically, our data for ovalbumin model antigens in this and a previous study [12], as well as similar findings for *Mycobacterium bovis* BCG overexpressing Ag85b [19], suggested that high in vivo expression levels enhance antigen immunogenicity. However, for autologous *Salmonella* antigens in vivo expression levels did not correlate with protectivity. Striking discrepancies between results for model antigens vs. autologous antigens have also been observed in other pathogens [38].

Some of the discrepancies could reflect technical issues. In particular, strong expression of foreign surface model antigens might induce subtle alteration in *Salmonella* in vivo properties such as increasing outer membrane vesicle shedding or alterations in protein secretion that could affect antigen presentation and immune recognition. Furthermore, model antigens might not be representative of autologous antigens that may have been shaped by host/pathogen co-evolution selecting for weak immunogenicity. Regardless of the actual causes of these discrepancies, our data indicated that in contrast to evidence from model antigens, protective *Salmonella* surface-associated antigens were not more immunogenic compared to internal antigens.

As an alternative explanation, surface-associated antigens might become more rapidly available for immune recognition compared to internal antigens that are only released after some pathogen damage. This could be relevant since early immune responses might facilitate infection control [32]. In the mouse typhoid fever model, however, a detectable fraction of *Salmonella* is rapidly killed early during infection as observed in this and previous studies [67,69] similar to events during *Mycobacterium* infection [79]. Consistent with these observations, CD4 T cell induction kinetics in the ovalbumin model system were similar for *Salmonella* strains with internal or surface-associated OVA-expression.

Instead, we propose an alternative explanation based on the observation that many live *Salmonella* resided alone, or together with other live *Salmonella*, in infected host cells with no dead *Salmonella* releasing their internal antigens. As a consequence,

Salmonella internal antigens remained inaccessible for antigen processing and presentation in these cells. In contrast, surface-exposed *Salmonella* antigens, or antigens released by outer membrane vesicle shedding, could be accessible for processing and presentation to cognate CD4 T cells for initiation of protective anti-*Salmonella* effector mechanisms (Fig. 8). In comparison, CD4 T cells recognizing internal *Salmonella* antigens would have limited impact on infection control because they miss many cells containing live *Salmonella* and instead direct their responses to host cells containing already dead *Salmonella*. According to this model, surface-associated antigens thus differ fundamentally from internal antigens because they are uniquely accessible in host cells containing only live *Salmonella*.

Surface-associated/secreted antigens have been shown to be crucial for CD8 T cell-dependent immunity to *Listeria* infection [31,33]. Our data suggested that such antigens might also be crucial for CD4 T cell mediated immunity to *Salmonella* and potentially other intracellular pathogens. Interestingly, some internal antigens have been shown to confer partial protection in infectious diseases caused by intracellular pathogens such as *Leishmania* [38] and *Mycobacterium* [37]. In these infections live and dead pathogens often co-occur in the same host microenvironments [80,81] suggesting that both internal and surface-associated antigens might be available for T cell recognition and initiation of antimicrobial immune effector mechanisms targeting both live and already dead pathogens [82]. We speculate that full protection might still require immune detection of all live pathogens including those that reside in microenvironments with yet no accessible internal antigens from dead pathogens. Further studies are required to test this hypothesis.

Conclusion

This study suggested novel *Salmonella* antigens that conferred partial protection against virulent *Salmonella* in a stringent typhoid fever model. High sequence conservation among relevant *Salmonella* serovars and cross-protection of serovar Typhi antigens against serovar Typhimurium challenge infection, suggested that some of these antigens might help to pave the way for a broadly protective vaccine against systemic *Salmonella* infection. In addition, our findings suggested that surface-associated antigens might represent particular promising antigens for both humoral and cellular immunity to *Salmonella*, since recognition of surface antigens uniquely enables detection and destruction of live *Salmonella* in relevant host microenvironments. This crucial importance of antigen localization could facilitate discovery of additional protective antigens for *Salmonella* and potentially other intracellular pathogens.

Materials and Methods

Ethics statement

All animal experiments were approved (license 2239, Kantonales Veterinäramt Basel-Stadt) and performed according to local guidelines (TschV, Basel) and the Swiss animal protection law (TschG).

Cloning, expression, and purification of *Salmonella* antigens

Antigens were PCR-amplified from *Salmonella enterica* serovar Typhi Ty2 (or *Salmonella enterica* serovar Typhimurium SL1344 [58] for *ompD*), cloned as His_6-fusions by conventional ligation into pET22b, or by Enzyme Free Cloning into plasmid pLICHIS [83], and overexpressed in *E. coli* BL21. GFP_His_6 was cloned as control antigen. Antigens were purified from washed inclusion bodies using immobilized metal ion affinity chromatography (Protino Ni TED 1000, Macherey Nagel) followed by ion exchange chromatography (Ion exchange spin columns, Pierce Thermo Scientific, cationic or anionic resins depending on antigen isoelectric point).

Proteome analysis of ex vivo sorted *Salmonella*

Salmonella expressing the green fluorescent protein (GFP) were sorted infected using flow cytometry as described [22]. Preparation of tryptic peptides and analysis by LC-MS/MS was done essentially as described [84] with some modifications. Given the limited sample material Protein LoBind tubes and pipette tips (Axygen) were used throughout the procedure. Frozen FACS sorted *Salmonella* pellets were resuspended in 15 µl lysis buffer (100 mM ammonium bicarbonate, 8 M urea, 0.1% RapiGest) and sonicated for 2×30 seconds. The released proteins were reduced and alkylated, and first digested for 4 hrs with sequencing grade LysC peptidase (10 ng/µl; Promega) before overnight trypsin digestion. The detergent was cleaved by adding 2M HCL and 5% TFA to final concentrations of 50 mM and 0.5% respectively, and incubating for 45 min at 37°C. Prior to centrifugation to remove the cleaved detergent (14,000×g, 10 min, 4°C) a mixture containing 32 heavy labeled reference peptides was added to the samples ($5*10^{-5}$ fmoles per *Salmonella* for expected "high" abundance proteins, $5*10^{-6}$ fmoles per *Salmonella* for expected "low" abundance proteins; Tab. S1). The recovered peptides were desalted on C18 reverse-phase spin columns (Macrospin columns, Harvard apparatus), dried under vacuum and subjected to LC-MS/MS using an LTQ-Orbitrap-Velos instrument (Thermo-Fischer Scientific). The amount of material analyzed in a single shot in the MS depended on the infection load, and corresponded to peptides derived from between $5*10^5$ and $2*10^6$ sorted *Salmonella*, plus contaminating mouse material which escaped detection in the cell sorter [22]. We analyzed samples from seven independently infected mice. In order to increase the number of *Salmonella* protein identifications, MS-sequencing was focused on previously identified peptides from *Salmonella* using the recently developed inclusion list driven workflow [84]. Each sample was analyzed twice in succession in the MS to verify technical reproducibility. Peptides and proteins were database searched against a decoy database consisting of the SL1344 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/*Salmonella*/), GFP_OVA, 204 frequently observed contaminants, all mouse entries from SwissProt (Version 57.12), and all sequences in reversed order (total 42502 entries) using the Mascot search algorithm. The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues); 2 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.5 Da for fragment ions. The false discovery rate was set to 1% for protein and peptide identifications. In addition to *Salmonella* proteins a substantial number of mouse proteins were identified in the samples as previously noted [22]. Absolute quantities were determined for those 18–20 "anchor" proteins that were detected along with a corresponding labeled AQUA peptide using the Trans-Proteomic Pipeline (TPP,V4.4.0). We then used the iBAQ method [40] to establish absolute quantities of all remaining protein identifications, with a linear model error of between 47 and 60%.

Construction of ovalbumin-expressing *Salmonella*

Translational fusions of the ovalbumin peptide containing amino acids 319 to 343 to various proteins with differential targeting in the *Salmonella* cell were constructed by PCR cloning. All fusion genes were cloned into a pBR322-derived plasmid

backbone [53] downstream of a *Salmonella* genome fragment containing the in vivo inducible *pagC* promoter [57] and ribosomal binding site 1 (AAGAA) or 2 (AGCAG) for low or high translation initiation efficiencies [12]. To generate *ova_aida*, coding sequence for the ovalbumin peptide (*ova*) was inserted between the signal peptide derived from cholera toxin B and the HA tag in plasmid pLAT260 [85]. A control plasmid coding for CTB_AIDA and GFP_OVA was also constructed. To generate *lpp_ova*, *lpp* without the C-terminal lysine codon that can cross-link to peptidoglycan [86], was amplified from *E. coli* DH5α and fused with *ova* and a C-terminal HA tag. To generate *ova_mglB*, *mglB* gene without the signal peptide sequence was amplified from *E. coli* DH5α and fused with a *ctB* signal sequence followed by *ova* and the HA tag. The construction of *gfp_ova* has been described [87]. The various plasmids were transformed into attenuated *Salmonella enterica* serovar Typhimurium aroA SL3261 [58].

Biochemical analysis

Ovalbumin expression was assessed by western blotting with a polyclonal antibody to ovalbumin (Sigma) that recognizes the OVA peptide comprising amino acids 319 to 343 [53]. *Salmonella* outer membranes were prepared by extraction with L-lauryl sarcosinate as described [85]. Periplasm was prepared by chloroform extraction as described [88]. Culture supernatants were sterile filtered (0,2 µm pore size) and subjected to TCA precipitation [89]. To assess ovalbumin surface accessibility, intact or lysed *Salmonella* cells were treated with 50 µg ml^{-1} trypsin at 37°C for 10 min. In addition, *Salmonella* were stained with an antibody to the HA tag, and examined by fluorescence microscopy.

Immune responses in convalescent mice

Female 8 to 12 weeks old 129/Sv mice were obtained from Charles River. Mice were orally infected with 10^9 CFU *Salmonella enterica* serovar Typhimurium SL1344 [58] from late log cultures using a round-tip stainless steel needle. Control mice were sham-infected. Mice were sacrificed 6 months after infection. Splenocytes were isolated and tested for antigen-specific CD4 T cell responses as described [41]. Unstimulated T cells from convalescent mice as well as antigen-stimulated T cells from naïve control mice showed only weak background responses (Fig. S1). Some antigens gave also weak responses for T cells from convalescent mice (depending on the individual mouse). Together, these data suggested that antigen-nonspecific background responses to *E. coli* contaminants that might have been present in trace amounts in our antigen preparations did not result in unspecific T cell responses in our assay. Plasma was tested for antigen-specific IgG responses using ELISA with an IgG calibration curve for absolute quantification.

Immunization and challenge experiments

Female, 8 to 12 weeks old BALB/c mice were obtained from Charles River. Groups of 5 mice were immunized subcutaneously with 10 µg antigen emulsified in complete Freund's adjuvant followed by a second immunization with incomplete Freund's adjuvant four weeks later. After additional four weeks, mice were orally infected with 6×10^5 CFU *Salmonella enterica* serovar Typhimurium SL1344 [58] from late log cultures using a round-tip stainless steel needle. Infected BALB/c were monitored twice daily and sacrificed when moribund.

Ovalbumin-specific CD4 T cell responses

BALB/c and DO11.10 mice [51] were bred in the Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Berlin, Germany) under specific-pathogen free conditions. Adoptive transfer of 4×10^6 DO11.10 T cells into syngenic age- and sex-matched BALB/c mice was performed one day before infection as described [53]. For infection, attenuated *Salmonella* strains carrying expression cassettes for various ovalbumin fusion proteins were grown to late log phase and harvested. Bacteria were washed twice and resuspended in LB containing 3% sodium bicarbonate. Doses containing ca. 10^{10} cfu in 100 µl were administered intragastrically to chimeric mice with a round-tip stainless steel needle. At various time points post infection, mice were anesthetized and sacrificed. DO11.10 T cell blast formation was determined by flow cytometry as described [53]. Aliquots of the same Peyer's patch preparations were treated with 0.1% Triton x-100 to release intracellular *Salmonella* for CFU determination by plating, and for quantitation of GFP_OVA *in vivo* expression levels by two-color flow cytometry as described [87]. Many TCR tg models show substantial clonal expansion upon antigen stimulation. However, in our *Salmonella* model we observe only weak and variable accumulation of tg CD4 T cells in infected tissues which might reflect the fact that even at peak *Salmonella* loads only about 1 ng antigen is present [87]. Instead, blastogenesis as measured by CD69 upregulation and increased forward scatter provides a sensitive antigen-specific readout.

Detection of intact *Salmonella* in infected tissues

BALB/c mice with *Salmonella* loads of 10^6 to 10^7 in spleen and liver were sacrificed. 10 µm cryosections were stained with polyclonal rabbit antibodies to *Salmonella* lipopolysaccharide (SIFIN) and anti-CD68 (abcam) followed by Alexa 546-conjugated goat anti-rabbit and Alexa 647-conjugated goat anti-rat antibodies (Invitrogen). Sections were examined by confocal microscopy (Leica, SP5).

Structural models and epitope prediction

Structural models for selected *Salmonella* outer membrane antigens based on solved structures of homologues were obtained from SWISS-MODEL [90] available at http://swissmodel.expasy.org. Linear B-cell epitopes were predicted using FBCPred [91] available at http://ailab.cs.iastate.edu/bcpreds/predict.html using an epitope length of 14 and 90% specificity. Peptides that bind to MHC II $I\text{-}A^d$ and/or $I\text{-}E^d$ were predicted using RANKPEP [92] available at http://imed.med.ucm.es/Tools/rankpep.html with a binding threshold yielding 85% sensitivity for detection of well-defined epitopes in MHCII haplotype databases (the default setting of RANKPEP).

Supporting Information

Figure S1 Representative antigen-specific CD4 T cell responses in convalescent and control mice. Cells were stimulated with various antigens (shown are examples for T2461 and T0937). CD4, CD154-double positive cells representing responding cells were gated (upper panels) and analyzed for IFNγ and IL-17 (lower panels). Background responses in control mice were subtracted form responses in convalescent mice and reported in Tab. 1.
(TIF)

Figure S2 Expression and localization of ovalbumin epitope fusion proteins in *Salmonella*. A) Anti-ovalbumin immunoblot of total *Salmonella* cell lysates (3×10^7 cfu) of strains expressing either low ("lo") or high ("hi") levels of ovalbumin fused to different proteins. Expected molecular weights were: GFP_OVA, 30 kDa; OVA_MglB, 38 kDa; Lpp_OVA, 11 kDa; OVA_AIDA, 67 kDa. **B**) Localization of various fusion proteins. OVA_MglB was detected in isolated periplasm fractions (Ppl.) in similar quantities as in whole cell lysates (Lys.). Lpp_OVA was detected in isolated outer

membrane fractions. It was unaccessible for trypsin degradation in intact *Salmonella* but readily digestible in isolated membrane fractions. Immunostaining of intact *Salmonella* with a fluorescent antibody showed no detectable signal. OVA_AIDA was detected in isolated outer membranes and accessible to trypsin digestion even in intact *Salmonella* suggesting surface localization. This was confirmed by immunostaining. **C**) Immunoblot of culture supernatants of 4.5×10^{11} CFU (TCA precipitation). Endogenous *Salmonella* proteins with apparent molecular weights of ca. 23 and 67 kDa, respectively, cross-react with the anti-ovalbumin polyclonal antibody (empty arrowheads). These bands were also detected in non-recombinant *Salmonella*. In addition, an OVA-containing protein of around 11 kDa was released from Lpp_OVA expressing *Salmonella* (black arrowhead), whereas a 30 kDa fragment was released from *Salmonella* expressing high amounts of OVA_AIDA.
(TIF)

Table S1 List of isotope labeled peptides used for protein quantification.
(XLS)

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Author Contributions

Conceived and designed the experiments: SB YW KR AKS DK AS NB DB. Performed the experiments: SB YW KR BC AKS AM AS DK NB. Analyzed the data: SB YW KR AKS DK AS NB DB. Wrote the paper: SB KR DB.

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2.2.3 Additional Results

Supplementary Figure 1 (Fig S1)

Representative antigen-specific CD4 T cell responses in convalescent and control mice.

Cells were stimulated with various antigens (shown are examples for T2461 and T0937). CD4, CD154-double positive cells representing responding cells were gated (upper panels) and analyzed for IFNγ and IL-17 (lower panels). Background responses in control mice were subtracted form responses in convalescent mice and reported in Tab. 1.

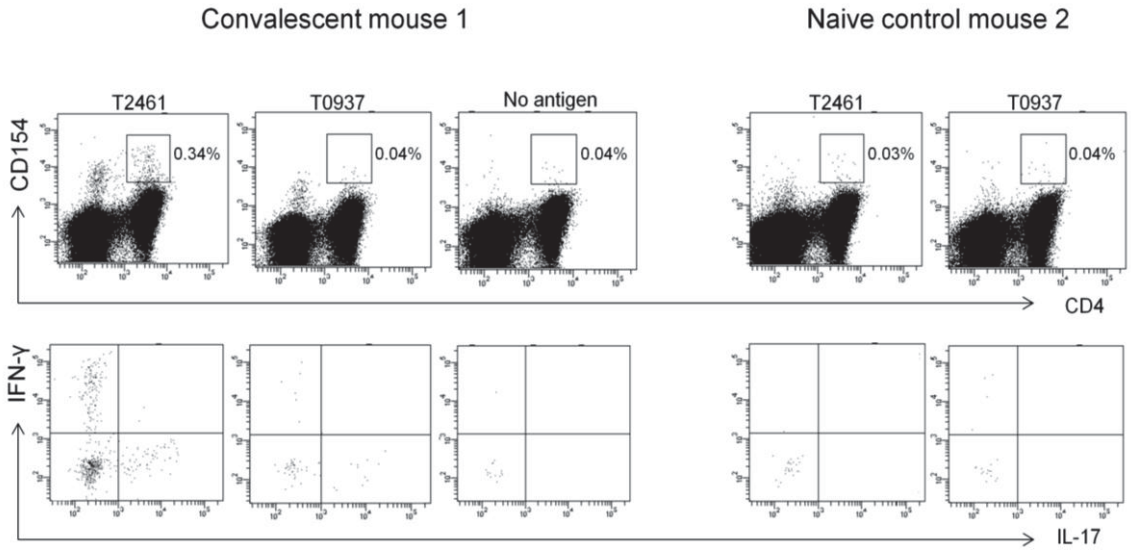
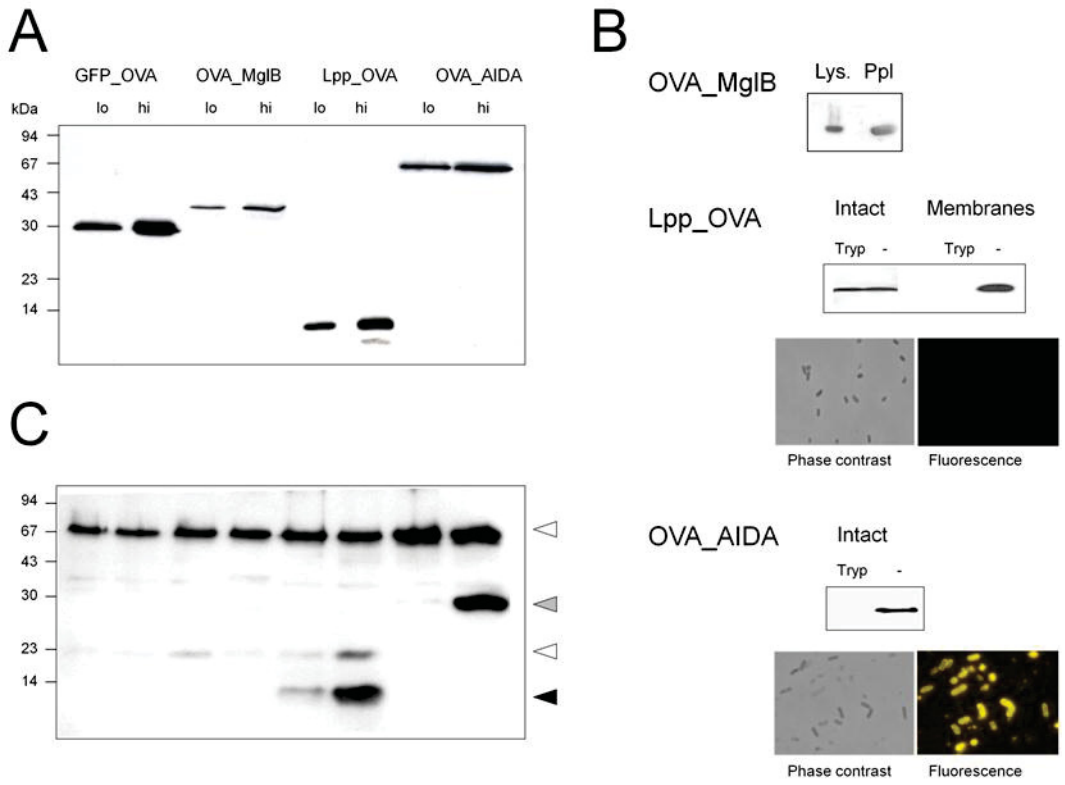


Figure S1

Supplementary Figure 2 (Fig S2)

Expression and localization of ovalbumin epitope fusion proteins in *Salmonella*. **A**) Anti-ovalbumin immunoblot of total *Salmonella* cell lysates (3×10^7 cfu) of strains expressing either low ("lo") or high ("hi") levels of ovalbumin fused to different proteins. Expected molecular weights were: GFP_OVA, 30 kDa; OVA_MglB, 38 kDa; Lpp_OVA, 11 kDa; OVA_AIDA, 67 kDa. **B**) Localization of various fusion proteins. OVA_MglB was detected in isolated periplasm fractions (Ppl.) in similar quantities as in whole cell lysates (Lys.). Lpp_OVA was detected in isolated outer membrane fractions. It was unaccessible for trypsin degradation in intact *Salmonella* but readily digestible in isolated membrane fractions. Immunostaining of intact *Salmonella* with a fluorescent antibody showed no detectable signal. OVA_AIDA was detected in isolated outer membranes and accessible to trypsin digestion even in intact *Salmonella* suggesting surface localization. This was confirmed by immunostaining. **C**) Immunoblot of culture supernatants of 4.5×10^{11} CFU (TCA precipitation). Endogenous *Salmonella* proteins with apparent molecular weights of ca. 23 and 67 kDa, respectively, cross-react with the anti-ovalbumin polyclonal antibody (empty arrowheads). These bands were also detected in non-recombinant *Salmonella*. In addition, an OVA-containing protein of around 11 kDa was released from Lpp_OVA expressing *Salmonella* (black arrowhead), whereas a 30 kDa fragment was released from *Salmonella* expressing high amounts of OVA_AIDA.


A
GFP_OVA
OVA_MglB
Lpp_OVA
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94
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B
OVA_MglB
Lys. Ppl
Lpp_OVA
Intact
Membranes
Tryp -
Tryp -
Phase contrast
Fluorescence
OVA_AIDA
Intact
Tryp -
Phase contrast
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94
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Figure S2

Discussion

3. Discussion

The discussion of the thesis is divided into two parts (Part A and Part B).

Part A: Discussion on the identification of suitable targets to eradicate persisting *Salmonella* (refer to section 3.1).

Part B: Discussion on the identification of predictive antigen properties that could help to develop a safe, enteric fever vaccine (refer to section 3.2).

Discussion

3.1 Part A - Identification of suitable targets to eradicate persisting *Salmonella*

Chronic infections pose a major obstacle for the treatment of infectious diseases. Complete eradication requires long–term chemotherapy causing poor patient compliance, emergence of antibiotic resistance, and failures to prevent relapse of the disease. Chronic infections are caused by persistent bacteria that are often drug tolerant, and may have limited or no replication and metabolic activities. As a consequence, most of the common antibiotics that target replicating bacteria fail to cure persistent infections. Hence, new targets for antibiotic development against persistent bacteria are required, but it is unclear if persisters have any essential maintenance requirements for survival that could provide opportunities for eradication through antimicrobial chemotherapy.

Indeed, the lack of well defined targets has been a major obstacle in the development of new antimicrobials against chronic infections [69]. On the other hand, few individual maintenance function requirements have been suggested in various pathogens like *Mycobacterium tuberculosis*, *E.coli* etc [73, 81, 83]. However, experimental evidence directly supporting these predictions is fragmentary and inconsistent because suitable *in vivo* models required for such studies are largely lacking.

Few *in vivo* models have actually been developed for *M. tuberculosis*, but it is controversial how well these models mimic relevant conditions during chronic infections [54]. For pathogens other than *M. tuberculosis*, there are no validated models to study persistency *in vivo*, representing a major challenge for drug development [136].

In this study, we established a practical *in vivo Salmonella* mouse infection model in which *Salmonella* with metabolic and virulence defects persisted at constant tissue loads. In this model, *Salmonella* subpopulation even survived chronic treatment with floroquinolone, the most potent antibiotic to eradicate persistent salmonellosis indicating stringency of our model. The *Salmonella* metabolic network contains more than 1200 different enzymes that could all represent potential antimicrobial targets. However, even during acute infection, only a very small fraction of these enzymes are important for *Salmonella* physiology and could thus qualify as potentially suitable targets [137]. So, persisters being antibiotic tolerant, and metabolically less active might shrink the list of potential targets even further. Evaluation of twelve major *Salmonella* defects revealed extremely relaxed requirements confirming limited targets for antimicrobial chemotherapy of chronic *Salmonella* infections. On the other hand, we identified few novel candidates that might be explored for their suitability to control chronic infections. In particular, a defect in the unsaturated fatty acid resulted in rapid clearance of *Salmonella* suggesting that this pathway might contain promising targets for antibiotic therapy. Requirement of unsaturated fatty acid synthesis suggests continuous internal turnover, damage, and/or loss of the membrane to the environment during persistency. Damage/loss of the membrane has been already proposed as a potential target for treating persistent infections [138]. As an alternate mechanism, membranes could also be continuously lost through shedding of outer membrane vesicles [139]. However, our data indicated that lipopolysaccharide synthesis (outer membrane component of *Salmonella*) may not be needed during persistence. Further studies are required to clarify the function of fatty acid synthesis during *Salmonella* persistency.

Interestingly, a study indicated that major gram–positive pathogens overcome growth inhibition by antimicrobials targeting the FASII (fatty acid synthesis type II present only in bacteria) pathway when unsaturated fatty acids are available from the host. Hence, it is important to note that both saturated and unsaturated host fatty acid might be sufficiently available in other infectious disease models especially for extracellular pathogens[140].

Additional targets for antimicrobial chemotherapy of chronic salmonellosis could be rapidly tested with our model. However, our data revealed extensive robustness of persisting *Salmonella* against perturbation suggesting a very limited target space (Fig 8). Possibly, target combinations might help to break the redundancy for persister survival but this remains to be tested in further studies.

To summarize, we have established a practical, highly stringent, *in vivo* persistency model that enabled us to test a number of promising target candidates. However, the data revealed that it is clearly very difficult to find suitable targets because persisting *Salmonella* had very limited maintenance requirements. On the other hand, our results also revealed few candidates that might be further analyzed for their suitability to control chronic infections.

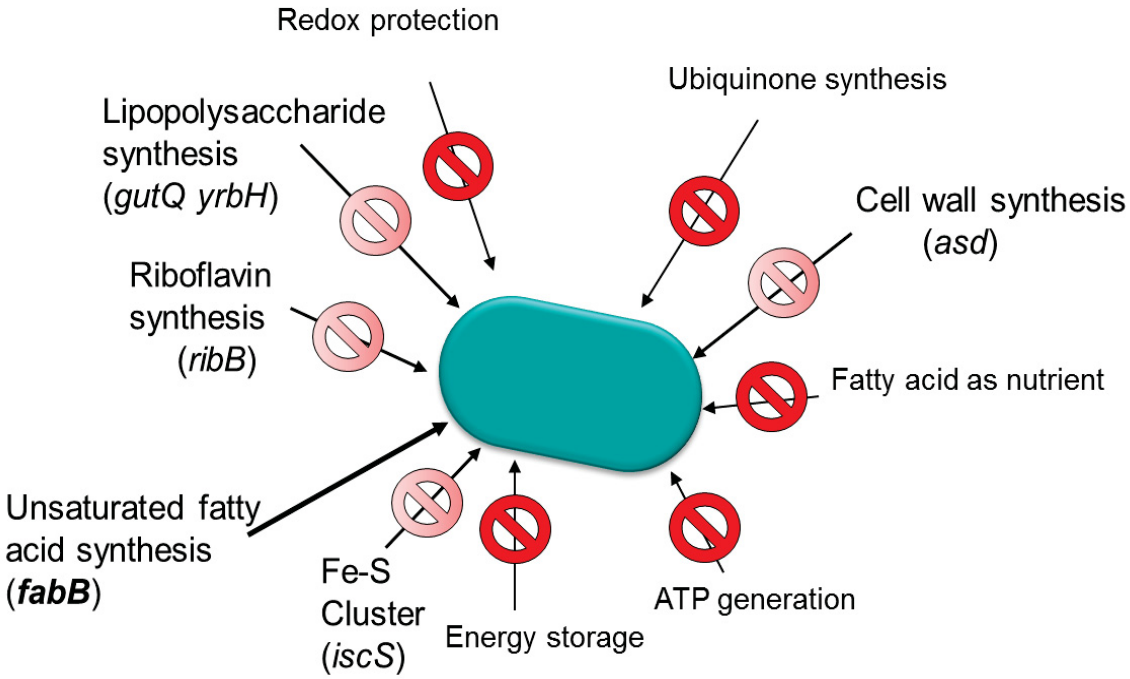

Figure 8. Targets against persistency. Picture showing most of the activities that are dispensable during persistency (deep red). Few genes had moderate persistent phenotype (light red). Only unsaturated fatty acid (*fabB*) synthesis had a more promising phenotype.

Discussion

3.2 Part B - Identification of predictive antigen properties that could help to develop safe, enteric fever vaccine

Enteric fever remains a significant cause of human death and disease throughout large areas of the world. Adding to the public health problem posed by enteric fever has been the increasing antibiotic resistance, and the emergence of *Salmonella* serovars that are not covered by currently available vaccines. Hence, there is an urgent need to develop a new vaccine with broad serovar coverage [1].

Both, humoral and $CD4^+$ T cell mediated immune response are required for protection against virulent *Salmonella* [125]. Vaccine development has been hampered by the slow, largely empirical search for protective antigens. The major challenge is the identification of protective antigens among thousands of candidates predicted from the genome sequences. However**,** protective antigens are generally rare, and antigen properties that are relevant for T cell recognition remain poorly uncharacterized [128]. Promising antigens could be identified by mass immunization/challenge experiments. Indeed, this approach was used to screen one hundred randomly selected antigens as DNA vaccines against *Leishmania major* infection [141]. However, this approach revealed only very few protective antigens, and required extensive animal experimentation. Alternatively, suitable antigen properties might facilitate prioritization of promising candidates. Antigen properties such as *in vivo* expression levels, sequence-based antigenicity, immunodominance in covalescent individuals, and antigen localization have previously been proposed to prioritize vaccine candidates. However, the relevance of these antigen properties for protective immunity to *Salmonella* remained unclear because *Salmonella* antigens with

differential properties have not yet been systematically compared in immunization/challenge experiments.

In this study, we did such experiments to determine relevant antigen properties in a *Salmonella* infected mouse typhoid fever model.

We compared 37 *in vivo* expressed *Salmonella* antigens for immunogenicity and protectivity in a mouse typhoid fever model. We identified two novel surface associated antigen candidates (IroN and CirA) that enabled prolonged survival in the immunization/challenge experiment. On the other hand, all identified antigens were only partially protective against challenge infection with virulent *Salmonella* suggesting a need for identification of more antigens. Unfortunately, among 37 tested *in vivo* expressed antigens that were highly immunogenic during infection, only five conferred protection reflecting that protective *Salmonella* antigens might be very rare. The results revealed that previously proposed antigen properties including high *in vivo* expression levels, immunodominance in covalescent individuals, and sequence based antigenicity failed to protect protective antigens. On the other hand, surface association appeared to be absolutely critical for antigen protectivity in contrast to the other antigen properties.

Antigen prioritization with relevant antigen properties might help to focus on a small number of promising candidates. In many infectious diseases, surface associated antigens are preferred as vaccine candidates. Several studies have suggested that surface associated model antigens might have higher immunogenicity compared to the internal model antigens [142, 143]. Indeed, our ovalbumin model also suggested superior immunogenicity of highly expressed surface antigens compared to the internal ones. However, these results were in marked contrast to humoral and cellular immune responses to autologous *Salmonella* antigens in covalescent mice.

Furthermore, the protective antigens were not immunodominant. These data clearly indicated that protective surface associated *Salmonella* antigens were not more immunogenic than internal antigens. However, surface associated antigens might have the advantage that they can be readily accessible for immune recognition compared to the internal antigens which are only released after the pathogen has been killed and degraded. Recognition of surface exposed antigens might thus be necessary to detect and kill live *Salmonella* that shield their internal antigens.

To conclude, our study identified novel *Salmonella* antigens that conferred partial protection against virulent *Salmonella* challenge. This might help to pave the way for protective vaccines against systemic *Salmonella* infection with broad serovar coverage. Additionally, our data also suggested a strategy for identification of protective *Salmonella* antigens that might be also relevant for other intracellular pathogens like *Mycobacteria*, *Chlamydia*, *Listeria* etc [144] that require both humoral and cellular immunity for protection.

The discussion (Part A and Part B) was mainly focussed on the two research publications.

In part A, I focussed on the results in Research publication I: Extensive in vivo resilience of persistent *Salmonella*.

In part B, I discussed about the data obtained in Research publication II: Immunity to intracellular *Salmonella* depends on surface associated antigens.

Appendix

4. Appendix

4.1 Abbreviations

ATP	Adenosine triphosphate
B cell	B lymphocyte
CD4$^+$ T cell	T cells expressing CD4 protein on their surface.
CD8$^+$ T cell	T cells expressing CD8 protein on their surface.
DFI	Differential fluorescence induction
E. coli	*Escherechia coli*
E. faecalis	*Escherechia faecalis*
FAS II	Fatty acid synthesis type II
IgA	Immunoglobulin A
IgM	Immunoglobulin B
IVET	*In vivo* expression technology
IVIAT	*In vivo* induced antigen technology
LPS	Lipopolysaccharide
M cells	Microfold cells
M. bovis	*Mycobacterium bovis*
M. tb	*Mycobacterium tuberculosis*
MDR	Multidrug–resistant
MHC	Major histocompatibility complex
Nramp	Natural resistance associated macrophage protein
NTS	Non-typhoidal salmonellosis
SCID	Severe combined immunodeficiency
S. typhi	*Salmonella enterica* serovar typhi
S. typhimurium	*Salmonella enterica* serovar typhimurium
SERPA	Serological proteome analysis
SPI 1	*Salmonella* pathogenecity island 1
SPI 2	*Salmonella* pathogenecity island 2
STM	Signature – tagged mutagenesis
T cell	T lymphocyte
TA	Toxin antitoxin

TCA	Tricarboxylic acid
TCR	T cell receptor
Th	T helper cell
VBNC	Viable but non culturable

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Curriculum Vitae

7. Curriculum Vitae

Somedutta Barat

Private address
J/K-6 Hatiara Road,Aswininagar
Baguiati – 700159. Kolkata. India

Email: barat.somedutta@gmail.com

Date of Birth: July 20th, 1984
Nationality: Indian

Education and Research Experiences

08/2007 – 03/2012	PhD research project Focal area infection biology, Biozentrum University of Basel, Switzerland. Supervisor: Prof. Dirk Bumann Committee members: Prof. Christoph Dehio and Prof. Urs Jenal. Title: *Target identification for prevention and therapy of Salmonella infections*
01/2007 – 07/2007	Joined Doctoral program IRTG 1273 Medizinische Hochschule Hannover (MHH) Germany Supervisor: Dr Dirk Bumann (Research group relocated to Biozentrum, CH)
09/2005- 01/2007	Master of Science,Molecular Genetics University of Leicester, United Kingdom. Supervisor: Prof. Wilhelm. J. Schwaeble, Dept of Infection, Inflammation, and Immunity Title: *Transgene expression of minigene constructs expressing two allelic variants of human complementary complement, C3 in a gene targeted C3 deficient mouse line*
06/2002 – 07/2005	Bachelor of Science, Biotechnology, University of Bangalore, PES Institute of Applied Sciences (PESIAS) India. a) Training in *In vitro culture of breast cancer (MCF-7) cell lines.* Chittaranjan National Cancer Institute, India.

Education and Research Experiences (continued)

b) Training in *analysis of pathological samples in hospital patients*,Woodlands hospital and Medical research centre, India.
c) Internal undergraduate project in *Terminator gene technology*, PES Institute of Applied Sciences (PESIAS)
Bangalore, India.

03/2000 – 04/2002 — Higher Secondary Studies
Serampore College
Kolkata, India.

Teaching experience

2007 – 2011 — Assistant and tutor for undergraduate students in Microbiology courses once a year.
Biozentrum, University of Basel, Switzerland.

Oral Presentations

Somedutta Barat, Benjamin Steeb, and Dirk Bumann
Molecular mechanisms of persistent *Salmonella* infections
69th Annual Assembly of SSM (Swiss Society for Microbiology)
ETH, Zurich, Switzerland (2010).

Publications

Somedutta Barat, Benjamin Steeb, Dirk Bumann
"Extensive in vivo resilience of persistent *Salmonella*".
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