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Influence of oral boost immunizations with recombinant *Salmonella* vaccine strains on the antigen-specific CD8 T-cell induction

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CONTENTS

LIST OF ABREVIATIONS

A	Amper
Abs	Antibodies
AEC	3-Amino-9-ethylcarbazole
Ag	Antigen
Alpha Mem	Alpha Modified Eagle Medium
Amp	Ampicillin
APC	Antigen presenting cell
APS	Ammonium persulphate
BHI	Brain Heart infusion
BSA	Bovine Serum Albumine
CDC	Center for disease control
CFU	Colony forming unit
ConA	Concanavalin A
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
dH_2O	Distilled water
DMF	*N*,*N*-dymethylformamide
DNA	Deoxyribonucleic acid
DMEM	Dulbeccos modified Eagle Medium
DMSO	Dimethylsulfoxid
EDTA	Ethylendiamintetraacetic acid
ER	Endoplasmic reticulum
EtOH	Ethanol
FCS	Fetal calf serum
Fig.	Figure
g	Gram
HRP	Horseradish peroxidase
i.d.	Intradermal
i.m.	Intramuscular
i.v.	Intravenous
IL	Interleukine

IFN-γ	Interferon gamma
kB	Kilobase
kDa	Kilodalton
Kan	Kanamycin
kV	Kilovolt
l	Liter
LB	Luria Bertani Medium
LD	Lethal dose
LD_{50}	50% of lethal dose
LLO	Listereolysin O
LPS	Lipopolysaccharide
M	Molar
MALT	Mucosa-associated lymphatic tissues
MHC	Major hiscompatibility complex
Min	Minute
mM	Milimolar
mm	Milimeter
mRNA	Messenger ribonucleic acid
µl	microliter
nm	nanometer
OD_{600}	Optical density at 600 nm wavelength
o/n	overnight culture
p60	Listeria protein
PAI	Pathogenicity island
PAMPs	Pathogen-associated molecular patterns
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pH	Power of hydrogen
PMN	Polymorphonuclear leukocyte
rpm	revolutions per minute

RT	Room temperature
s.c.	subcutaneous
SCVs	*Salmonella*-containing vacuole
SDS	Sodium dodecyl sulphate
Sec	second
SPI-1	*Salmonella* pathogenicity island 1
SPI-2	*Salmonella* pathogenicity island 2
Spp.	Species
Sm	Streptomycin
TAE	Tris-Acetat-EDTA Buffer
TCA	Trichloracetic acid
TEMED	*N,N,N',N'*-Tetramethylendiamine
T_H	T helper lymphocyte
Tris	Tris-hydroxymethyl-aminomethane
T3SS	Type Three Secretion System
Tween 20	Polyoxyethylensorbitanmonolaureate
Yop	*Yersinia* outer protein
WHO	World Health Organisation

A. INTRODUCTION

1. Infectious diseases and vaccination

According to the World Health Organization (WHO), infectious diseases are still the major cause of death worldwide, since a third of global deaths are due to microbial agents (see Fig. 1) (209).

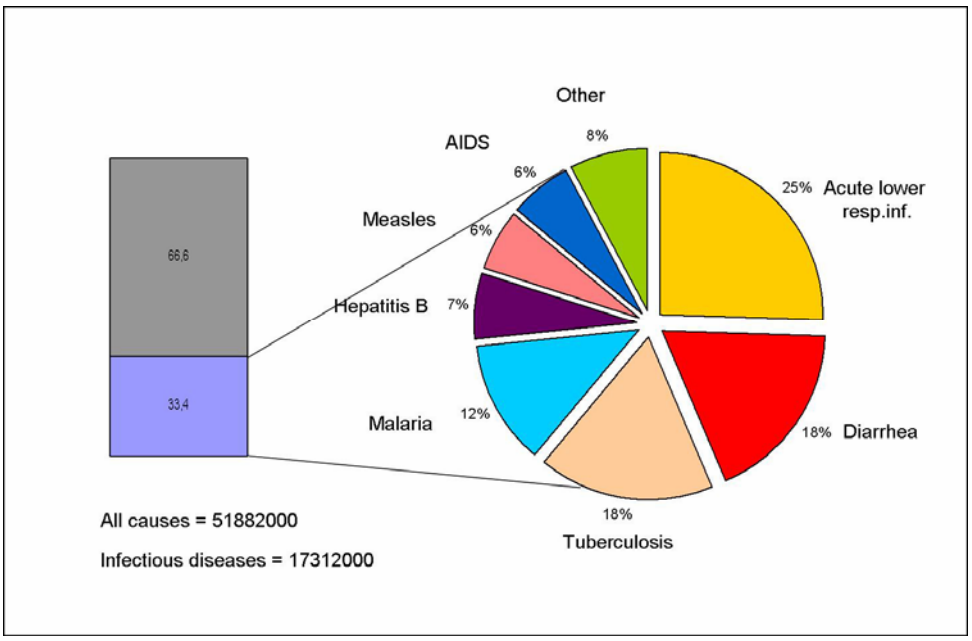

Fig. 1. Global deaths due to selected infectious diseases.

Combating infectious diseases involves prophylaxis and/or therapy. Treatments options for infectious diseases include administration of antibacterial, anti-viral and anti-parasitic drugs as well as symptomatic treatment for example, rehydration therapy in case of diarrhoea. Although drug therapy minimizes the percentages of mortality and morbidity by shortening the period that an infected individual remains infectious to others, there are still several infectious diseases without effective treatment and in addition, multiple drug resistances have emerged in the last decades. In conclusion, infectious diseases still remain a major threat to global health and for this reason, preventive measures are required to keep infectious diseases under control. Prophylactic strategies against infectious diseases

include improvement of public health and the use of vaccines. On one hand, the strength of sanitation conditions has dramatically reduced the toll of water- and food-borne diseases as well as the risk of vector-borne diseases (71). On the other hand, the implementation of extensive routine immunizations has led to eradicate infectious diseases and has prevented suffering, disability and death on a large scale (see Table 1). In fact, it has been the only method that accomplished the global eradication of smallpox in 1977 after a 10-year campaign carried out by the WHO (48). In addition, polio infections have been fallen by more than 99%, and some 5 million people have escaped paralysis. Measles has been also eliminated from the Americas and illness and mortality attributed to diphtheria, tetanus and pertussis have significantly decreased.

Table 1. Baseline 20th century annual morbidity (case of disease) before the vaccine became available and 1998 morbidity from 8 diseases with vaccine recommended before 1990 for universal use in children (reprinted from MMWR 48:243-248, 1999 (25)).

Disease	Baseline 20th century annual morbidity	1998 morbidity	% Decrease
Smallpox	48164[a]	0	100
Diphteria	175885[b]	1	100[c]
Pertussis	147271[d]	6279	95.7
Tetanus	1314[e]	34	97.4
Poliomyelitis (paralytic)	16316[f]	0[g]	100
Measles	503282[h]	89	100
Mumps	152209[i]	606	99.6
Rubella	47745[j]	345	99.3
Meningitis	20000[k]	54[m]	99.7

[a] Average annual number of cases during 1900-1904.

[b] Average annual number of reported cases during 1920-1922, 3 years before vaccine development.

[c] Rounded to nearest tenth.

[d] Average annual number of reported cases during 1922-1925, 4 years before vaccine development.

[e] Estimated number of cases based on reported number of deaths during 1922-1926, assuming a case-fatality rate of 90%.

[f] Average annual number of reported cases during 1951-1954, 4 years before vaccine licensure.

[g] Excludes one case of vaccine-associated polio reported in 1998.

[h] Average annual number of reported cases during 1958-1962, 5 years before vaccine licensure.

[i] Number of reported cases in 1968, the first year reporting began and the first year after vaccine licensure.

[j] Average annual number of reported cases during 1966-1968, 3 years before vaccine licensure.

[k] Estimated number of cases from population-based surveillance studies before vaccine licensure in 1985.

[m] Excludes 71 cases of *Haemophilus influenzae* disease of unknown serovar.

2. Current vaccine situation: registered vaccines

As vaccines have been shown to be the most successful and cost effective intervention to prevent infectious diseases (72), it has become very reasonable to develop effective vaccines for old, new and re-emerging pathogens for which vaccines are not yet available or the current vaccines are not satisfactory enough due to low efficacy (no life-long protection), poor biological stability (cold chain requirement) and/or high costs (the price per dose is more than 10 US dollar).

Since 1796, when Edward Jenner (1749-1823) put variolation (inoculation of cowpox virus in humans to induce protection against smallpox) in practice, several new vaccines have been introduced against various pathogenic organisms (see Table 2). Ten of these vaccines have been recommended for use only in selected populations at high risk because of area of residence, age, medical conditions or risk behaviours and other 11 have been proposed for use on all US children (25). As a result, recommendation for routine vaccination against smallpox was rescinded in 1971 in the United States because of its low rate in population (23) and its practice ceased totally in 1983 (24, 210).

Composition and route of application have an impact on the safety and effectiveness of present vaccines. Based on their composition, vaccines can be divided into two groups: a) those composed of inactivated (killed) organisms and purified products derived from them, and b) attenuated live vaccines. Killed vaccines, toxoid vaccines (inactivated toxins) and subunit vaccines are not able to replicate in the vaccinee and lead mainly to humoral immune responses. Attenuated live vaccines are capable of replicating in the host, thus mimicking a wild-type infection to a certain extent. Contrary to killed vaccines and their purified components, attenuated live vaccines induce a more complex immune response, involving both, the humoral and cellular arm of the immune system (1, 88).

The route of vaccine administration (subcutaneous, intramuscular, intradermal or oral) is also very relevant for the type of immunity induced. Parenterally applied vaccines only induce a systemic immune response. They require high costs of production and delivery, and are associated with the potential risk of contamination through syringes and needles. In addition, potential vaccines need professional personnel for their administration and are painful for the vaccinee. In contrast, mucosally targeted vaccines simulate the natural infection route of the majority of wild-type pathogens, which enter into the host via the lungs, the intestinal or the genital tract. Oral immunization elicits a local mucosal response

(immunoglobulin A production) and therefore, a blockade of colonization in the early phases of infection and posterior systemic spread. Because oral vaccines are cheaper to produce and do not require a cold chain for the formulation's safety and stability, their production, storage and distribution is easier and thus they are more accessible in both, developed and developing countries. Their simple and painless administration has been associated with a major acceptance by the public, making oral vaccines particularly suitable for mass immunization programs (41, 123, 124).

Table 2. List of vaccine-preventable diseases by year of vaccine development or licensure, by type of vaccine and recommended administration route in the United States (remodelled from MMWR 48:243-248, 1999(25)).

Pathogen	Disease	Year	Type of vaccine	Route
Smallpox virus	Smallpox	1798	Attenuated vaccinia virus	Subcutan (s.c.)
Rabies virus	Rabies	1885	Inactivated virus	Intramuscular (i.m.) or intradermal (i.d.)
Salmonella typhi and paratyphi	Typhoid	1896	inactivated bacteria or (Ty21a oral) live bacteria	s.c. or (Ty21a) oral
Vibrio cholera	Cholera	1896	Inactivated bacteria	s.c. or i.d.
Yersinia pestis	Plague	1897	Inactivated bacteria	i.m.
Corynebacterium diphterae	Diphteria	1923	Inactivated toxin (Toxoid)	i.m.
Bordetella pertussis	Whooping cough	1926	Inactivated bacterial component and toxoid	i.m.
Clostridium tetani	Tetanus	1927	Inactivated toxin (Toxoid)	i.m.
Mycobacterium tuberculosis	Tuberculosis	1927	Live attenuated bacteria (Bacillus of Calmette Guérin)	oral
Influenza virus	Flu	1945	Inactivated virus or viral components	i.m.
Yellow fever virus	Yellow fever	1953	Live virus	s.c.
Poliovirus	Poliomyelitis	1955	(IPV) Inactivated viruses of all 3 serovars (OPV) live viruses of all 3 serovars	(IPV) s.c. (OPV) oral
Measles virus	Measles	1963	Live attenuated virus	s.c.
Mumps virus	Mumps	1967	Live attenuated virus	s.c.
Rubella virus	Rubella	1969	Live attenuated virus	s.c.
Bacillus anthracis	Anthrax	1970	Inactivated bacteria	s.c.
Neisseria meningitidis	Meningitis	1975	Bacterial polysaccharides of serovars A/C/Y/W-135	s.c.
Streptococcus pneumoniae	Pneumonia	1977	Bacterial polysaccharides of 23 pneumococcal types	i.m. or s.c.
Adenovirus virus	Adenovirus	1980	Live virus	oral
Hepatitis B virus	Hepatitis B	1981	Inactive viral antigen	i.m.
Haemophilus influenza type b	Meningitis and epiglotitis	1985	Bacterial polysaccharide conjugated to protein	i.m.
Japanese encephalitis virus	Japanese encephalitis	1992	Inactivated virus	s.c.
Hepatitis A virus	Hepatitis A	1995	Inactivated virus	i.m.
Chickenpox virus	Varicella	1995	Attenuated live virus	s.c.
FMSE	Lyme disease	1998	Inactivated virus	i.m.
Rotavirus	Diarrhea and dehydration	1998	Attenuated live virus	oral
Papilloma virus	Cervix carcinom	2002	Inactivated virus	i.m.

Future directions in the challenging task of improving old vaccines and designing new ones should firstly imply a better knowledge about each pathogen, in particular about its mode of transmission, mechanism of replication and pathogenesis and secondly, the need for a detailed understanding of humoral and cellular components of the host immune system to protect against the pathogen.

3. Live attenuated bacterial vaccines

As mentioned above, among all available vaccine types, oral vaccines are the most suitable for carrying out mass immunization programs. There are current strategies for developing oral vaccines based on either attenuated bacterial pathogens or non-pathogenic commensal microorganisms to express heterologous antigens (41, 114, 124). Until now, the most exploited bacterial strains for this aim have been *Listeria monocytogenes* (89, 146, 207), *Salmonella* spp. (21, 39, 103, 178), *Vibrio cholera* (47, 205), *Shigella* spp. (99), *Mycobacterium bovis* (BCG) (29, 77), *Yersinia* spp. (118, 140, 167, 185, 186, 197, 213) and *Bacillus anthracis* (16, 181, 182) as avirulent pathogens and *Streptococcus gordonii* (37, 122, 142, 143, 158), *Lactobacillus* spp. and *Staphylococcus* spp. (190) as commensal candidates.

Live vaccines are able to induce a long-lasting humoral and cellular immune response against the infection and the disease caused by the vector itself. In addition, the microbial vaccine carrier can be used to express foreign (heterologous) antigens from other infectious agents or tumors. This vaccination strategy has been refined during the last two decades, especially due to a better understanding of pathogen-host interactions (113, 188).

4. Pathogen-host interactions

The immune system is composed of the innate immune system that recognizes pathogen-associated molecular patterns (PAMPS) and the adaptive immune system that identifies specific antigens (126). Although both immune systems work synergically and coordinately, the innate immune system provides a first line of defence at the beginning of an infection. In contrast, the adaptive immune system requires nearly four days in order to become activated. The innate immune system responds to all pathogens in the same way,

whereas the adaptive immune system has the capacity of generating specific responses and immunological memory (88).

The immune response elicited against infectious agents depends mainly on pathogen location in the host body during the microbial life cycle (see Fig. 2). Based on the site of replication, infectious organisms are divided into two groups: extracellular and intracellular microorganisms. Extracellular pathogens are found in spaces like the interstice, the blood, the lymph and on epithelial surfaces. For the control of extracellular organisms and their products, antibodies provide the most important adaptive mechanism of host defence. B cells recognize soluble antigens by their superficial immunoglobulins and present them to CD4 helper T cells, which can in turn stimulate the B cells to release specific antibodies. Specific antibodies directly neutralize antigens or recruit other cells and molecules that will destroy pathogens via phagocytosis and complement, respectively. Intracellular pathogens, in contrast, must invade host cells in order to replicate. Such pathogens are further subdivided into those that replicate freely in the cytosol of host cells, like viruses and certain bacteria (*ricketsiae, listeriae, shigellae and chlamydiae*) and those that replicate in vesicles (endosomes or phagosomes), like the majority of bacteria and parasites. Neutralizing antibodies, whose production relies on T helper 2 (T_H2) cells, play the most important role in preventing intracellular pathogens from invading host cells. Once inside the cell, such pathogens are not accessible to antibodies anymore and for this reason, other mechanisms must be activated in order to delete and eliminate them. For intravesicular pathogens, the response is mediated by CD4 T helper 1 (T_H1) lymphocytes, which secrete IFN-γ and TNF-α and activate macrophages that are able to kill intracellular organisms (119). Protection against cytosolic pathogens is achieved through cytotoxic T cells (CTL), namely CD8 T lymphocytes, which remove sites of pathogen replication by destroying infected cells (69, 97).

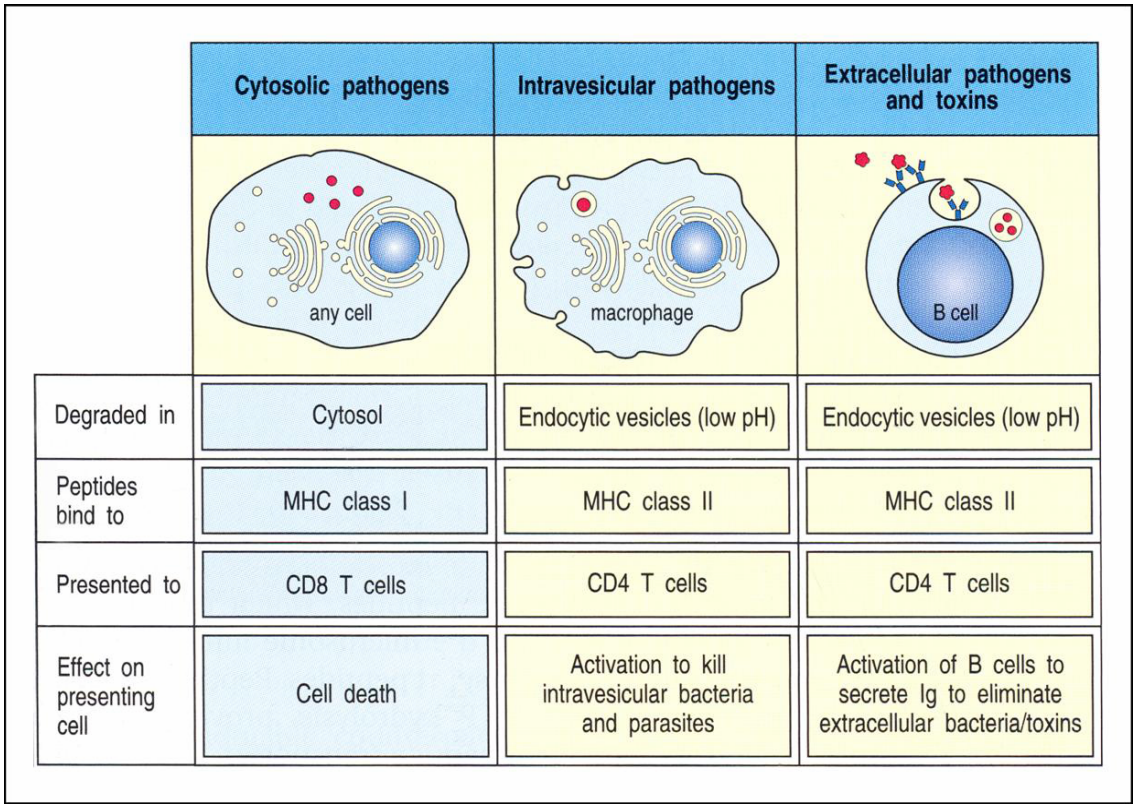

Fig. 2. Habitat of pathogens dictates the induction of different T cell populations. The two left panels correspond to bacteria, which are found in intracellular compartments. However, in the left panel, viruses and some bacteria replicate freely in the cytosol and because of this location, the antigens are presented by MHC class I molecules to CD8 T cells which kill target cells whereas in the center panel, other bacteria and some parasites survive and proliferate into endosomes, where antigens are degraded and presented to CD4 by MHC class II molecules. The right panel illustrates extracellular bacteria and their derived proteins, which may enter into the vesicular system of B cells or other cell types by endocytosis. Such cells are able to present antigens to CD4 helper T cells, which stimulate B cells to produce specific antibodies against a determined antigen (Figure reprinted from Immunobiology, C.A. Janeway, P. Travers, M. Walport, M. Shlomchick, 5th edition, Garland Publishing).

5. Antigen processing and presentation to T cells

Historically, most traditional vaccination programs have focused on inducing high titers of antibodies for protection against pathogens. Nevertheless, it has been demonstrated that T cells play a crucial role when controlling and protecting the host from various pathogenic organisms such as Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), *Mycobacterium tuberculosis* and *Plasmodium falciparum* among others (44, 96, 97).

Antigens of pathogenic microorganisms can be detected by T cells because infected cells display peptide fragments derived from pathogenic proteins on their surfaces. Again,

depending on the intracellular location of the pathogen, two different pathways of protein degradation will take place and that will lead to presentation and activation of different sorts of T lymphocytes. Classically, CD8 T lymphocytes respond to cytoplasmatically-derived protein antigens from viruses and cytoplasmatic bacteria and CD4 T lymphocytes respond to exogenous bacterial and parasitic proteins taken up by antigen-presenting cells (APC). Antigenic peptides are presented to CD8 or CD4 T cells by MHC class I and class II molecules, respectively.

The processing of exogenous antigens and presentation to CD4 T cells by MHC class II molecules involves the internalization of extracellular pathogens and their products into the endosomal system (see Fig. 3). Professional APCs like dendritic cells, B lymphocytes and macrophages are efficient in taking up extracellular material by macropinocytosis, phagocytosis or endocytosis (79, 108, 164). In the endosome, antigenic proteins are hydrolyzed in small peptides of 10-30 amino acids. This degradation is a result of endosome maturation, which implies decrease in pH of the compartment leading to activation of proteolytic enzymes like Cathepsin S (116). The endosome containing the hydrolyzed antigens fuses with exocytic vesicles, which hold the MHC class II molecules. These exocytic vesicles are generated in the endoplasmic reticulum (ER), where self-peptides and endogenous peptides are found. To avoid that those peptides attach in the groove of MHC class II molecules, a protein termed invariant chain binds MHC class II molecule, thereby occupying the peptide binding pocket. After fusion of the exocytic vesicle and endosome, the invariant chain is proteolytically cleaved, leaving a 24 residue peptide named class II-associated invariant chain peptide (CLIP). The removal of CLIP from the peptide binding pocket of the MHC class II molecule is facilitated by a non-polymorphic MHC class II-like protein called HLA-DM. This allows the loading of antigenic peptides to MHC class II molecules. The complex of antigenic peptides and the MHC class II molecule moves in vesicles to the cell surface and is presented to CD4 T lymphocytes. After CD4 T-cell priming, CD4 T cells are activated and can differentiate into either T helper 1 (T_H1) or T helper 2 (T_H2) cells that secrete specific subsets of cytokines. In general, T_H1 cells secrete the cytokines IFN-γ and TNF-α, which are able to activate macrophages. T_H2 cells release the cytokines IL-4, IL-5, IL-13 and IL-10 and are responsible for B cell activation and antibody production (119).

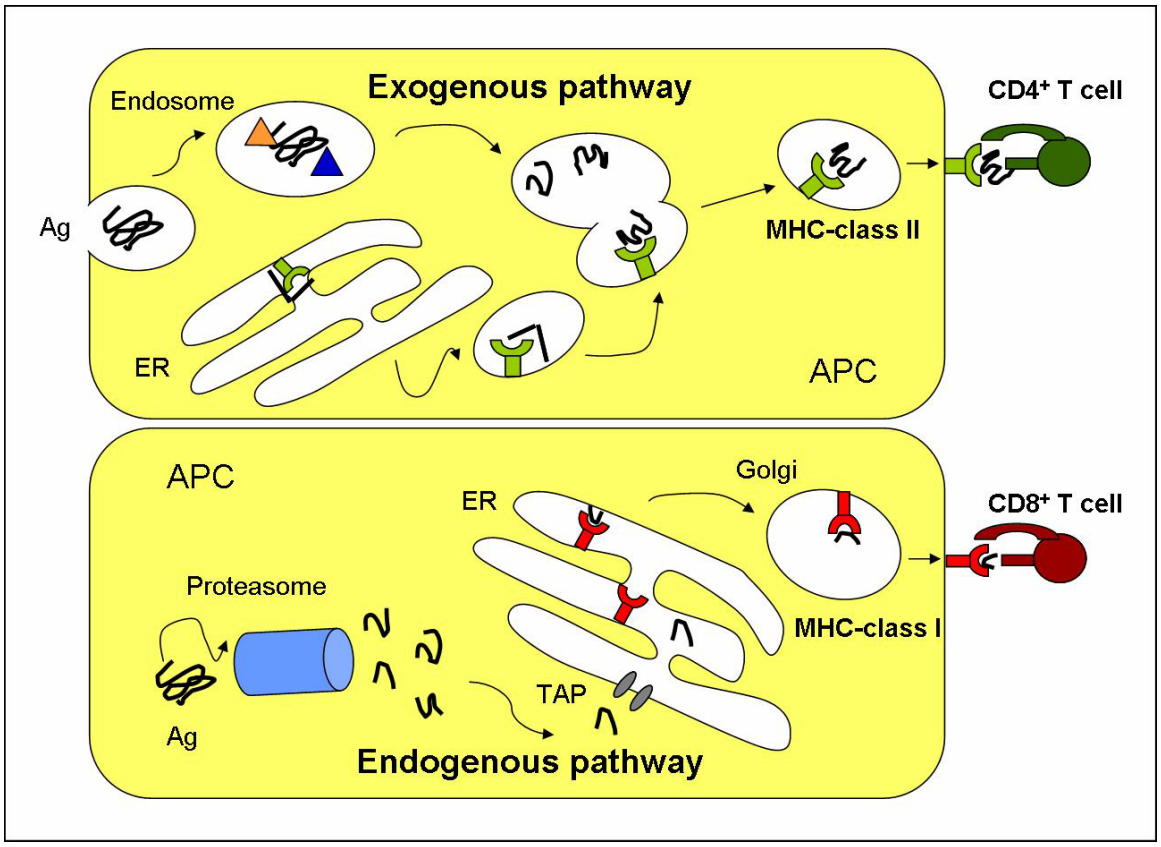

Fig. 3. Presentation of exogenous antigens to CD4 T lymphocytes and endogenous antigens to CD8 T lymphocytes. Two different pathways are used in order to present non-self antigens. The peptides that bind MHC class II and are recognized by CD4 T cells are internalized from extracellular spaces by professional APCs. In contrast, endogenous peptides are produced in the cell's cytoplasm and are loaded onto MHC class I molecules which are presented to CD8 T cells.

A distinct pathway of proteolysis is implicated for endogenous antigens (see Fig. 3). Here, the proteins derived from pathogens present in the cell cytoplasm are digested by a multiprotein enzyme complex called proteasome. The proteasome recognizes ubiquitinated proteins from the cytoplasm and generates peptides of 6 to 30 residues, which need to come in contact with MHC class I molecules. MHC class I molecules are produced in the ER of almost all nucleated cells (220). In order to gain access to the MHC class I compartment, hydrophobic and basic cytoplasmic peptides are transferred from the cytoplasm to the ER by ATP-dependent peptide transporters TAP-1 and TAP-2. In the ER, the translocated peptides are trimmed by ER aminopeptidase associated with antigen processing (ERAAP). Now, the peptides are 8-12 amino acids in length and are suitable for binding MHC class I molecules (160, 189). For assembly and stabilization of the complex (peptide-MHC class I molecule), some chaperones like tapasin, calreticulin and calnexin are demanded. Once MHC class I molecules are loaded with optimised antigenic peptides,

the complex is transported within an exocytic vesicle to the cell surface (87), where they will be presented and recognized by the T cell receptor (TCR) of CD8 T cells (195, 220). After CD8 T-cell priming, clonal expansion of antigen-specific CD8 T cells occur, which trigger the elimination of infected cells by lysis or apoptosis.

6. *Salmonella* spp.

*Salmonella s*pp., named after Daniel Elmer Salmon, are Gram-negative, peritrichous flagellated, facultative anaerobic and facultative intracellular rods belonging to the family of *Enterobacteriaceae*. *Salmonella* spp. are found in environmental sources including soil, water and food and are able to infect both humans and a broad spectrum of animals by the oro-faecal route.

All members of the genus *Salmonella* share important traits that do not exist in other closely related species like *Shigella* spp. and *E. coli* (see Fig. 4). About 160-100 million of years ago, *Salmonella* spp. acquired the first *Salmonella* pathogenicity island (SPI-1) by horizontal gene transfer (62). Genes encoded by SPI-1 mediate invasion of host cells (e.g. intestinal epithelial cells). According to DNA relativeness, presence or absence of *Salmonella* pathogenicity islands (SPIs) and specificity to hosts, *Salmonella* spp. are classified into 2 species and 7 subspecies. The two species comprise *S. bongori*, which colonizes cold-blooded animals and *S. enterica*, which evolved further into 7 species and is able to colonize both, cold- and warm-blooded hosts. *S. bongori* and *S. enterica* diverged from each other 40-35 million years ago, when *S. enterica* adopted a second *Salmonella* pathogenicity island (SPI-2) necessary for growth and survival in macrophages (8, 9, 34). *Salmonella* spp. are also further divided in the Kauffmann-White scheme into over 2400 different serovars, which classifies strains on the basis of serological identification of their H-(flagellar), O-(lipopolysaccharide) and Vi-(capsular) antigens (15).

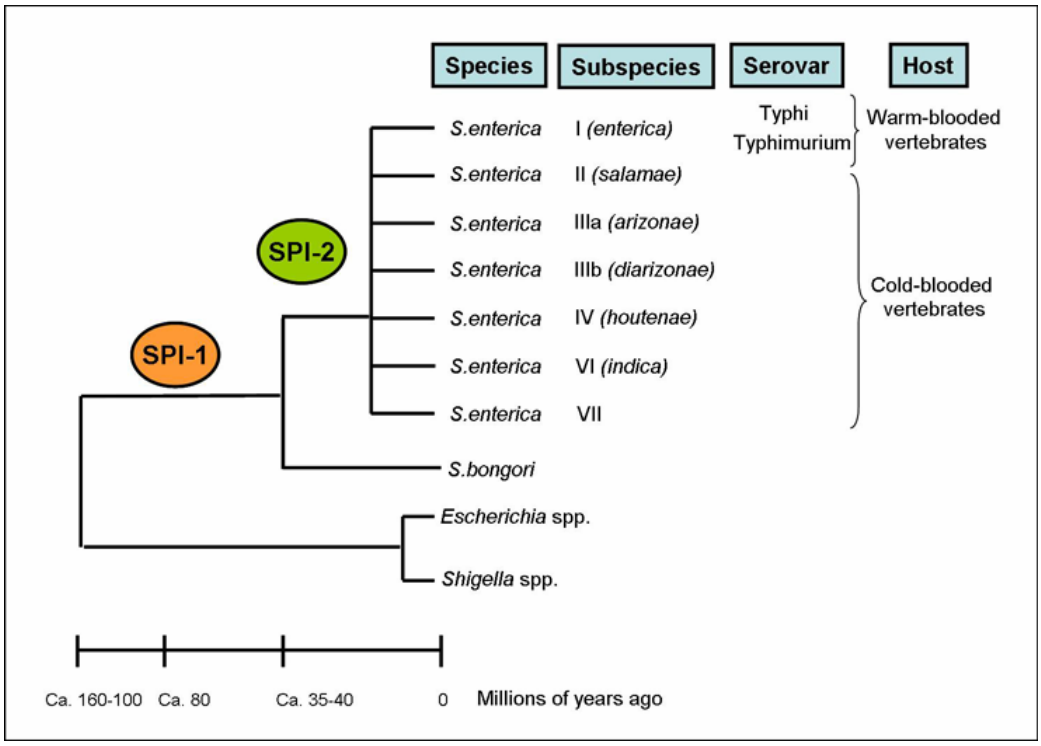

Fig. 4. Dendogram showing phylogenetic relationships among *Salmonella* species and subspecies. *Salmonella* spp. diverged from *Shigella* and *Escherichia* spp, when *Salmonella* acquired the *Salmonella* pathogenicity island 1 (SPI-1) 160-100 million years ago. This pathogenicity island 1 mediates intestinal invasion, an ability shared by all species of *Salmonella* (*S. bongori* and *S. enterica*). About 40-35 million years ago, *S. enterica* adopted also a second *Salmonella* pathogenicity island (SPI-2) by horizontal gene transfer, which is responsible for systemic disease. Subspecies are further divided according to host adaptation. There is another classification based on the antigenic profile of the strains (Kauffmann-White scheme) that has identified more than 2400 different serovars.

In humans, *Salmonella* spp. provoke two diseases called salmonellosis (64). Depending on the serovar, salmonellosis ranges from a localized gastroenteritis to a systemic infection. Gastroenteritis is a self-limiting disease caused mainly by two serovars, Enteritidis and Typhimurium (later referred as *S. enteritidis* and *S. typhimurium*, respectively). The typical symptoms include abdominal cramps, nausea, vomits, diarrhoea and fever. In a small fraction of infected people, the bacteria can enter the bloodstream and cause septic shock. This is most likely to happen in immunocompromised people and elderly. According to the WHO, *S. typhimurium* causes up to 1.3 billions cases per year worldwide, leading to 3 millions deaths. In contrast, typhoid fever is a systemic infection elicited by either *Salmonella enterica* serovar Typhi or serovar Paratyphi A, B or C (later referred as *S. typhi* or *S. paratyphi*, respectively). The symptomatic comprises high fever, flushed appearance, anorexia, chills, convulsions and delirium. The WHO also estimates that the annual global

incidence of typhoid fever is around 16.6 million cases per year, and accounts for 600,000 deaths (149, 150).

Whether the infection results in a self-limited gastroenteritis or in typhoid fever is determined by genetic factors of the host species and the *Salmonella* serovar. For example, *S. typhi* causes a systemic disease in humans whereas *S. typhimurium* remains confined in the intestine. Moreover, it is noteworthy to mention that *S. typhimurium* produces a systemic typhoid-like disease in mice, and for this reason it serves as an experimental model of typhoid fever (174, 218).

6.1. Pathogenesis of *Salmonella* mediated by type III secretion systems

Infectious agents cause disease in humans and animals by releasing adhesins, toxins, enzymes and mediators of motility, which interact with host cells and stimulate several cellular functions. These proteinaceous virulence determinants are produced in the bacterial cytoplasm and have to cross either the plasma membrane and the thick cell wall layer of Gram-positive bacteria or the double-membrane system of Gram-negative bacteria that sandwiches the peptidoglycan and the periplasmic space between them in order to gain access to the extracellular environment. For this purpose, bacteria have evolved different protein secretory mechanisms. To date, five different pathways have been identified and numbered from I to V. The prototypical example of a type I secretion system is the α-hemolysin (HlyA) export apparatus of *E. coli* (58). The type II secretion system is examplified with the secretion of the lipoprotein PulA of *Klebsiella oxytoca* (90, 159, 171, 172, 192). The type IV secretion system is ancestrally related to the bacterial conjugation machinery and it has been found in different species like *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Legionella pneumophila* and *Helicobacter pylori* (27, 28). The type V or autotransporter system has been observed to secrete VacA by *H. pylori*, the SphB1 by *B. pertussis* and AspA/NalP of *Neisseria meningitidis* (67). Finally, the type III secretion system (T3SS) has been identified in several Gram-negative pathogens like *Yersinia* spp., *Salmonella enterica*, *Shigella flexneri*, *E. coli*, *Ralstonia solancearum*, *Pseudomonas syringae*, and *Chlamydia trachomatis* (83, 155). The type III secretion proceeds through a needle-complex composed of more than 20 different proteins which share several homologies with those involved in flagellar assembly (see Fig. 5) (104, 105, 134). Whereas the structural components of the T3SS are highly conserved among the different pathogenic species, the secreted effector proteins are divergent and perform various

biological functions (105). However, the most striking feature of the T3SS is the ability to target effector proteins directly into eukaryotic cells. This phenomenon is triggered when a bacterium comes in contact with an eukaryotic cell. Nevertheless, not all T3SS are contact dependent and some effector molecules secreted by T3SS are released into the external environment (33). Controversy exists about the mechanism of effector molecule recognition and targeting to the T3SS. One hypothesis suggests that the signal resides at the 5-terminal region of the mRNA, which may target the ribosome-RNA complex to the T3SS, thereby permitting temporal coupling of translation and secretion (4). A second proposal corroborates that the N-terminal 20 amino acids serve as a binding site for cytoplasmic chaperones which specifically target the effector molecules to the T3SS (117). Notwithstanding the differences in these hypotheses, it is apparent that the region encoding the first 20 amino acids (either the untranslated mRNA or the first 20 amino acids of the polypeptide) is essential for secretion and the process is highly regulated (83).

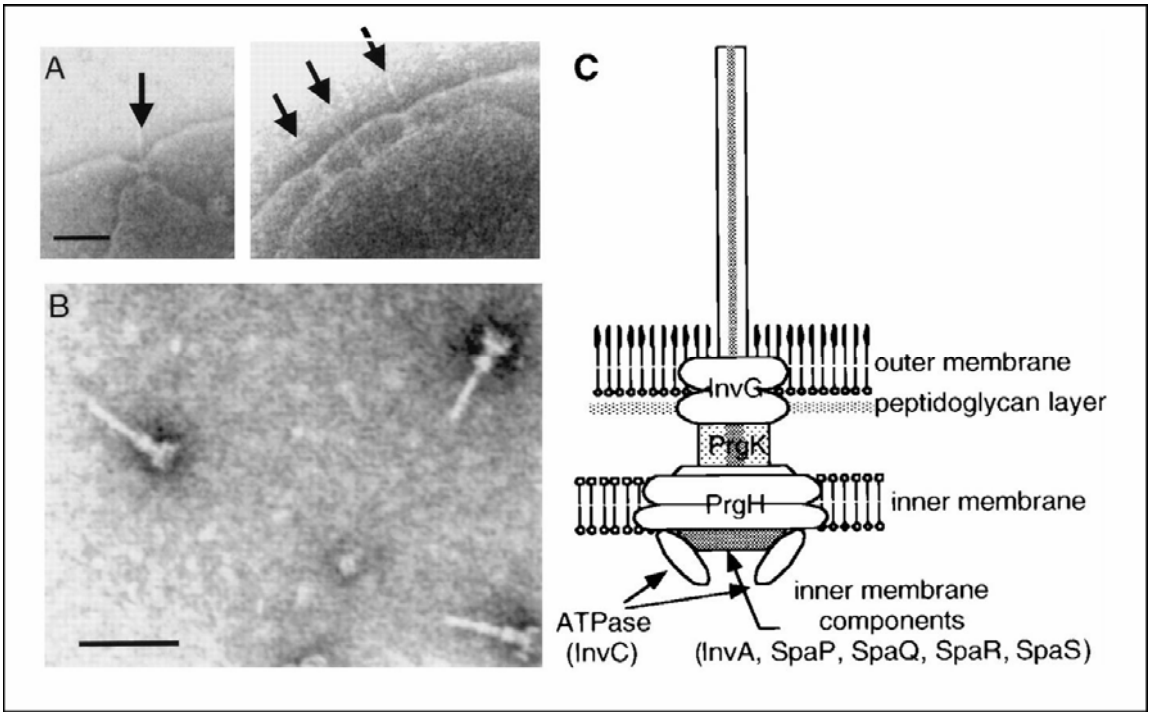

Fig. 5. The needle complex of *S. typhimurium* type III secretion system (T3SS). The T3SS is a multiprotein apparatus capable of injecting bacterial proteins into the cytoplasma of host cells. For this aim, the injectisome spans both the inner and the outer membranes of the bacterial envelope and also the eukaryotic membrane. This system resembles the flagellar basal body (Figure from Galán, J.E. and Collmer, A., 1999).

Salmonella spp. employ T3SS for pathogenesis (51-54). Two T3SS are encoded in two different *Salmonella* pathogenicity islands, which operate in an independent, but coordinate fashion. The *Salmonella* pathogenicity island 1 (SPI-1) is required for the initial step of invasion (51) whilst the *Salmonella* pathogenicity island 2 (SPI-2) acts at later stages and is responsible for systemic infection (68).

6.1.1. Early stages of *Salmonella* spp. infection: intestinal invasion

Salmonella spp. enter humans and animals by contaminated water or food (see Fig. 6). Following ingestion, bacteria pass through the stomach, where they have to resist the low pH, and reach the distal ileum and the caecum (22). Once in the intestinal lumen, *Salmonella* attach actively to the host cell surface through adhesion molecules like fimbriae (10). Due to cell contact and environmental factors like low oxygen concentration and high osmolarity (6), the *Salmonella* pathogenicity island 1 (SPI-1) becomes activated and using the needle complex, *Salmonella* injects several effector proteins into the cytosol of host cells (52). The SPI-1 encoded effector proteins promote in first place massive cytoskeletal rearrangements and the formation of pseudopods or so-called membrane ruffles. These structures enclose the bacterium actively leading to their internalization by macropinocytosis (49). Invasion occurs mainly in M cells that cover the Peyer's patches (91), but also in enterocytes and secretory cells (173). The active engulfment results either from direct interaction of the effector proteins SipC and SipA with components of the cytoskeleton of the host cell (66) or from interference of the effector proteins SopE, SopE2 and SopB with host cell signalling pathways that induce actin rearrangements. This latter mechanism involves the activation of Cdc42 and Rac1 directly by the potent guanine nucleotide exchange factors (GEFs) SopE and SopE2, and indirectly by the SopB effector protein (52, 219). In addition, SPI-1 proteins procure an accumulation of host cell membrane cholesterol at the site of *Salmonella* entry for an efficient invasion (57). The actin cytoskeleton changes induced by *Salmonella* are reversible. In fact, after bacterial invasion, the infected cells return to their normal architecture of the cytoskeleton. This reversion is mediated by a GTPase-activating protein (GAP) called SptP that acts towards Cdc42 and Rac1 (50). Apart from this active invasion of non-phagocytic cells, there is an alternative route of *Salmonella* uptake that involves bacterial transport and dissemination in spleen, liver and bone marrow by CD18+ cells like dendritic cells and macrophages

(201). In second term, SPI-1 proteins induce more cellular signal cascades which mediate other cellular responses. For example, SopA and SopD trigger localized inflammation and fluid secretion, responsible for diarrhoeal symptoms (92, 206). SipA also mediates local inflammation by the production of IL-8. Simultaneously, IL-8 and other chemoattractants are implicated in PMNs (polymorphonuclear leukocytes) transmigration in the gut lumen (60, 110). The pro-inflammatory response and the subsequent recruitment of phagocytic cells to the site of infection may facilitate systemic spread of the bacteria (68). Finally, the SPI-1 system induces apoptosis in macrophages at the early stages of infection, since SipB binds and activates caspase-1 (70, 137).

6.1.2. Later stages of *Salmonella* spp. infection: survival and replication inside phagocytes

After bacterial internalization, *Salmonella* spp. remain localized in a membrane-bound vacuole, referred to as *Salmonella*-containing vacuole (SCV) (see Fig. 6). *Salmonella* spp. are capable of adapting their new environment in order to proliferate and survive inside host cells (56, 133). SPI-2 is precisely assigned for this task and for this reason, it is induced intracellularly in response to vacuolar acidification and, magnesium, calcium and phosphate starvation (36). Up to now, many genes belonging to SPI-2 have been identified, but no specific effector protein has been elucidated for the main functions (75). The normal maturation process of phagosomes containing pathogens involves interaction with the endosomal system (75, 100). The effector protein SpiC modifies the normal endocytic trafficking by blocking fusion between lysosomes and SCVs (196). However, fusion with lysosome and late endosomes has also been observed (145). SifA is required for the formation of Sifs (*Salmonella*-induced filaments) which maintain the integrity of the SCV membrane and establish an optimal environment for *Salmonella* replication (12, 166). Similar to SPI-1, SPI-2 initiates actin polymerisation that leads to the formation of an actin meshwork around the SCV, that also contributes to the integrity of the SCV membrane (128, 129). In order to survive, SPI-2 effector proteins have to evade both the innate immune system by avoiding respiratory burst (reactive oxygen and nitrogen intermediates) and acidification (55, 200), and the adaptive immune system by inhibiting antigen presentation through MHC class I and II to T cells (193). Another function of the SPI-2 is to induce delayed macrophage death by apoptosis. The effector proteins responsible for

this activity have not yet been identified, but they facilitate bacterial colonization of the spleen and the liver, either by releasing bacteria upon lysis or apoptotic cells by neighbouring macrophages (198).

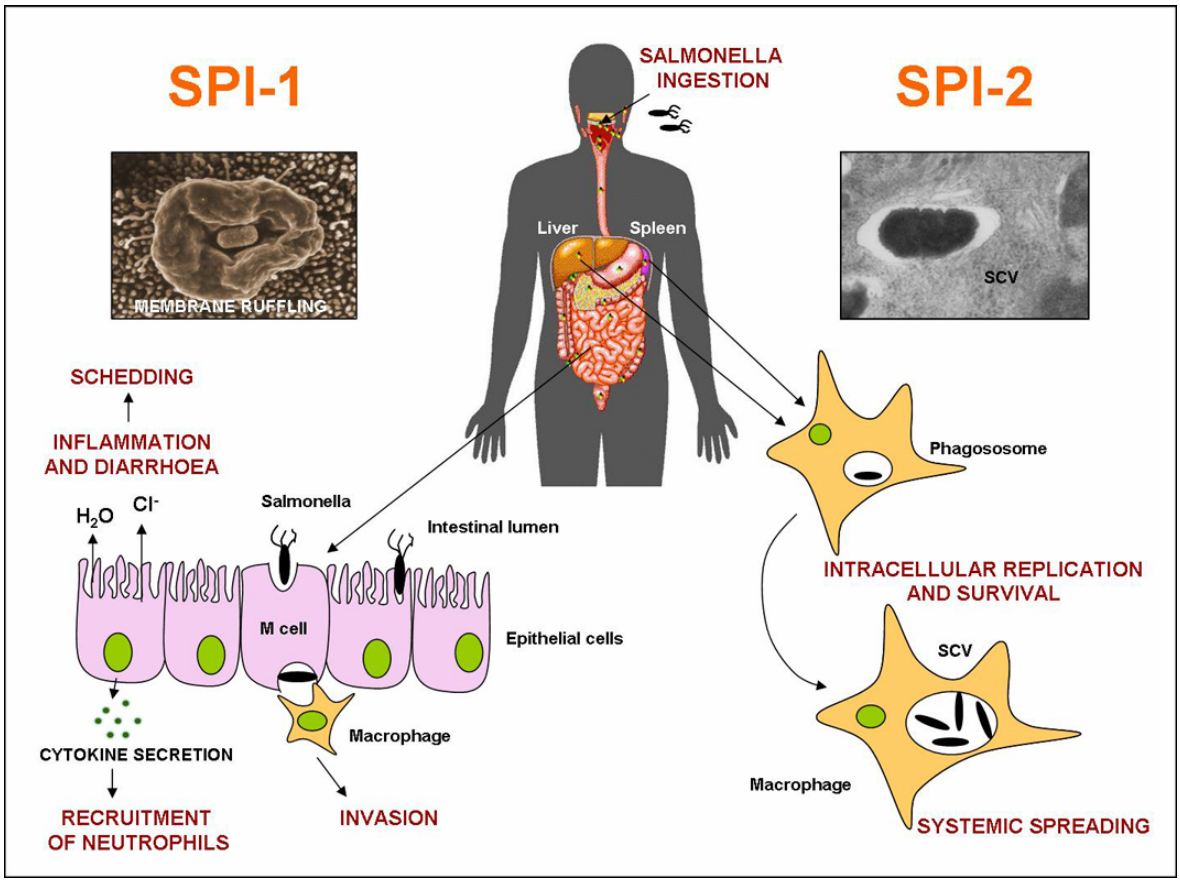

Fig. 6. Schematic representation of host-pathogen interactions during pathogenesis of *Salmonella* spp. infections. SPI-1 function is required for the initial stages of salmonellosis, i.e. entry of *Salmonella* into non-phagocytic cells by active penetration into the gut epithelium. SPI-1 also mediates fluid and electrolyte accumulation, and inflammation in the intestinal lumen that lead to diarrhoea and consecutive shedding of bacteria. Finally, SPI-1 is responsible for secretion of pro-inflammatory cytokines that recruit and attract PMNs to the site of infection. In contrast, SPI-2 acts at later stages of infection and is necessary for both growth and survival in host phagocytes and for systemic spread into different host organs (Figure remodelled from Hansen-Wester *et al.*, 2001).

6.2. Live attenuated *Salmonella* spp. as oral vaccine carrier for heterologous antigens

Avirulent *Salmonella* spp. have been extensively studied for their capacity to serve as vaccine carriers for foreign antigens (19, 21, 46, 63, 112, 124, 177, 187). In this approach, *Salmonella* spp. express and carry recombinant DNA from viral (45, 169), bacterial (86,

101, 168), parasitic (26) and even tumoral origin (125, 139). Since *Salmonella*-based vectors are capable of mimicking the infection in a mitigated way, involving colonization of the gut wall, restricted replication and blood-spreading, they are able to trigger an intense mucosal and systemic immune response that provides both cellular and humoral immunity against a wide range of pathogens (38, 76, 79, 144). A prerequisite of attenuated vaccines is to ensure safety and prevent unwanted side effects. One of the main problems of employing attenuated live *Salmonella* spp. as an antigen delivery system, is the potential risk of reversion to virulence which could compromise its use in people suffering from any immunodeficiency. In order to find the optimal balance between attenuation and immunogenicity, construction of several safe, genetically stable, defined and non-reverting mutants have been performed. This was made possible by the improvement in molecular manipulation and knowledge of *Salmonella* genetics (20). Up to now, many vaccine candidates have been engineered by inactivating genes involved in biochemical pathways (59, 74, 78, 191), global regulatory systems (35, 154) or virulence (85, 98, 132). Besides, it has been shown that two independent and distantly located mutations in a vaccine strain may significantly reduce the possibility of reversion to the virulence state (98). An additional and controversial problem regarding the use of *Salmonella* spp. as a vaccine carrier could be the potential restriction for repeated administrations with the same *Salmonella* serovar. Some researchers have reported that the immune response to heterologous antigens could be compromised when previous exposition to the vaccine carrier has existed (5, 102, 162, 203). In contrast, other investigators have shown that pre-existing anti-vector immunity is able to enhance the specific immune response to homologous and heterologous antigens (7, 11, 208).

6.3. The use of T3SS for heterologous antigen delivery

After invasion, *Salmonella* spp. reside within SCVs. This confinement leads mainly to MHC class II-restricted antigen presentation and therefore to peptide-specific CD4 T-cell priming (212). In contrast, the ability to generate MHC class I-restricted antigen presentation and subsequently a CD8 T-cell stimulation remains limited. In attempt to circumvent this problem, Rüssmann *et al.* have used the T3SS to target heterologous antigens into the cytosol of APCs, resulting in an efficient CD8 T-cell induction (169). The first type III effector protein used as a carrier molecule to induce an antigen-specific CD8 T-cell response was the *Salmonella* protein tyrosine phosphatase (SptP), which was fused

to the immunodominant CD8 epitope of the nucleoprotein from the murine lymphocytic choriomeningitis virus ($LCMVNP_{118-126}$) or the influenza virus ($IVNP_{366-374}$), respectively (169). However, the use of SptP to deliver foreign antigenic peptides to the MHC class I presentation pathway was restricted to small protein fragments of 45-55 amino acids (169). Because a versatile antigen delivery system should be capable of targeting large protein fragments derived from diverse pathogens, our laboratory has focused its research on identifying other type III effector proteins that could be used in *Salmonella* for this purpose.

The best characterized T3SS-protein from *Yersinia* is the *Yersinia* outer protein E (YopE), a GTPase-activating protein that disrupts eukaryotic cytoskeleton dynamics and inhibits phagocytosis by macrophages (13, 153, 165). The 25-kDa YopE molecule contains an N-terminal secretion sequence of 11-15 amino acids and a translocation domain of at least 50 residues that can be delivered by the type III secretion machinery of attenuated *Salmonella* (168).

Our laboratory showed that mice orally vaccinated with a single dose of attenuated *S. typhimurium* expressing the translocated $YopE_{1-138}$ fused to immunodominant CD8 epitopes of *L. monocytogenes* $p60_{217-225}$ (murein hydrolase) or LLO_{91-99} (listeriolysin) revealed high numbers of IFN-γ-producing cells reactive with $p60_{217-225}$ and LLO_{91-99}. This CD8 T-cell response was sufficient to protect mice against a challenge infection with wild-type *L. monocytogenes* (86, 168).

B. THE AIMS OF THIS STUDY

There are conflicting reports concerning the impact of the pre-existing anti-*Salmonella* immunity on the efficacy of *Salmonella*-based vaccines. For this reason, the goal of this work was to:

a) determine whether oral boost immunizations with recombinant *S. typhimurium* expressing translocated YopE/p60 via its T3SS could enhance the p60-specific CD8 T-cell response.

b) examine whether anti-listerial immunity induced by prime immunization with recombinant *Salmonella* expressing YopE/p60 contributes to a more rapid clearance of the vaccine carrier after subsequent immunizations of mice.

c) analyze whether a short-term colonization of the *Salmonella* vaccine carrier due to anti-vector immunity could prevent an efficient p60-specific CD8 T-cell response.

d) evaluate whether the use of two different *Salmonella* serovars for prime and boost immunizations would circumvent the anti-vector immunity, therefore augmenting the frequencies of antigen-specific T cells.

C. MATERIALS AND METHODS

I. MATERIALS

1. Laboratory equipment and accessoires

All the equipment used for performing this work is mentioned below.

Table 3. Equipment used in this work.

Equipment	Type	Company
Analyticscale	Kern 440-33	Sartorius, Gottinga, Germany
Cell strainer	70 µm Nylon	Becton Dickinson, Heidelberg, Germany
Centrifuge	Eppendorf 5810R	Eppendorf, Hamburg, Germany
	Sorvall super T-21	Sorvall, Langenselbold , Germany
	Eppendorf 5417C	Eppendorf, Hamburg, Germany
Centrifugal filter devices	Amicon ultra 14 ml cut off	Millipore, Schwalbach, Germany
Culture tube	Falcon 50 ml, polypropylene conical tube	Becton Dickinson, Heidelberg, Germany
	Falcon 15 ml, polypropylene conical tube	Becton Dickinson, Heidelberg, Germany
	Falcon 14 ml, polypropylene round-bottom tube	Becton Dickinson, Heidelberg, Germany
CO_2-Incubator	Cytoperm 2	Heraus, Hanau, Germany
Electrode Assembly	Mini-PROTEAN II Power-Pac 200	Bio-rad, Munich, Germany
Electroblot apparatus	Trans-Blot	Bio-rad, Munich, Germany
Electrophoresis chamber		Peqlab, Erlangen, Germany
Electroporation apparatus	Gene pulser II	Bio-rad, Munich, Germany
Electroporation cuvette		Bio-rad, Munich, Germany
Freezer	Profi line	Liebherr, Bulle, Switzerland

Equipment	Type	Company
Fridge	Profi line	Liebherr,Bulle , Switzerland
Heat block	TR-L 288	Liebisch, Bielefeld, Germany
Homogenisators	10 ml and 30 ml	Wheaton, Millville, USA
Homogenisator machine	MM 2000	Retsch, Wuppertal, Germany
Incubator	Function line	Heraus Instruments, Hanau, Germany
Incubator with shaker	Certomat BS-1	B.Braun Biotech International, Melsungen, Germany
Laminar flow	UVF 6.18.S	BDK, Sonnenbühl-Genkingen, Germany
Microscope	Axiovert 25	Zeiss, Jena, Germany
Microscope	Light microscope (slides)	Zeiss, Jena, Germany
Microwave		LG Electronics Deutschland GmbH, Willich, Germany
Magnetic stirrer	RCT basic	Ika Labortechnik, Staufen, Germany
pH-meter	Accumet basic	Fisher Scientific, Schwerte, Germany
PCR cycler	Gene Amp System 9700	Perkin Elmer Applied Biosystems, Darmstadt, Germany
Pipette	10 µl, 100 µl and 1000 µl Research	Eppendorf, Hamburg, Germany
Pipette	20 µl and 200 µl	Gilson, Bad Camberg, Germany
SDS-PAGE apparat	PROTEAN II	Bio-rad, Munich, Germany
Silent screen plate	96 well clear w/o membrane lid byodime B	Nalgen Nunc, Wiesbaden, Germany
Spectrophotometer	Spectronic 20	Spectronic instruments, Rochester, USA
Speedvac	DNA120	Savant Thermo Electron, Langenselbold, Germany
Laminar Flow		BDK, Sonnenbühl-Genkingen, Germany
Syringe	10 ml	B.Braun, Melsungen, Germany
Syringe filters	Acrodisc 25 mm	PALL, Ann Arbor, U.S.A
Thermomixer	Comfort	Eppendorf, Hamburg, Germany
Transiluminator		Bio-Rad, Munich, Germany
Vortex apparatus	Vortex-2 Genie G-560E	Scientific industries si, Bohemia, N.Y., USA

Equipment	Type	Company
Water bath	WB/OB7-45	Memmert, Schwabach, Germany
Orbital shaker	OMV ROM	Fröbel Labortechnik, Lindau, Germany

2. Chemicals

Reagents were purchased from the following companies:
Becton Dickinson (Heidelberg, Germany), Biozym (Hameln, Germany), Boehringer (Mannheim, Germany), Difco (Detroit, USA), Gibco (Gaitersburg, USA), Merck (Darmstadt, Germany), Sigma-Chemie (Steinheim, Germany), Seromed-biochrom (Berlin, Germany), Serva (Heidelberg, Germany), Fluka (Steinheim, Germany), Roth (Karlsruhe, Germany), PAN Biotech (Aidenbach, Germany), ICN Biomedicals (Aurora, Ohio, USA).

3. Commercial kits

The Commercial kits, termed below, were used for DNA purification.

Table 2. Commercial Kits for DNA purification.

Kit	Company
DNAsy Tissue Kit	Qiagen, Hilden, Germany
Qiagen Spin Miniprep	Qiagen, Hilden, Germany

II. METHODS

1. Bacteria and plasmids

1.1. Bacterial strains

The genotype and the source of the bacterial strains and plasmids used in this work are put down on a list in the following tables.

Table 4. Strains used in this study.

Organism	Strain	Description	Resistance	Source/Reference
***Salmonella enterica* serovar Typhimurium**	SB824	ΔaroA sptP::Kan^R	Kanamycin	Rüssmann *et al*., 1998
***Salmonella enterica* serovar Dublin**	BRD620	ΔaroA ΔaroD		Roberts *et al*., 1999
Listeria monocytogenes	Sv1/2a EGD	Wild type		Hess *et al.,* 1996
	10403s	Wild type		Laboratory Busch, TU, Munich, Germany
Escherichia coli	K6060	araD139 Δ (ara-leu)7697 ΔlacX74 ΔphoA20 galK galE recA1 rpsE argE (Am) rpoB thi		Stratagene, La Jolla, USA
	TOP10	F-,*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80lacZΔM15, ΔlacX74, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL, (Str^R), endA1, nupG	Streptomycin	Invitrogen, Karlsruhe, Germany
	SCS110	rpsL (Str^r) thr leu endA thi-1 lacY galK gal Tara tonA tsx dam dcm supE44 Δ(lac-proAB) [F' traD36 proAB lacl^q ZΔM15]	Streptomycin	Stratagene, La Jolla, USA

1.2. Plasmids

Table 5. Plasmids used in this study.

Plasmid	Promoter	Resistance	Plasmid-encoded protein	Source/Reference
pHR241	*lac*	Ampicillin	SycE,YopE$_{1-138}$/p60$_{130-477}$/M45	Rüssmann *et al*., 2001
pHR231	*lac*	Ampicillin	SycE,YopE$_{1-138}$/LLO$_{51-363}$/M45	Rüssmann *et al*., 2001

2. Bacterial cultivation and storage conditions

2.1. Media

The following broths and agars were used for bacterial cultivation and storage.
After their preparation, they were immediately sterilized by autoclave (121ºC and 1 bar for 20 minutes).

Table 6. Broth composition used in this work.

Broth	Components	Source
Luria-Bertani (LB) medium	10 g Bacto tryptone 5 g Yeast extract 5 g NaCl Adjust to 1 l $H_2O_{dest.}$ and to pH 7.4-7.6 with NaOH	Miller, 1972
0.3M NaCl LB medium	10 g Bacto tryptone 5 g Yeast extract 17.5 g NaCl Adjust to 1 l $H_2O_{dest.}$ and to pH 7.4-7.6 with NaOH	Leclerc *et al*., 1998
BHI medium	52 g Brain-Heart-Infusion (BHI) Adjust to 1 l $H_2O_{dest.}$ and to pH 7.4-7.6 with NaOH	Fluka, Steinheim, Germany
Peptone broth	20 g/l Peptone 50 ml Glycerol Adjust to 1 l LB medium	

Table 7. Agars used in this work.

Agar	Composition	Source
Luria-Bertani agar	10 g Bacto tryptone 5 g Yeast extract 5 g NaCl 15 g Agar Adjust to 1 l $H_2O_{dest.}$ and to pH 7.4-7.6 with NaOH	Miller, 1972
MacConkey agar CM7	20 g/l Peptone 10 g/l Lactose 5 g/l Bile salts 5 g/l NaCl 0,075 g/l Neutral red 12 g/l Agar Adjust to pH 7.4-7.6 with NaOH	Oxoid, Wesel, Germany

2.2. Antibiotics

Antibiotics were added to broths and agars to select the different bacterial strains. The addition of antibiotic in broths was applied just before preparation of the culture and in the case of agars was added before the agar solidified. The concentrations and solvents used for antibiotics are defined in Table 8. All antibiotics were prepared under the flow and were sterilised through syringe filters (0.45 µm) (Pall corporation, Ann Arbor, USA).

Table 8. Antibiotics used in this work.

Antibiotic	Abbreviation	Solvent	Stock solution mg/ml	Final concentration µg/ml
Ampicillin	Amp	$H_20_{dest.}$	100	100
Kanamycin	Kan	$H_20_{dest.}$	50	50
Streptomycin	Sm	$H_20_{dest.}$	100	100

2.3. Cultivation and storage conditions

S. typhimurium, S. dublin, L. monocytogenes and *E. coli* were cultured aerobically in LB broth for 12-16 hours at 37ºC and 200 rpm. Overnight cultures were always fresh prepared and when required, antibiotics were included in their appropriate concentrations.
For bacterial storage, a single colony was picked and transferred into 10 ml liquid media. Cultures were grown overnight with the aforementioned conditions and on next day, they were centrifuged at 4000 rpm (3220 rcf) and 4ºC for 10 minutes. Bacterial pellets were resuspended with 2 ml peptone broth under the sterile bank. The bacteria were stored at -80ºC.

3. Molecular biological methods

3.1. Isolation of genomic DNA

The isolation of genomic DNA from Gram-positive bacteria was performed according to DNeasy Tissue Kit, Quiagen (Hilden, Germany).
A single colony of *L. monocytogene* EGD was cultured in 3 ml LB medium overnight at 37ºC and 200 rpm. On next day, 1 ml of the overnight culture was centrifuged in a table centrifuge at 7500 rpm (6000 rcf) for 10 minutes and by 4°C. Afterwards, the supernatant was discarded and the bacterial sediment was resuspended in 180 µl enzymatic lysis buffer. The lysis took place at 37ºC for 30 min. 25 µl of 20 mg/ml proteinase K and 200 µl Buffer AL were added into the suspension and was mixed by vortexing and incubated at 70ºC for 30 min. Then, 200 µl EtOH (96-100%) were added to the sample and the sample was mixed thoroughly by vortexing. The whole sample was applied into the DNeasy spin column and this was placed in a 2 ml collection tube already provided. The sample was centrifuged for 1 min at 8000 rpm (6800 rcf) and the flow-through and collection tube was discarded. The DNeasy spin column was placed this time in a new provided 2 ml collection tube and 500 µl Buffer AW2 were added to the sample. Afterwards, it was centrifuged for 3 min at full speed to dry the DNeasy membrane. The flow-through and collection tube were removed and the DNeasy spin column was placed in a clean 1.5 ml microcentrifuge tube. 200 µl Buffer AE were directly pippeted onto the DNeasy membrane and was left for

2 min. Finally, the DNeasy membrane was centrifugated for 1 min at 8000 rpm (6800 rcf). This step was repeated twice and the elution was stored at -20°C.

3.2. Plasmid isolation

Plasmids were isolated from bacteria using FastPlasmid Mini Kit, Eppendorf (Hamburg, Germany) for screening of clones and Qiagen Spin Miniprep Kit, Qiagen (Hilden, Germany) for cloning and sequencing.

According to FastPlasmid Mini Kits, 2 ml of fresh bacterial culture were centrifuged at 14000 rpm (20800 rcf) for 1 min. The supernatant was removed and the pellet was resuspended with 400 µl ICE-COLD Complete Lysis Solution and mixed by constant vortexing at the highest setting for a full 30 seconds. Then, the lysate was incubated at room temperature for 3 min and transferred to a Spin Column Assembly which was centrifugated for 1 min at maximum speed (14000 rpm or 20800 rcf). Subsequently, 400 µl of Diluted Wash Buffer were added to the Spin Column Assembly and the Spin Column Assembly was centrifugated for 1 min at maximum speed. All the flow-through was decanted and it was again centrifugated for 1 min at 14000 rpm (20800 rcf) to dry the Spin Column Assembly. Finally, the Spin Column Assembly was placed into a collection tube and 50 µl of Elution Buffer were directly added to the center of the Spin Column membrane. The Spin Column was centrifuged for 60 seconds at maximum speed and the eluted DNA, which was in the collection tube, was stored at -20°C.

In case we wanted to work with a plasmid for cloning aims, we used Qiaprep Spin Miniprep Kit of Qiagen (Hilden, Germany). 1-5 ml overnight cultures were prepared for plasmid purification. On next day, the bacterial cultures were centrifugated at 4000 rpm (3220 rcf) for 10 min. The supernatant was thrown and the pellet was resuspended with 250 µl Buffer P1 and transferred to a microcentrifuge tube. 250 µl of Lysis Buffer P2 were added and the tube was gently mixed by inverting 4-6 times. The lysis reaction was proceeded during 5 min. 350 µl Buffer N3 were added to neutralize the lysis. The tube was gently inverted and centrifuged for 10 min at 13000 rpm (17900 rcf). After this step, the supernatant was applied into the Qiaprep spin column by pipetting and it was centrifuged for 1 min at maximal speed. The flow-through was discarded. Then, the Column was washed with 500 µl Buffer PB and centrifuged again for 1 min. The flow-through was thrown off. The Column was washed once more by adding 750 µl Buffer PE and was centrifugated for 60 seconds. The flow-through was dropped and the Column was

centrifugated for 1 min to remove residual wash buffer. Finally, the Spin Column was replaced in a clean 1.5 ml microcentrifuge tube. 50 µl $H_2O_{dest.}$ were added into the Spin Column and were standing for 1 min and afterwards were centrifugated for 1 min. The eluted DNA was ready for use or stored at -20ºC.

3.3. Determination of DNA concentration

The quality and concentration of genomic DNA was determined by measuring the absorbance at 260/280 nm wavelength in quartz crystal cuvette and by agarose gel electrophoresis.

3.4. Agarose gel electrophoresis

DNA molecules were separated by agarose gel electrophoresis according to their molecular sizes and conformations. In addition, DNA molecules migrate to the positive pole since DNA is negative-loaded. The final concentrations of agarose used for the gels were comprised between 0.8% and 1.2% (w/v) depending on the expected fragment size.

Table 9. 50x TAE buffer composition.

Components	Quantity
Tris Base	242 g
Glacial acetic acid	57.1 g
0.5 EDTA	100 ml
$H_2O_{dest.}$	Adjust to 1 l and pH 8

40 ml 50% TAE (see Table 9) in 2 l $H_2O_{dest.}$ were required to obtain 1% TAE, which was used for the preparation of agarose gel and running electrophoresis buffer. The agarose mixture was cooked in the microwave until the solution was transparent and homogeneous. Afterwards, 2 µl ethidiumbromid (10 mg/ml) were added to the solution and this mixture was applied into the chamber. When the gel solidified, the samples were mixed with ¼vol. loading buffer (see Table 10) and were put into the gel slots. As size standard marker was used 1Kb Plus Ladder from Invitrogen (Karlsruhe, Germany).

Table 10. 4x Loading buffer composition.

Components	Quantity
Sucrose	40%
Xylene cyanol	0.25%
Bromophenol blue	0.25%

The samples migrated at 90V for 1 hour. Afterwards, the agarose gel was displayed in the Transiluminator.

3.5. Preparation of competent cells

Competent cells were prepared as described by Hanahan (65). One single colony of *E. coli* or *Salmonella* spp. was picked up in 5 ml LB broth with its appropriate antibiotic and was incubated overnight at 37ºC and 200 rpm. On next day, a 200-500 ml LB broth culture from the overnight culture (1:50) was prepared and incubated until OD_{600nm} of 0.6. Culture was centrifugated at 4ºC and 4000 rpm (3220 rcf) for 15 min. The following steps were performed on ice. All supernatants were discarded and all the sediments were resuspended with 20 ml LB medium. Cells were washed twice with 20 ml $H_2O_{dest.}$ and twice with 20 ml 10% glyclerol in $H_2O_{dest.}$. After the washing, the sediment was resuspended with 2-3 ml 10% glycerol and 70 µl aliquots of the bacterial suspension was put into 1.5 ml Eppendorf tubes. All the aliquots were stored at -80ºC.

3.6. Electroporation

Following the standard protocols of Sambrook (170), competent bacterial cells (see chap. 3.5) were put on ice and mixed with 1-3 µl plasmid DNA. The mixture was transferred into an electroporation cuvette and transformed with a BIO-RAD gene pulser II at 1.8 kV, 25 µF and 200 Ohms. After the electroporation, the mixture was added to 1 ml LB medium and incubated for 1 hour at 37ºC and 200 rpm. 100 µl and pellet of transformed bacteria were plated on selective agar plates and incubated at 37°C for 12-16 hours.

4. Biochemical standard methods

4.1. Protein release

A single colony was picked in 3 ml LB broth with its appropriate antibiotic and the culture was grown overnight at 37ºC and 200 rpm. On next day, 10 ml 0.3M NaCl LB medium (see Table 6) were inoculated with 500 µl of the overnight culture and incubated until OD_{600nm} = 0.6-0.8. Afterwards, the culture was centrifugated at 4000 rpm (3220 rcf) and 4ºC for 15 min. Two fractions were obtained: the supernatant, which contained the secreted proteins and the sediment, which corresponded to the bacterial cells. The supernatant fraction was filtrated through a 0.45 µm filter (Pall corporation, Ann Arbor, USA) and placed into a new 14 ml polypropylene tube. 1 ml TCA (99.5%) was added to the supernatant, mixed by vortexing and left on ice at least for 2 hours. Thereinafter, the solution was centrifugated for 30 min at 10000 rpm (11920 rcf) and 4ºC. The supernatant was discarded and the sediment was resuspended in 1 ml PBS by a cell scraper. After this, 4 ml aceton were added. The solution was vortexed and was incubated for 1 hour on ice. Then, it was centrifugated again for 30 min at 10000 rpm (11920 rcf) and 4ºC. The sediment was resuspended with 1 ml acetone and transferred to a new 1.5 ml eppendorf tube. Later on, it was centrifugated at 14000 rpm (20800 rcf) and 4ºC for 3 min. The supernatant was carefully removed by aspirating with a narrow pipet tip and the pellet was dried for 2 min in a Speedvac and disrupted by pipeting up and down with 50 µl 50 mM Tris pH8.

The sediment fraction of the 10 ml 0.3M NaOH LB medium was resuspended with 1 ml PBS and centrifugated for 3 min at 14000 rpm (20800 rcf) and 4ºC. The supernatant was removed and the sediment was resuspended with 500 µl 1x Laemmli (see Table 16), which lyses bacteria. Both, supernatant and whole cell lysate fraction, were either directly used for Western blot analysis or stored at -20ºC.

4.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Like DNA molecules, proteins can be as well separated in an electric field through electrophoresis (107). The polypeptide molecules are negative-loaded and migrate to the positive pole (anode) proportionally to their size and molecular weight.

Proteins were treated with Sodiumdodecylsulfate (SDS) and β-mercaptoethanol at 95ºC for 5 min. β-mercaptoethanol was used to reduce the disulfide bond between cysteine and SDS, a negative charged detergent, was necessary to break the secondary-, tertiary- and quaternary-structures of the proteins and to impart a negative charge to all proteins in order that proteins migrate through the gel based only on the protein size and not on the charge. Both substances were components of the protein Loading buffer (see Table 11).

Table 11. Components of 5% Loading buffer (SDS reducing buffer).

Components	Quantity
250 mM TrisCl pH6.8	2.5 ml
500 mM Dithiothreitol (DTT)	0.8 g
10% Sodium-dodecyl-sulfate (SDS)	1 g
50% Glycerol	5 ml
0.5% Bromphenol blue	0.05 g
$H_2O_{dest.}$	Fill up to 10 ml

The preparation of polyacrylamide gels was carried out in Mini protean gel electrophoresis chamber. Two glass slides were put one in front of the other at the same height and were clamped in the chamber. Afterwards, the chamber was poured with separating gel (see Table 13) up to 2 cm under the border of the glasses. 1 ml of 2-butanol was added to accelerate the polymerisation. After 1 hour the 2-butanol was removed and the stacking gel (see Table 13) was added. The comb was put to form the slots for the samples and was left for 30 min. When it was compact, the slots were loaded with the samples and the protein marker.

Table 12. Quantity of samples and protein marker that was required for the SDS-PAGE.

	Quantity	Loading buffer's quantity
Sample	25 µl	6 µl
Protein marker	5 µl	3 µl

Table 13. Composition of separating gel and stacking gel.

Components	5% Stacking gel	10% Separating gel
$H_2O_{dest.}$	2.2 ml	3 ml
30 % Acrylamide, 0.8% Bisacrilamyde	630 µl	2.5 ml
4x TrisCl/SDS	940 µl $_{pH6.8}$	1.9 ml $_{pH8.8}$
30% APS	25 µl	50 µl
TEMED	10 µl	10 µl

Table 14. Compositon of 4x TrisHCl/SDS for stacking and separating gel.

Components	1.5M TrisCl/ 0.4% SDS $_{pH8.8}$	0.5M TrisCl/ 0.4% SDS $_{pH6.8}$
Trisbase	18.2 g	6.05 g
SDS[a)]	0.4 g	0.4 g
$H_2O_{dest.}$	Add to 100 ml	

Both solutions were adjusted to its correspondent pH with 1N HCl.

[a)] SDS was added after having autoclaved the solution.

Finally, the chamber was filled up with 1x Laemmli buffer (see Table 16). Voltage of 50V was applied for 30 min at the beginning and was increased to 120V.

Table 15. 10x Laemmli.

Components	Quantity
Glycine	720.5 g
Tris base	154.5 g
SDS	50 g
$H_2O_{dest.}$	1 l

Table 16. 1x Laemmli buffer.

Components	Quantity
10x Laemmli	100ml
$H_2O_{dest.}$	900ml

4.3. Western-blot

According to Towbin (194), when the electrophoresis finished, the polyacrylamide gels were taken out of the glass slides and were transferred onto a Whatman filter paper which was previously soaked in transfer buffer (see Table 17). The uncovered side of each gel was overlaid with a sheet of pre-wetted nitrocellulose membrane and was ensured that air bubbles between the gel and the membrane were excluded. Another Whatman filter paper was laid over the membrane. Afterwards, pre-wetted sponges were added at both sides forming a sandwich. The sandwich was placed in a plastic support, which was inserted into a MiniTrans-blot Electrophoretic Transfer Cell containing Transfer buffer (see Table 17). The transfer took place for 90 min at a constant current of 0.3A.

Table 17. Transfer buffer.

Components	Quantity
Trisbase	9.09 g
Glycine	43.2 g
Methanol	600 ml
$H_2O_{dest.}$	Fill up to 3 l

After the transfer, the nitrocellulose membrane was blocked by incubating with 30 ml Blocking buffer (see Table 18) for 30 min at room temperature. Then, the Blocking buffer was removed and the primary antibody (anti-M45) (see Table 19) was added for 1 hour. The membrane was washed three times for 10 min with Wash buffer (see Table 18) and then was incubated for 1 hour at room temperature with the secondary antibody (Goat anti-mouse IgG (H+L) horseradish peroxidase) (see Table 19). The membrane was washed three times as described above and was developed using a chemiluminescence kit (Super Signal® West Pico, Pierce, Illinois, USA). The signals were detected using Kodak films.

Table 18. Blocking/Wash buffer.

Components	Quantity
PBS	500 ml
Tween 20	1 ml
Dry milk powder	15 g

Table 19. Antibodies used for Immunoblot.

Antibody	Type	Origin	Concentration	Source
Anti-M45	Monoclonal	Mouse	1:1000	Genovac, Freiburg, Germany
Goat anti-mouse IgG (H+L) Horseradish peroxidase	Monoclonal	Mouse	1:5000	Pierce, Illinois, USA

5. Mice infection experiments

5.1. Mice

Specific-pathogen-free female BALB/c mice, 6-8 weeks old, were purchased from Harlan-Winkelmann (Borchem, Germany). For the experiments, mice were housed in groups of five under standard barrier conditions in individually ventilated cages (Tecniplast, Buguggiate, Italy) and equipped with steel grid floors and autoclaved filter paper or with mulch. All mice were kept under specific-pathogen-free conditions (positive-pressure cabinet) and were provided with food and water *ad libitum*.
Animal experiments were approved by German authorities and performed according to the legal requirements.

5.2. Infection of mice

5.2.1. Oral prime immunization with *Salmonella* spp.

The day before immunization, a single colony of different strains of *S. typhimurium* or *S. dublin* was picked in 3 ml LB medium with its adequate antibiotic and was incubated overnight at 37ºC and 200 rpm. On next day, 1 ml of the o/n culture was inoculated in 50 ml 0.3M NaCl LB medium (1:50) and incubated until OD_{600} of 0.6-0.8. In previous experiments the correlation between OD_{600nm} and CFU was determined. According to this correlation, a defined volume of culture was taken and centrifugated at 4000 rpm (3220 rcf) and 4ºC for 15 min. The bacterial sediment was resuspended with PBS, pH 7.4. Each

mouse was orally prime-immunized with $5x10^8$ *Salmonella* spp. in 50 µl PBS. Inoculum was determined by plating serial dilutions of bacterial suspension on LB plates with selective antibiotic. The plates were incubated overnight at 37ºC and the CFU was determined on next day.

5.2.2. Boost immunization with *Salmonella* spp.

Boost immunizations were carried out orally at day 30 and 60 after prime immunization. The mice dose and procedures were the same as in prime immunization (see chap. 5.2.1).

5.2.3. Immunization with *L. monocytogenes*

On the immunization day, 5 ml BHI Medium were infected with 20µl of *L. monocytogenes* 10403s. The culture was incubated at 37ºC and 200 rpm until OD_{600} = 0.5-1. Previous experiments had determined the CFU by OD_{600} = 0.1. Serial dilutions were performed to obtain the end concentration of $5x10^3$ for *Listeria* infection and $1x10^4$ for lethal dose. The number of bacteria inoculated to mice was determined by plating the inoculum and its dilutions on BHI agar. The plates were incubated at 37ºC overnight and the CFU was determined on next day.

5.2.4. Challenge with *L. monocytogenes*

Challenge infection was carried out with *L. monocytogenes* 10403s 6 weeks after prime immunization with recombinant *Salmonella.* Immunized mice were challenged intravenous with a lethal dose of $1x10^4$/ 200µl. Three days after the challenge, mice were sacrificed and the bacterial load was determined in spleens by plating the organs on BHI plates. The plates were incubated 16 hours at 37ºC.

5.3. Collection of samples from mice

At determined time points post immunization, mice were sacrificed by CO_2 asphyxiation and samples from the intestinal tract, mesenteric lymph nodes, Peyer's patches and spleens were collected in cold PBS for analysis.

5.3.1. Intestinal contents

Intestinal contents from the cecum of mice immunized with *Salmonella* spp. were weighed before resuspending them in 500 µl of 4ºC PBS, pH 7.4. The numbers of CFU per 1 g from intestinal content were determined by plating serial dilutions on MacConkey agar plates containing selective antibiotic.

5.3.2. Organs

Mesenterial lymph nodes, Peyer's patches and spleens were aseptically removed from mice infected with *Salmonella* spp. Each organ was put into 2 ml Eppendorf tube with 500 µl sterile PBS containing 0.5% Tergitol and 0.5% BSA. Sterile steel balls were added in the tubes and were placed in the homogenisator machine. The organs were homogenized for 3 minutes at 80% intensity and serial dilutions were plated on MacConkey agar and LB agar with its appropriated antibiotic. The plates were incubated at 37ºC for 16 hours. The number of bacteria was determined as colony forming units (CFU).

6. Immunological methods

6.1. Enzyme-linked immunospot assay (ELISPOT-assay)

The frequency of activated T lymphocytes in mice with attenuated *Salmonella* spp. was determined by IFN-γ-specific Elispot assay.

A day before the experiment started, a nitrocellulose-backed 96-well microtiter plate of Nalgen Nunc international (Wiesbaden, Germany) was coated with 50 µl Coating buffer (see Table 20) and was incubated o/n at 4ºC.

Table 20. Composition of Coating buffer.

Components	Quantity	Source
$H_2O_{dest.}$	4 ml	
2.93 g $NaHCO_3$ / 100 ml $H_2O_{dest.}$	500 µl	Merck Kga, Darmstadt, Germany
1.59 g Na_2CO_3/ 100 ml $H_2O_{dest.}$	500 µl	Merck Kga, Darmstadt, Germany
Rat anti-mouse IFN-γ-monoclonall antibody	10 µl	Clone RMMG-1, Biosource, Camarillo, USA

6.1.1. Preparation of single cell suspension

Spleens were aseptically removed from mice and were placed in a falcon tube with 5 ml DMEM medium (PAN Biotech, Aidenbach, Germany) with 10% FCS and 1% Penicillin/Streptomycin. Spleens were homogenized and filtered with cell strainer (70µm nylon) from Becton Dickinson (Heidelberg, Germany). Afterwards, the cell suspension was applied into a new 50 ml Falcon tube and centrifugated at 20ºC and 1200 rpm (290 rcf) for 10 min. The supernatant was discarded and the sediment was resuspended in the bench.

6.1.2. Lysis of erythrocytes

2 ml of 0.15M NH_4Cl solution, pH 7.4 (see Table 21) were applied to the sediment and were left for 5 min. After the lysis of erytrocytes, 5 ml DMEM medium were added and were centrifugated for 10 min at 1200 rpm (290 rcf) and 20ºC. The supernatant was removed and the sediment was resuspended. 5 ml T-cell medium (see Table 22) were added and the number of cells was determined.

Table 21. Composition of the 0.15M NH_4Cl solution for lysis erythrocytes.

Components	Quantity
NH_4Cl	8 g
$H_2O_{dest.}$	1 l and adjust to pH 7.4

Table 22. Composition of T-cell medium.

Components	Quantity	Source
1M HEPES	5 ml	Sigma-Aldrich Chemie, GmbH. Steinheim, Germany
Penicillin/Streptomycin	5 ml	Seromed Biochrom, Berlin, Germany
Non-essential aminoacids	5 ml	Seromed Biochrom, Berlin, Germany
Fetal calf serum	50 ml	PAN Biothech, Aidenbach, Germany
β-mercaptoethanol	500 µl	GIBCO, Praisley, Scottland
Alpha MEM Eagle medium	500 ml	PAN Biothech, Aidenbach, Germany

For determining the numbers of cells, 10 µl of cell suspension were added to 90 µl Trypan blue solution (0.5%) (Sigma-Aldrich, Irvine, UK). 10µl aliquot of the mixture was put into a Neubauer counting chamber and viable cells were counted under a light microscope.

6.1.3. Stimulation with peptides and development of spots

$1.5x10^5$ splenocytes in 100 µl per well were stimulated for 6 hours in round-bottomed microtiter plates in the presence of a 10^{-4}M concentration of the CD8 T-cell epitope $p60_{217-225}$ or LLO_{91-99} (see Table 23). As positive control, cells were incubated with Concanavalin A, a lecithin that stimulates unspecifically T cells. As negative control, cells were only incubated with T-cell medium. After 6 hours incubation, 100 µl of each probe were transferred to Elispot (Nitrocellulose) plates and were incubated for 12-18 hours. On next day, the wells were washed ten times with Wash buffer (see Table 25) and were incubated with Biotin-labeled rat anti-mouse IFN-γ mAb (clone XMG1.2; Pharmingen, San Diego,

USA) (see Table 24) for 2 hours at room temperature. Then, the plate was washed five times with Wash buffer and incubated with Horseradish peroxidase-streptavidin conjugate (Dianova, Hamburg, Germany) for 2 hours at RT. Thereafter, the wells were washed again 5 times and were developed with a Dye solution (see Table 26) for 30 min at RT. The reaction was stopped with tap water. The wells were dried and the spots were counted.

Table 23. p60 and LLO T-cell epitopes.

Peptide	Type	Sequence	Source
$p60_{217-225}$	CD8	KYGVSVQDI	Jerini Biotools, Berlin, Germany
LLO_{91-99}	CD8	GYKDGNEYI	Jerini Biotools, Berlin, Germany

Table 24. Antibodies used for ELISPOT.

Antibody	Clone	Origin	Concentration	Source
Anti-IFN-γ	RMMG-1	Mouse	1:500	Biosource, Sollingen, Germany
Biotin-coupled-anti-IFN-γ	XMG1.2	Mouse	1:500	Pharmingen, Hamburg, Germany

Table 25. Composition of Wash buffer.

Composition	Quantity
PBS	500 ml
Tween 20	1.25 ml

Table 26. Composition of Elispot Dye solution.

Composition	Quantity	Source
DMF	2.5 l	Merck, Darmstadt, Germany
AEC	1 tablet	Sigma-Aldrich Chemie, Steinheim, Germany
50 mM Natriumacetate	47.5 ml	Roth, Karlsruhe, Germany
30% H_2O_2	25 µl	Merck, Darmstadt, Germany

7. Statistical analysis

The statistical significance of the results was checked with the non-parametric Tukey multiple comparison test at the 0.05 significance level. All tests were performed using WINKS statistical analysis software (Texasoft, Cedar Hill, USA).

D. RESULTS

Our laboratory has reported that the single oral immunization of mice with recombinant *S. typhimurium* expressing translocated *Yersinia* outer protein (YopE) fused to the immunodominant antigen p60 or LLO from *L. monocytogenes* results in a strong induction of p60- or LLO-specific CD8 T cells and confers protection against a lethal wild-type *Listeria* challenge infection (86, 168).

There are conflicting reports concerning the impact of prior vector priming on the immunogenicity of recombinant *Salmonella*-based vaccines. Some data indicated that prior exposure to *Salmonella* enhanced antibody responses to a foreign antigen delivered orally by *Salmonella* (7, 208). These findings were contradicted by studies reporting that prior exposure to *Salmonella* can dramatically reduce serum antibody responses to a foreign antigen (5, 102, 162, 203). However, the first goal of the present study was to determine whether (i) oral boost immunizations result in an enhanced p60-specific CD8 T-cell response and whether (ii) anti-listerial immunity induced by the first immunization with recombinant *Salmonella* expressing YopE/p60 contributes to a more rapid clearance of the vaccine carrier after subsequent immunizations of mice.

1. Influence of boost immunizations on the antigen-specific CD8 T-cell induction

For this study, the pWSK29 derivative plasmid pHR241 was employed. Plasmid pHR241 is a low-copy-number expression vector that encodes SycE, the specific chaperone of YopE, the N-terminal translocation domain of YopE ($YopE_{1-138}$) and the p60 murein hydrolase of *L. monocytogenes*. The $p60_{130-477}$ fragment bears an amino acid exchange in the cystein 396 for alanine to avoid any bacterial cell-lysing activity (216). The chimeric protein was tagged at the C-terminus with 18 amino acids of M45 (MDRSRDRLPPFETETRIL), which is derived from the E4-6/7 protein of adenovirus.

The transcription of the hybrid *yopE* gene fusion is controlled under the *lac* promoter, which is constitutively active in *Salmonella*.

Fig. 7. Scheme of plasmid pHR241. Plasmid pHR241 bears the genetic information for SycE, the specific chaperone of YopE, the translocated chimeric $YopE_{1-138}$ fused to $p60_{130-477}$(cys→ala) and the epitope-tag M45. Transcription of the gene fusion is achieved under the control of the *lac* promoter, which is constitutively active in *Salmonella*.

Plasmid pHR241 was transformed into *S. typhimurium* SB824 and *S. dublin* BRD620 by electroporation. Strain SB824 (169) was engineered by introducing the s*ptP::Kan* mutant allele from strain SB237 (95) into the *aroA* mutant strain SL3261 (74) by P22HT*int* transduction. Strain BRD620, an *aroA aroD* mutant, was kindly provided by Mark Roberts (162).

1.1. Determination of colonization and persistence of *S. typhimurium* SB824 (pHR241) in BALB/c mice after single oral application

We evaluated the ability of the attenuated strain SB824 (pHR241) to colonize and persist in murine organs after oral administration. Strains with mutations in the prechorismate pathway (*aro* mutants) are defective in the production of aromatic compounds including (aromatic) amino acids, which are essential for bacterial growth. Aromatic *Salmonellae* mutants have been found to be highly attenuated in mice (74). For this investigation, BALB/c mice were orally immunized with $5x10^8$ CFU of SB824 (pHR241). On days 7, 14, and 21 mice were sacrificed and the number of bacteria present in Peyer's patches and spleens was determined by viable count (see Fig. 8). Seven days after the orogastric inoculation, bacteria were recovered from Peyer's patches and spleens ($6x10^2$ and $4x10^2$ CFU, respectively). By day 14 post administration, bacteria were still present in both organs. However, the amounts of SB824 (pHR241) were significantly decreased as compared to day 7 after inoculation ($4x10^1$ CFU in spleens and $1x10^1$ CFU in Peyer's patches). By day 21 post immunization, the mutant strain was almost undetectable in spleens and totally cleared from Peyer's patches. Bacteria could not be reisolated anymore from these organs on day 28 after oral administration (data not shown).

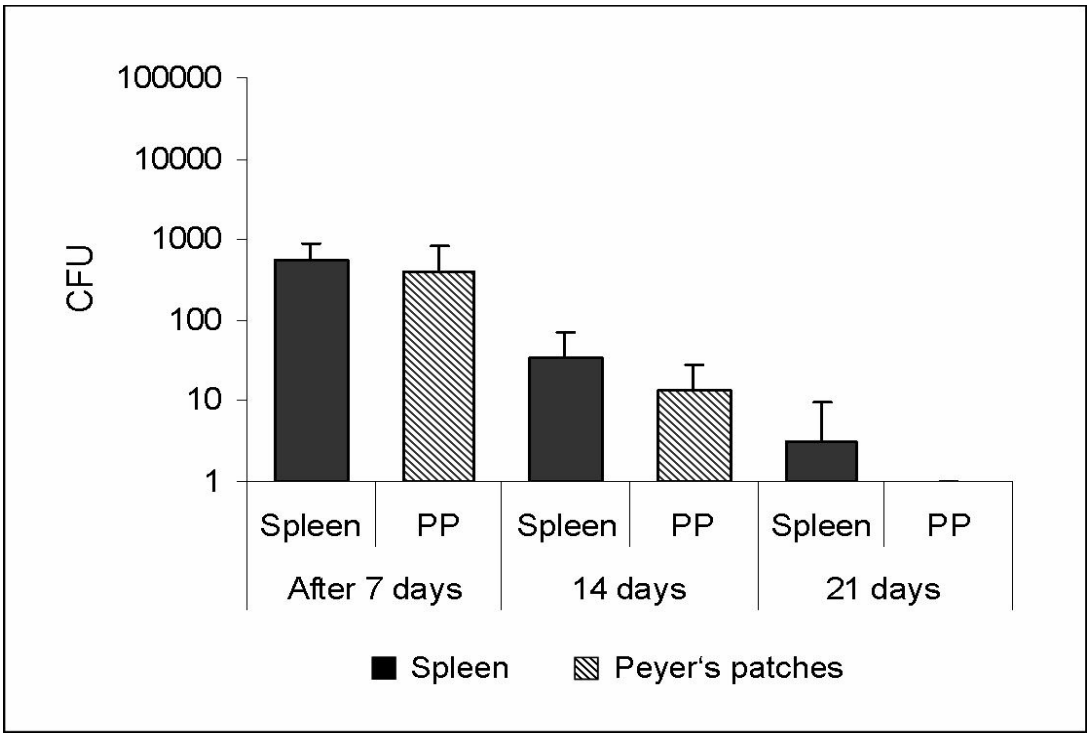

Fig. 8. Time course of colonization and persistence of *S. typhimurium* SB824 (pHR241) in mice organs. BALB/c mice were orally immunized with $5x10^8$ CFU of SB824 (pHR241). On days 7, 14, and 21 post infection mice were killed and viable bacteria were determined as CFU in spleens and Peyer's patches. The results and standard deviations from 15 individual mice are indicated.

1.2. Time course of colonization of recombinant *Salmonella* strains after boost immunizations

BALB/c mice were orally immunized one, two, or three times with the attenuated *aroA* mutant strains SB824 or SB824 (pHR241) expressing chimeric $YopE_{1-138}/p60_{130-477}$/M45 (168), and the kinetics of colonization and persistence of the bacteria *in vivo* were investigated. The immunization and sampling schedule is shown in Fig. 9. The time course of colonization was determined by counting the numbers of viable bacteria, as CFU, in the cecum, mesenteric lymph nodes, and spleens on days 2, 4, and 7 after the respective oral immunization.

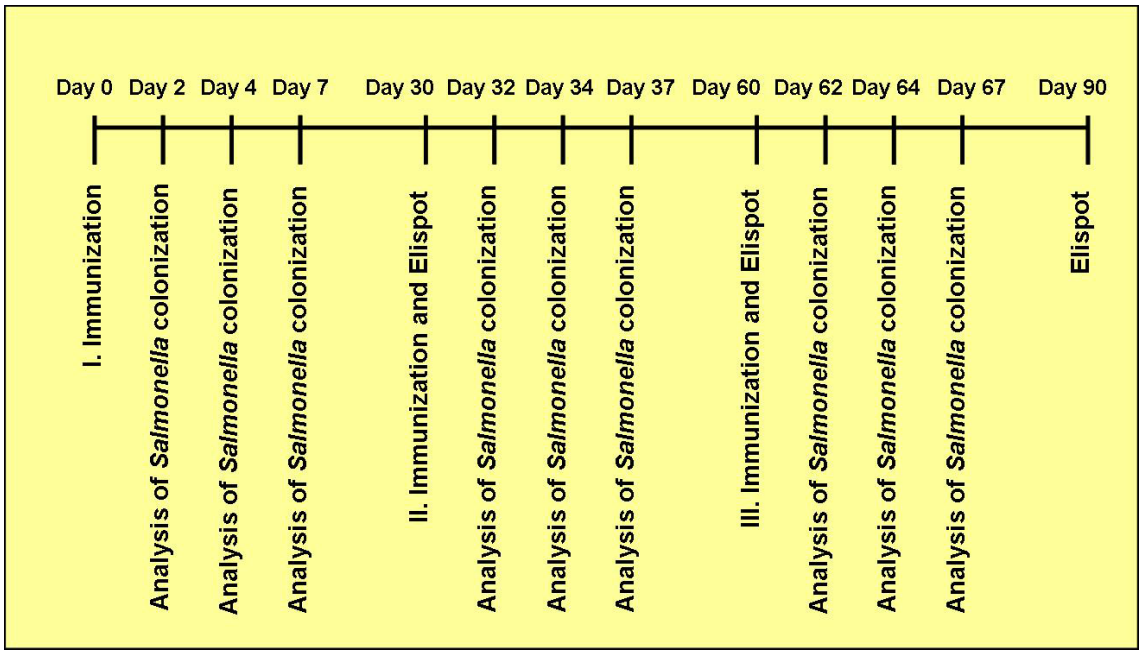

Fig. 9. Immunization, sampling and Elispot schedule. BALB/c mice were orally immunized at the days indicated (0, 30, and 60). Samples of the cecum, mesenteric lymph nodes, and spleens were collected and analyzed for the presence of *Salmonella* 2, 4, and 7 days after the respective immunizations. Elispot assays were performed on days 30, 60, and 90.

The results are summarized in Fig. 10. Two days after the first oral immunization, both strains colonized the intestine but were not detectable in mesenteric lymph nodes and spleens (see Fig. 10A). The time course of colonization of both strains, SB824 and SB824 (pHR241) was progressive, with dissemination of the bacteria into mesenteric lymph nodes on day 4 post immunization, and into spleens on day 7 post immunization, respectively (see Fig. 10A). Surprisingly, the colonization profiles of both strains did not differ significantly, indicating that the plasmid-mediated expression of hybrid YopE/p60/M45 did not diminish *Salmonella´*s ability to persist in the host. It is noteworthy that pHR241 was remarkably stable *in vivo*, with >97% of the bacterial population retaining the recombinant plasmid 7 days after immunization (data not shown).

Two days after the second oral immunization (day 32), both strains colonized the cecum and were already detectable in mesenteric lymph nodes and spleens at comparable levels (see Fig. 10B). *Salmonella* disseminated faster into the latter two organs as compared to the time course of dissemination after the first immunization (see Fig. 10A). SB824 and SB824 (pHR241) were also present in intestines, mesenteric lymph nodes, and spleens four days after the second immunization (day 34) (see Fig. 10B). However, by day 37, all mice had cleared both recombinant strains indicating an efficient *Salmonella*-specific immune response.

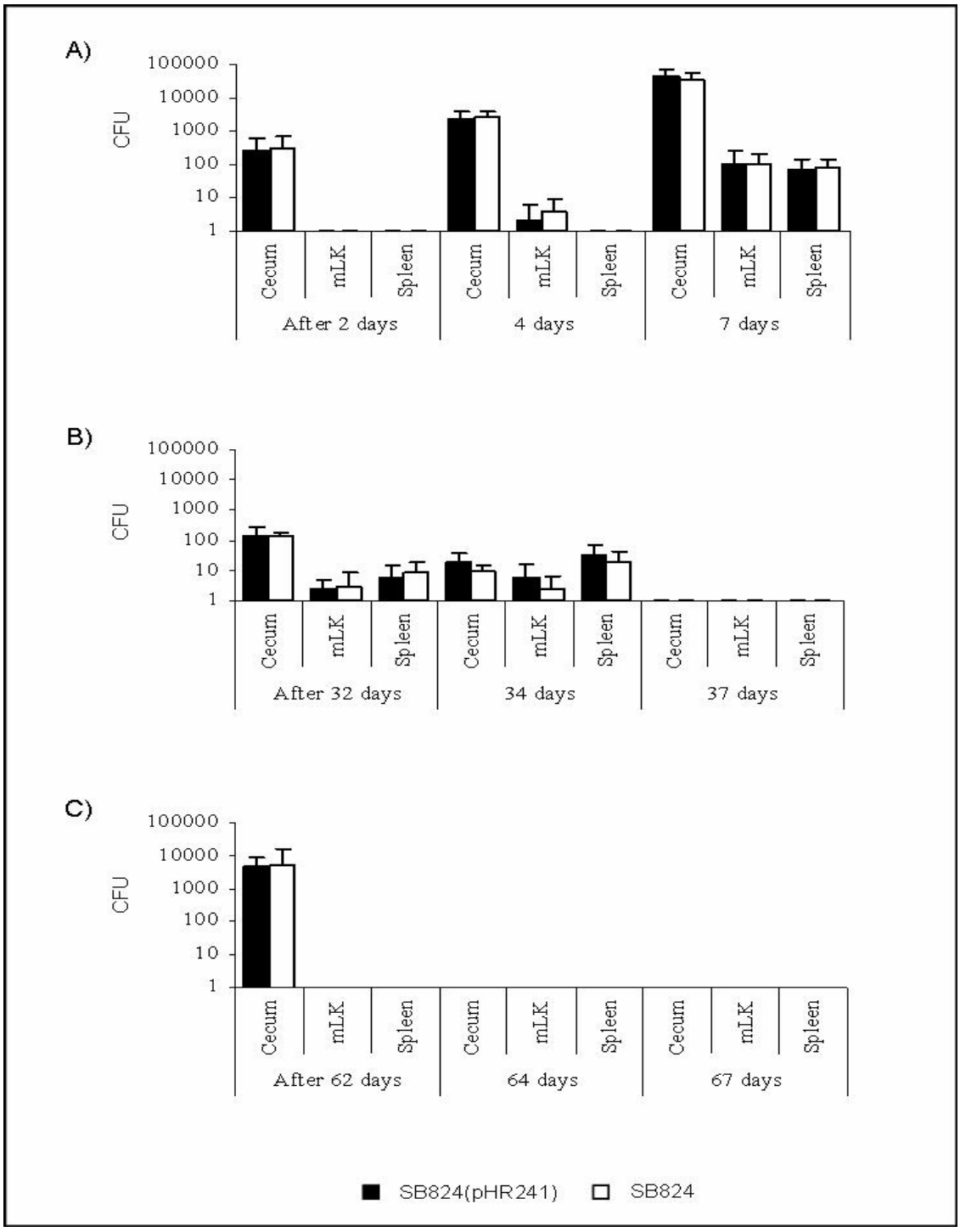

Fig. 10. Time course of colonization and persistence in mice organs. Mice were orally immunized with either *S. typhimurium* SB824 (pHR241) expressing YopE/p60 (filled bars) or SB824 (open bars). A) Mice received a single immunization on day 0. Two, 4, and 7 days later mice were killed and the numbers of bacteria present in cecum, mesenteric lymph nodes and spleens were determined as CFU. B) Mice received a prime immunization on day 0 and a boost immunization on day 30. On days 32, 34, and 37 mice were killed and the numbers of bacteria present in the respective organs were determined as CFU. C) Mice received a prime immunization on day 0 and two boost immunizations on days 30 and 60. On days 62, 64, and 67 mice were killed and the numbers of bacteria present in the respective organs were determined as CFU. The results and standard deviations of 15 individual mice per group are indicated. Comparing same time points, values for SB824 and SB824 (pHR241) do not differ significantly ($P > 0.05$).

Two days after the third oral immunization (day 62), both strains colonized the intestine but were not detectable in mesenteric lymph nodes and spleens (see Fig. 10C). *Salmonella* was rapidly cleared on the following days by all mice, thus preventing further dissemination. By day 64 and day 67, SB824 and SB824 (pHR241) could not be recovered from any organ investigated in this study.

Taken together, at a given time point post inoculation, no significant differences in the ability of both *Salmonella* strains to colonize, disseminate and persist in various mouse organs were observed. Particularly after the third immunization, mice revealed a rapid clearance of the vaccine carrier strains within 4 days.

1.3. CD8 T-cell responses after boost immunizations

Recently, we have used the p60 protein of *L. monocytogenes* as a model antigen for the construction of hybrid YopE proteins to be delivered by the *Salmonella*-T3SS because the p60 protein is remarkable conserved among all *Listeria* species (17, 86, 168). After invasion of host cells and the escape from the phagosome, *Listeria* constitutively secretes the murein hydrolase p60 (157). Subsequently, p60 is directed to the MHC class I antigen processing pathway, leading to presentation of antigen-derived peptides to CD8 T cells (148). Analysis of T cells from *Listeria*-infected BALB/c mice revealed that the immunodominant listerial nonamer peptide $p60_{217\text{-}225}$ is presented to cytotoxic CD8 T lymphocytes in the context of the H2-K^d MHC class I molecule (147, 148). The previously described low-copy-number plasmid pHR241 (see Fig. 1) bears the genetic information for a YopE/p60 hybrid protein (168). The N-terminal 138 amino acids of YopE containing the secretion and translocation domains (175, 184) were fused to $p60_{130\text{-}477}$ and the resulting chimeric protein was tagged at its C-terminus with an adenoviral M45 epitope (141). Constitutive expression of the respective gene fusion led to the production of a hybrid protein that was shown to be translocated into the cytosol of macrophages by *S. typhimurium* (168). Furthermore, it was demonstrated that a single oral immunization with an attenuated *Salmonella aroA* mutant strain expressing translocated YopE/p60 resulted in the induction of a p60-specific CD8 T-cell response and animal protection against a virulent *L. monocytogenes* challenge (168, 202).

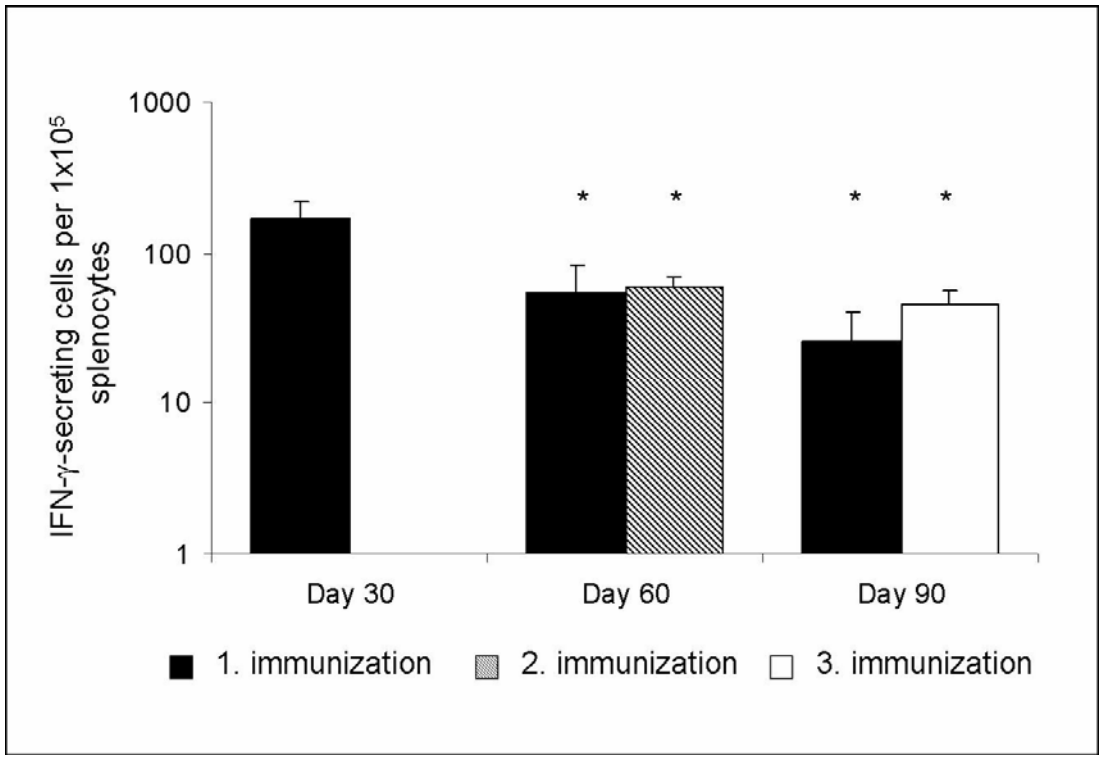

Fig. 11. Frequency of p60-specific CD8 T cells in spleens of mice orally immunized with *S. typhimurium* strain SB824 (pHR241) expressing translocated YopE/p60. Mice received either a single (prime) immunization (filled bars) on day 0, a prime immunization and a boost immunization on day 30 (hatched bar), or a prime immunization and two boost immunizations on days 30 and 60 (open bar). T cell frequencies were determined by Elispot assay. The frequencies of cells reactive with $p60_{217-225}$ are shown as the number of reactive cells per 10^5 splenocytes. The standard deviations of three cultures from 10 individual mice per group are indicated. Asterisks indicate values that differ significantly ($P < 0.05$) from that of mice sacrificed on day 30 (filled left bar).

The question was asked whether boost immunizations on day 30 and day 60 after prime immunization could augment the frequency of antigen-specific CD8 T cells. The frequency of $p60_{217-225}$-specific CD8 T cells was calculated as the number of IFN-γ spots generated per $1x10^5$ spleen cells in the presence of the corresponding synthetic peptide. Mice immunized with a single dose of SB824 (pHR241) translocating YopE/p60 revealed high numbers of IFN-γ-producing cells reactive with $p60_{217-225}$ 30 days after inoculation (see Fig. 11, filled left bar). In comparison, the frequency of $p60_{217-225}$-specific CD8 T cells was significantly lower in the same group of mice 60 days after immunization (see Fig. 11, filled middle bar). Mice that had received a second immunization on day 30 did not show significant higher numbers of antigen-specific CD8 T lymphocytes by day 60 (see Fig. 11, hatched bar) as compared to mice that were immunized with a single dose (see Fig. 11, filled middle bar). A further but not significant decline of $p60_{217-225}$-specific CD8 T cell

frequencies was found in all mice 90 days after prime immunization. Thus, even a third immunization (see Fig. 11, open bar) on day 60 could not enhance the p60-specific CD8 T-cell response as compared to mice that had obtained only the prime immunization (see Fig. 5, filled right bar).

Taken together, mice orally immunized with a single dose of SB824 (pHR241) reached the highest numbers of IFN-γ–producing cells reactive with $p60_{217-225}$ on day 30 post immunization. After this time point, the frequencies of $p60_{217-225}$-specific CD8 T cells were declining despite mice received one and two boost immunizations.

2. Influence of short-term colonization on CD8 T-cell priming

As demonstrated above, we found that the ability of recombinant *Salmonella* to colonize the intestine, mesenteric lymph nodes, and spleen were markedly impaired after boost immunizations probably due to anti-vector immunity.

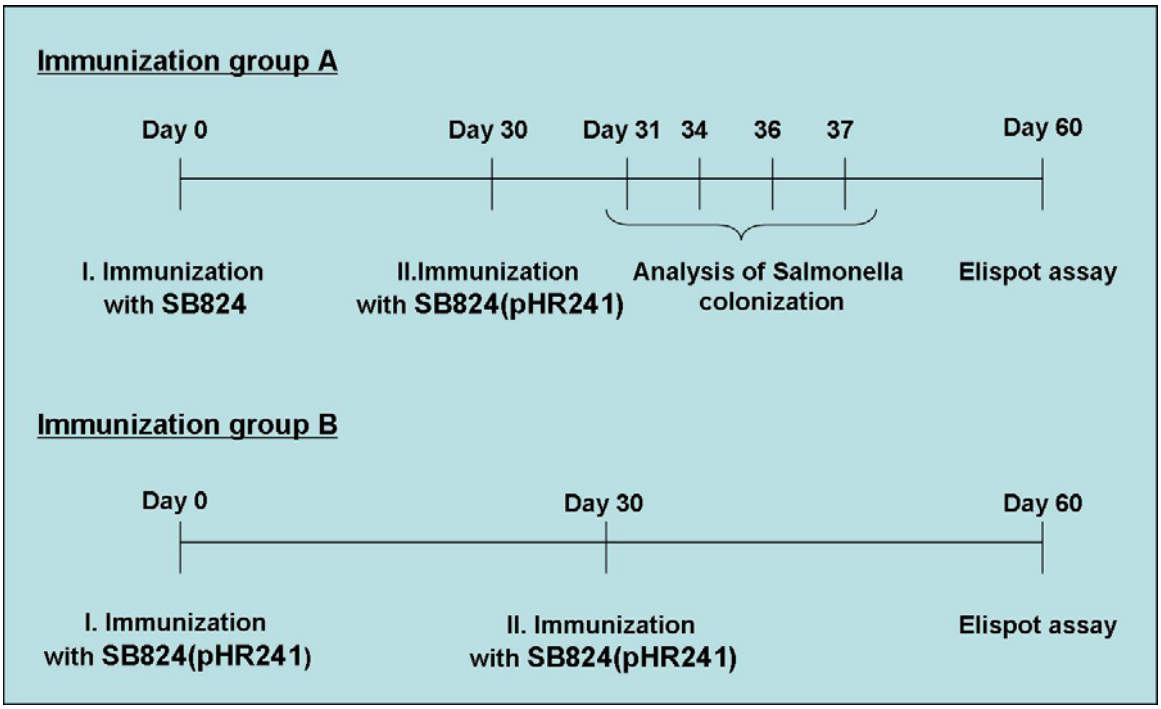

Fig. 12. Immunization, sampling and Elispot schedule. Two groups of BALB/c mice were orally immunized on day 0 with either SB824 (immunization group A) or with SB824 (pHR241) (immunization group B). On day 30, both groups of mice were boost-immunized with SB824 (pHR241) and by day 60, Elispot assays were carried out for the purpose of determining the frequency of $p60_{217-225}$-specific CD8 T cells. Samples of cecum, Peyer's patches, mesenteric lymph nodes and spleens were collected and analyzed in group A for the presence of viable *Salmonella* 1, 4, 6, and 7 days after the second immunization.

In a next set of experiments, we were interested to identify, whether a short-term colonization is able to induce a $p60_{217-225}$-specific CD8 T-cell response. Therefore, two groups (A and B) of BALB/c mice were prime immunized with $5x10^8$ CFU of either SB824 or SB824 (pHR241), respectively (see Fig. 12).

By day 30, mice of both groups were boost-immunized with SB824 (pHR241) and 30 days later, the frequency of $p60_{217-225}$-specific CD8 T cells was determined by Elispot assay. In immunization group A, the colonization and persistence of SB824 (pHR241) was investigated on days 31, 34, 36, and 37 (1, 4, 6, and 7 days after the second immunization) (see Fig. 13). Results from the respective experiments with mice of immunization group B are demonstrated in Fig. 10B.

2.1. Time course of colonization of SB824 (pHR241) after prime immunization with SB824

Fig. 13 summarizes the kinetics of colonization and persistence of bacteria in mice of immunization group A. Bacteria were able to colonize cecum, Peyer's patches, mesenteric lymph nodes and spleens from mice on day 1 post boost immunization. The bacterial load in cecum was $1.5x10^4$ CFU. In contrast, few numbers of bacteria were found in the Peyer's patches, mesenteric lymph nodes and spleens. Four days after the second immunization (day 34), SB824 (pHR241) was present in all organs investigated. The amount of bacteria in Peyer's patches, mesenteric lymph nodes and spleens was comparable to the numbers of bacteria on day 1 post boost immunization. However, the bacterial load in cecum was significantly reduced (3 $\log_{10}$ lower). As expected, the colonization and persistence profiles of bacteria in these organs were comparable with the amount of bacteria collected in the former study after the second immunization with either SB824 or SB824 (pHR241) (see Fig. 10B). This confirms that expression of hybrid YopE/p60 by pHR241 does not alter the course of colonization and persistence of bacteria in the organs of mice.

In the experiments described in chapter 1.2, viable SB824 and SB824 (pHR241) were analyzed on days 32, 34 and 37 and it was evident that bacteria disappeared from all organs between days 34 and 37. To find out the exact time point of bacterial clearance, day 36 was added to the investigation. By day 6 after boost immunization (day 36), bacteria were still detectable in all organs and no significant differences in bacterial loads were observed in comparison to day 34. As previously shown in Fig. 10B, on day 37 bacteria could not be isolated from the organs investigated.

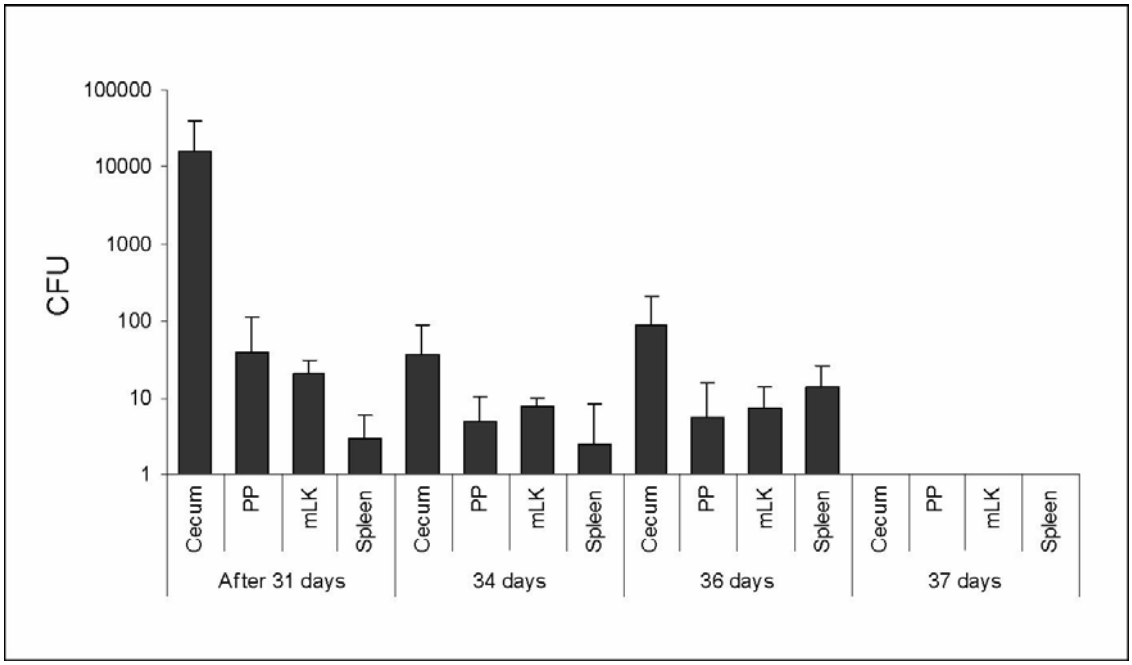

Fig. 13. Kinetics of colonization and persistence in mice organs 1, 4, 6, and 7 days after boost immunization. BALB/c mice were prime immunized with the *aroA S. typhimurium* mutant strain SB824 on day 0 and boost-immunized with SB824 (pHR241) expressing YopE/p60 on day 30 (Fig. 12, immunization group A). Mice were sacrificed on day 31, 34, 36 and 37. Cecum, Peyer's patches, mesenteric lymph nodes and spleens were removed from mice, homogenized and plated on MacConkey agar with selective antibiotic. The viable bacteria were determined as CFU. Columns represent the standard deviation of 15 individual mice.

2.2. Impact of 6-days colonization on p60-specific CD8 T-cell induction

It has been shown above that SB824 (pHR241) was only able to colonize and persist in mice organs for 6 days after prior exposure to SB824. We asked whether a 6-days colonization is sufficient to induce a $p60_{217-225}$-specific CD8T-cell response. To answer this question, two groups of BALB/c mice were immunized on day 0 with either *S. typhimurium* SB824 (see group A, Fig. 12) or SB824 (pHR241) (group B, Fig. 12). Four weeks later, both groups received a second immunization with SB824 (pHR241), expressing the hybrid YopE/p60 protein. By day 60, mice were sacrificed and an Elispot assay was carried out to determine the frequency of $p60_{217-225}$-specific CD8 T cells *in vivo*. Mice immunized twice with SB824 (pHR241) translocating YopE/p60 revealed high numbers of IFN-γ-producing cells reactive with $p60_{217-225}$ (see Fig. 14, open bar). In contrast, mice that were prime immunized with SB824 and subsequently boosted with

SB824 (pHR241) showed significantly lower numbers of $p60_{217-225}$-reactive CD8 T cells (see Fig. 14, filled bar).

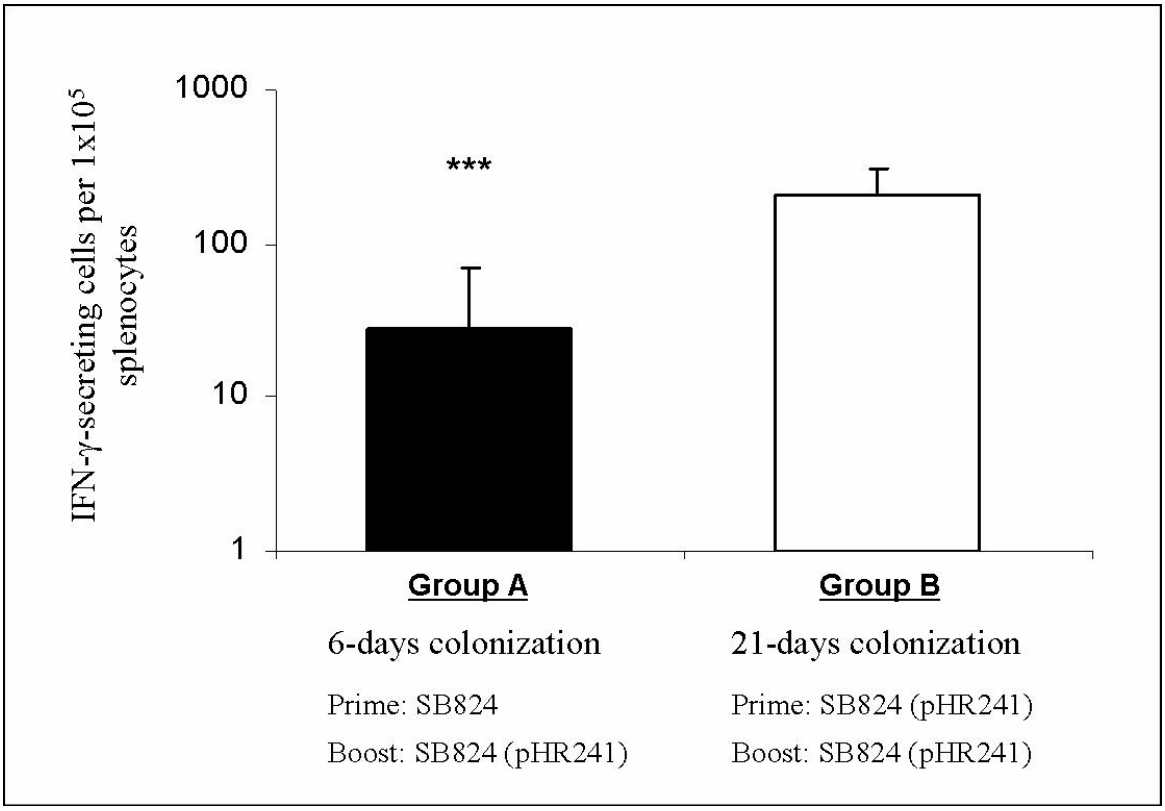

Fig. 14. Frequency of p60-specific CD8 T cells in spleens of mice after 6-days and 21-days colonization. Two groups of mice were prime immunized with either *S. typhimurium* SB824 (group A, filled black bar) or SB824 (pHR241) expressing the hybrid protein YopE/p60 (group B, open bar). On day 30, both groups received a second immunization with SB824 (pHR241). By day 60, an Elispot assay was performed. The standard deviations from 15 individual mice are indicated. Asterisks show values that differ highly significantly ($P < 0.001$) from that of mice immunized twice with SB824 (pHR241).

In conclusion, this demonstrates that a long-term colonization of the vaccine strain and therefore a prolonged display of the heterologous protein leads to a superior induction of antigen-specific CD8 T cells.

2.3. Efficacy of short-term versus long-term colonization on vaccine-induced protection

To compare the contribution of translocated p60 displayed for 6 days (see Fig. 12, group A) versus 21 days (see Fig. 12, group B) on vaccine-induced protection, mice were intravenously (i.v.) challenged with $1x10^4$ CFU of *L. monocytogenes* 10403s 6 weeks after

boost immunization with *S. typhimurium* expressing hybrid YopE/p60 proteins. CFU were determined in spleens 3 days after the challenge. Spleens of uninfected mice (negative control) and mice of group A (6-days colonization) were colonized with $5.2x10^6$ and $5.2x10^5$ CFU of *Listeria*, respectively (see Fig. 15, hatched bar and filled black bar, respectively). In contrast, mice of group B (21-days colonization) showed a pronounced decrease of the bacterial load in spleens ($2.5\ x10^1$ CFU) as compared to non-immunized mice or mice of group A (see Fig. 15, open bar). The display of translocated p60 for 21 days led to a similar level of protection as in *Listeria*-immune mice (positive control) (see Fig. 15, open bar and filled grey bar, respectively).

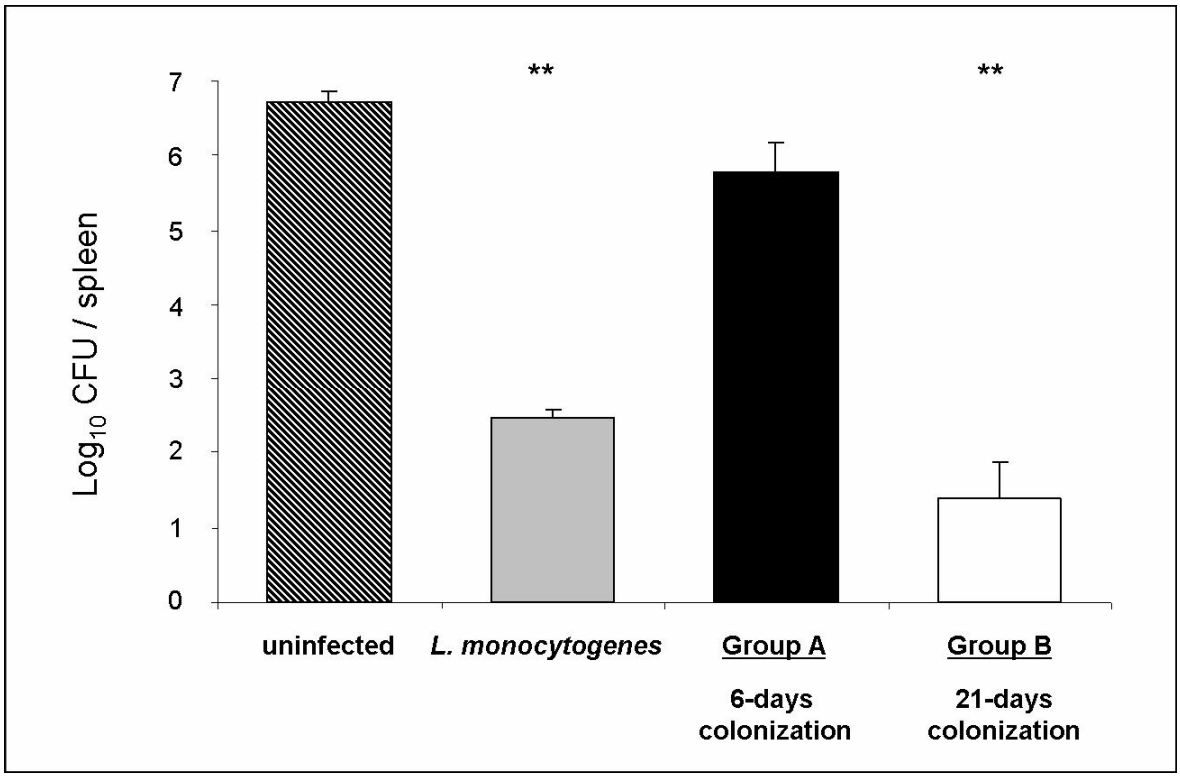

Fig. 15. Efficacy of 6-days colonization versus 21-days colonization to induce protective immunity against listeriosis in mice orally prime immunized with either *S. typhimurium* SB824 (group A) or SB824 (pHR241) (group B) and boost immunized with SB824 (pHR241). Positive control mice recieved a sublethal intravenous dose of $1x10^3$ CFU of *Listeria* 6 weeks before the challenge infection (grey filled bar). Negative control mice were uninfected (hatched bar). Mice were i.v. challenged with $1x10^4$ *L. monocytogenes* 10403s 6 weeks after immunization. The bacterial load of spleens with *L. monocytogenes* was determined 72 hours post-infection. The standard deviations from 15 individual mice are indicated. Asterisks differ significantly form that of negative groups ($P < 0.05$).

In conclusion, mice colonized for 21 days with SB824 (pHR241) were totally protected against the lethal challenge with *Listeria* in comparison to mice colonized only for 6 days with the recombinant *Salmonella* vaccine strain. In the latter group of mice, the low frequency of p60-specific CD8 T cells was not able to confer protection against the *Listeria* challenge infection (see Fig. 14).

3. Use of different *Salmonella* serovars for prime-boost immunizations

Above it was demonstrated that prior exposure to a *S. typhimurium* vaccine strain resulted in a more rapid clearance of bacteria after the second immunization, thus shortening the time of antigen display to 6 days.

Vindurampulle *et al*. showed that the oral application of attenuated *S. dublin* and *S. stanley* strains, harboring mutations in the *aroA* gene, induced anti-lipopolysaccharide (LPS) serum IgG and intestinal IgA antibody titers (203). Structurally, LPS consists of two components: a variable, antigenically active polysaccharide (O-antigen) and a highly-conserved lipid (lipid A). Antibodies directed against the LPS O-antigen confer protection to *Salmonella* infection (81).

The ambition of our *Salmonella*-based vaccination strategy is to use recombinant *Salmonella* strains for boost immunizations against the same or a different antigen. In order to circumvent the problem of pre-existing immunity to *Salmonella*, we decided to employ two different *Salmonella* serovars for prime and boost immunizations. According to the Kauffmann-White scheme, there are more than 2400 different serovars of *S. enterica* (80-82, 115, 179).

In addition to *S. typhimurium* strain SB824, we have chosen the *S. dublin aroA aroD* strain BRD620 for our studies (162). *S. dublin* belongs to serogroup D and expresses the O-antigens O1, O9 and O12. In contrast, *S. typhimurium* belongs to serogroup B and bears the O-antigens O1, O4, O5 and O12 (109). Despite two common O-antigens, we selected these serovars because of the fact that several studies have been published demonstrating the effectiveness of *S. dublin* and *S. typhimurium* as vaccine carriers in the mouse model (30, 74, 121, 156, 163, 183).

3.1. *In vitro* expression and secretion of YopE/p60 by *S. dublin* BRD620

In a first set of experiments, the ability to express and secrete the hybrid YopE/p60 protein was directly compared in *S. typhimurium* SB824 and *S. dublin* BRD620. For this purpose, bacteria were cultivated in 0.3M NaCl LB medium in order to activate the T3SS of *Salmonella* and to induce protein secretion. Two different fractions were examined by immunoblotting for the presence of YopE/p60: (i) the bacterial whole-cell-lysate fraction (fraction 1) and (ii) the supernatant fraction containing secreted proteins (fraction 2). The chimeric protein was detected by using a monoclonal antibody directed against M45.

As shown in Fig. 16, *S. typhimurium* SB824 carrying the low-copy-number vector pHR241 revealed a detectable amount of YopE/p60 in both, the bacterial lysate and the surrounding medium.

The same amount of hybrid protein was observed in fraction 1 and 2 of *S. dublin* BRD620 (pHR241), indicating that both mutant strains express and secrete comparable levels of YopE/p60.

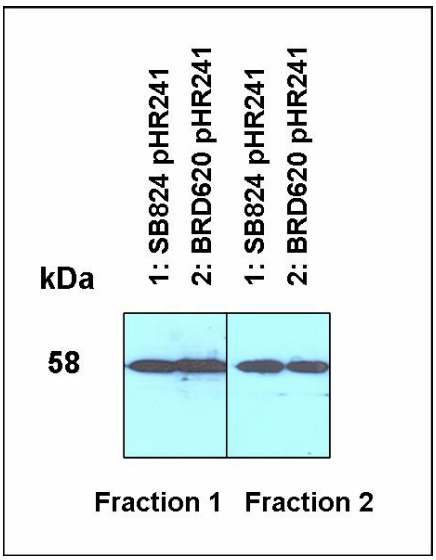

Fig. 16. Western Blot showing the expression and secretion of hybrid $YopE_{1-138}/p60_{130-477}$/M45 proteins by either *S. dublin* or S. *typhimurium*. Both strains were able to express and secrete comparable levels of hybrid YopE/p60/M45. Lane 1 represents *S. typhimurium* SB824 (pHR241) and lane 2 represents *S. dublin* BRD620 (pHR241). Fraction 1 contains the bacterial whole-cell-lysate and fraction 2 contains the proteins secreted into the culture supernatant.

3.2. Determination of colonization and persistence of *S. dublin* BRD620 (pHR241) in BALB/c mice after single oral application

We were interested in studying the kinetics of colonization and persistence of *S. dublin* BRD620 (pHR241) in mice organs in order to compare them with the colonization and persistence profiles of *S. typhimurium* SB824 (pHR241). Vindurampulle *et al.* reported that a *S. dublin aroA* mutant was able to colonize and persist in the gut-associated lymphoid tissue (GALT) of BALB/c mice for 8 days. From this time point on, bacteria were slowly cleared and disappeared from GALT at day 21 (204).

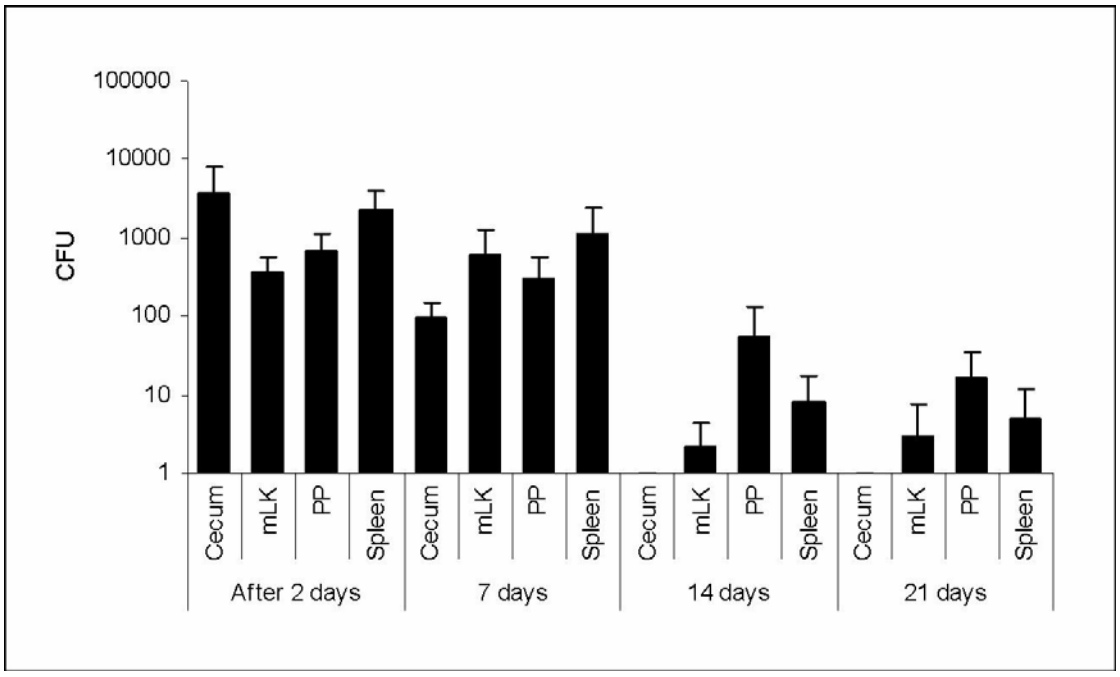

Fig. 17. Time course of colonization and persistence of *S. dublin* BRD620 (pHR241) in intestinal organs and spleens. Mice were orally immunized with *S. dublin* BRD620 (pHR241). Two, 7, 14, and 21 days post immunization, mice were killed and cecum, Peyer's patches, mesenteric lymph nodes and spleens were aseptically removed and homogenized. Afterwards, the numbers of bacteria present in respective organs were determined as CFU. The standard deviations from 10 individual mice are indicated.

For our study, we orogastrically administered $5x10^8$ CFU of *S. dublin* BRD620 (pHR241) to BALB/c mice. Mice were killed and the abilitiy of colonization was analyzed in cecum, mesenteric lymph nodes, Peyer's patches and spleens on day 2, 7, 14, and 21 post immunizations. As demonstrated in Fig. 17, two days after oral immunization, bacteria were detected in cecum ($3.7x10^3$ CFU), Peyer's patches ($6.5x10^2$ CFU), mesenteric lymph nodes ($3.6x10^2$ CFU) and spleens ($2.2x10^3$ CFU). Five days later (day 7), *S. dublin* was

still present in all organs investigated. However, the recovery rate of BRD620 (pHR241) in cecum was two fold lower whereas the colonization in Peyer's patches, mesenteric lymph nodes and spleens showed no differences as compared to day 2. By day 14 after oral immunization, bacteria were not detectable in cecum anymore. Nevertheless, bacteria were still found in Peyer's patches, mesenteric lymph nodes and spleens, but in extremely low amounts. *S. dublin* was still present in Peyer's patches, mesenteric lymph nodes and spleens on day 21. The numbers of viable bacteria in these organs were equivalent to the numbers on day 14.

Taken together, these results revealed that the colonization and persistence profiles of *S. dublin aroA aroD* BRD620 (pHR241) are comparable with the profiles of *S. typhimurium aroA* SB824 (pHR241) (see Fig. 8).

3.3. CD8 T-cell response after a single oral immunization with BRD620 (pHR241)

To investigate whether BRD620 (pHR241) is capable of inducing a p60-specific CD8 T-cell response, BALB/c mice were orally immunized with a single dose of either *S. dublin* BRD620 (pHR241) or *S. typhimurium* SB824 (pHR241). The latter strain was used as positive control. Thirty days later, the frequency of p60-specific CD8 T cells was determined by Elispot assay. As shown in Fig. 18, *S. dublin* BRD620 (pHR241) was able to induce a $p60_{217-225}$-specific CD8 T-cell response (see Fig. 18, hatched bar). However, this response was significantly lower when compared with the response triggered by SB824 (pHR241) (see Fig. 18, filled bar).

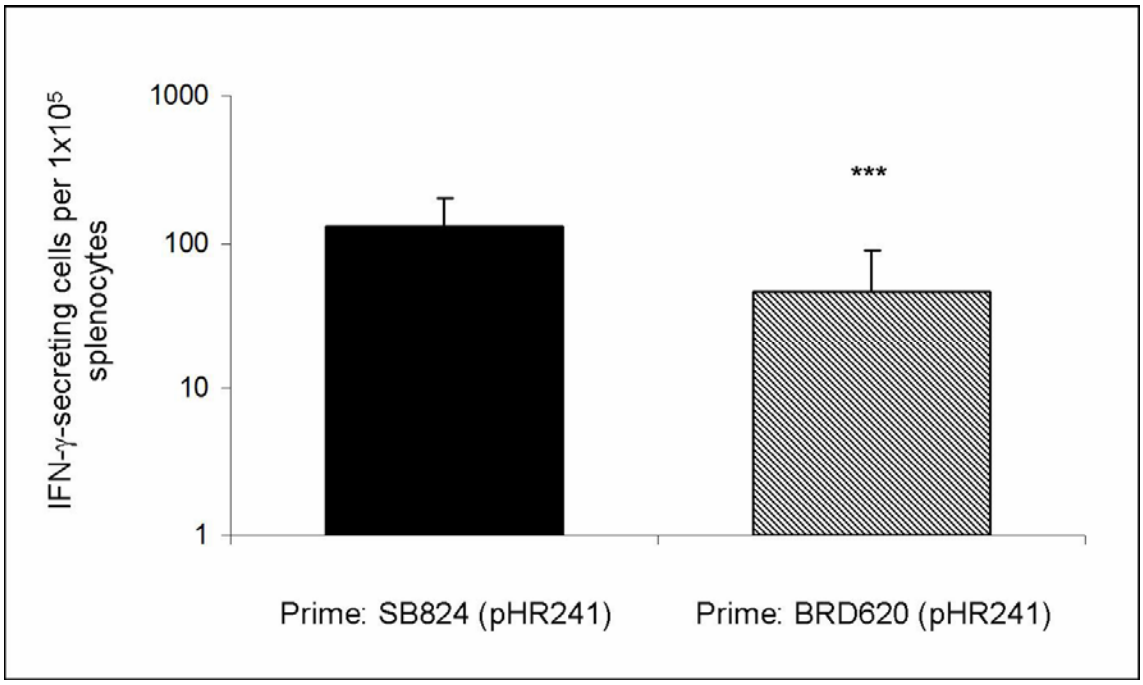

Fig. 18. Frequency of p60-specific CD8 T cells in spleens of mice orally immunized with either *S. typhimurium* SB824 (pHR241) or S. *dublin* BRD620 (pHR241). Mice received a single dose of either *S. typhimurium* SB824 (pHR241) (filled bar) or *S. dublin* BRD620 (pHR241) (hatched bar). On day 30, mice were sacrificed and the frequency of cells reactive with $p60_{217-225}$ was determined by Elispot asssay. The standard deviations of 10 individual mice per group are indicated. Asterisks show values that differ highly significantly ($P < 0.001$) from that of mice immunized *with S. typhimurium* SB824 (pHR241).

3.4. Time course of colonization of recombinant *Salmonella* strains after heterologous boost immunization

For this study, two groups of BALB/c mice were prime immunized with either *S. dublin* BRD620 (pHR241) (see Fig. 19, group A) or with *S. typhimurium* SB824 (pHR241) (see Fig. 19, group B) on day 0. Thirty days later, both groups were boost-immunized with *S. typhimurium* SB824 (pHR241). Mice from immunization group A were sacrificed 2, 4, 7, and 14 days after this boost immunization (days 32, 34, 37 and 44) for collecting cecum, Peyer's patches, mesenteric lymph nodes and spleens of mice and for analyzing the bacterial loads of SB824 (pHR241) in these organs. By day 60, an Elispot assay was performed in both groups to determine the frequency of p60-specific CD8 T cells.

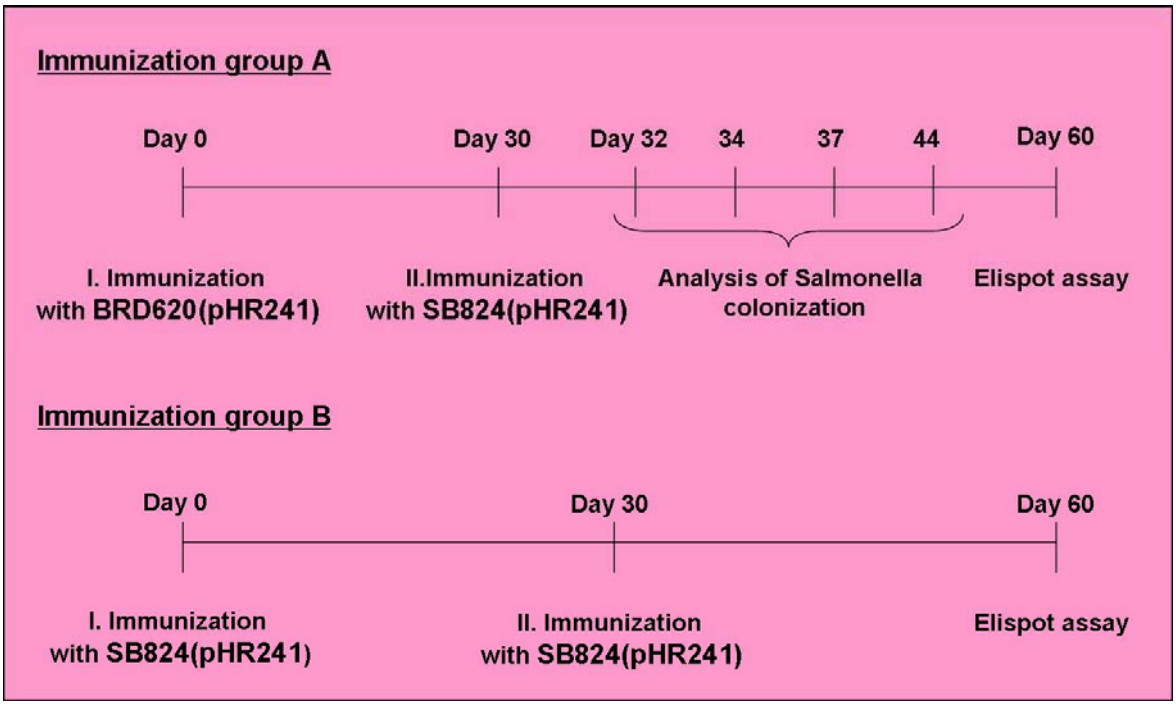

Fig. 19. Immunization, sampling and Elispot schedule. Mice of immunization group A were prime immunized with BRD620 (pHR241), a *S. dublin aroA aroD* mutant strain, and then boost-immunized with *S. typhimurium* SB824 (pHR241) on day 30. Mice of immunization group B were immunized twice with *S. typhimurium* SB824 (pHR241) and were used as a positive control of the study. On days 32, 34, 37, and 44, mice of immunization group A were sacrificed to analyze the bacterial loads in cecum, Peyer's patches, mesenteric lymph nodes and spleens. By day 60, an Elispot assay was performed with spleens from mice of both groups.

As shown in Fig. 20, two days after the second immunization (day 32), in mice of immunization group A, SB824 (pHR241) was present in cecum, Peyer's patches, mesenteric lymph nodes and spleens. The colonization profile at this time was indistinguishable from mice of immunization group B (see Fig. 10B). By day 4 post second immunization (day 34), all organs investigated revealed bacterial growth of the recombinant *Salmonella* strain. Three days later (day 37), bacteria were cleared from cecum, but were still detectable in Peyer's patches, mesenteric lymph nodes and spleens, where they reached a maximum peak. By day 44, bacteria were recovered from Peyer's patches in similar quantities as on days 34 and 37. In contrast, the bacterial load in spleens of mice was very low and in cecum and mesenteric lymph nodes the vaccine strain was not detectable anymore.

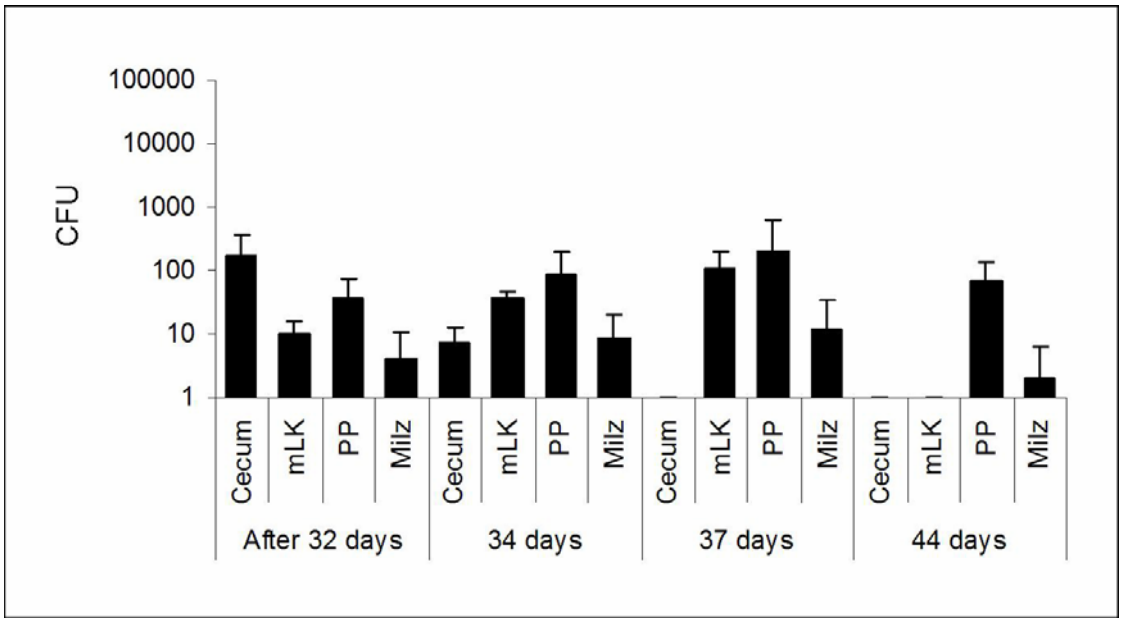

Fig. 20. Kinetics of colonization and persistence of *S. typhimurium* after prime immunization with *S. dublin* BRD620 (pHR241) in murine organs. Mice were orally immunized with *S. dublin* BRD620 (pHR241). On day 30, mice were boost-immunized with *S. typhimurium* SB824 (pHR241). On day 2, 4, 7, and 14 post second immunization, mice were sacrificed and cecum, Peyer's patches, mesenteric lymph nodes and spleens were aseptically removed and homogenized. The numbers of SB824 (pHR241) present in respective organs were determined as CFU. The results and standard deviations from 10 individual mice are indicated.

In conclusion, the application of a heterologous prime-boost protocol (immunization group A; priming with *S. dublin* and boosting with *S. typhimurium*) resulted in persistence of SB824 (pHR241) for at least 14 days after boost immunization. This is a significant difference to the results obtained from immunization group B (priming and boosting with *S. typhimurium*) where SB824 (pHR241) was cleared from all organs 6 days after the boost immunization.

3.5. CD8 T-cell response after heterologous prime-boost immunization

In further experiments, the question was asked whether a heterologous boost immunization could augment the frequency of p60-specific CD8 T cells. For this purpose, two groups of BALB/c mice were orally prime immunized on day 0 with either *S. typhimurium* SB824 (pHR241) (see Fig. 19, group B) or *S. dublin* BRD620 (pHR241) (see Fig. 19, group A). On day 30, a second dose of *S. typhimurium* SB824 (pHR241) was orally applied to mice of both groups. Mice from group A revealed high numbers of $p60_{217\text{-}225}$-specific CD8 T

lymphocytes by day 60 (see Fig. 21, hatched bar). The frequency of p60-specific CD8 T cells detected in spleens of mice from group B showed no significant difference (see Fig. 21, filled bar). It is important to mention that the frequency of $p60_{217-225}$-specific CD8 T lymphocytes in mice of group A was significantly increased as compared to mice that have received a single immunization of BRD620 (pHR241) (see Fig. 18).

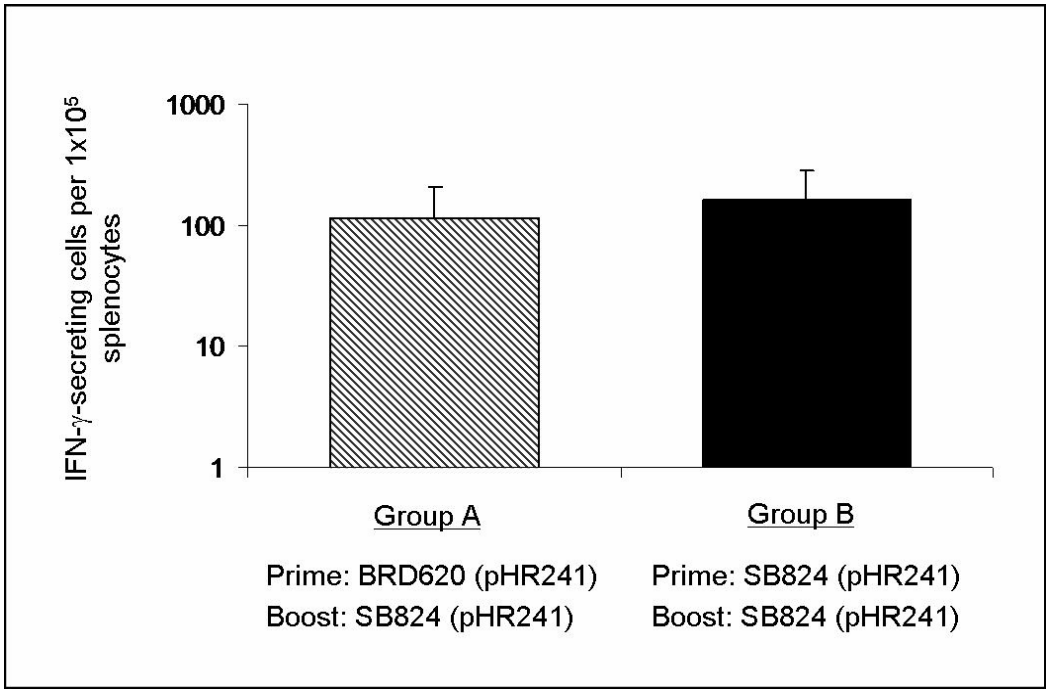

Fig. 21. CD8 T-cell responses after heterologous boost immunization. Mice received a prime immunization on day 0 with either *S. dublin* BRD620 (pHR241) (group A, hatched bar) or *S. typhimurium* SB824 (pHR241) (group B, filled bar). By day 30, both groups of mice were boost-immunized with *S. typhimurium* SB824 (pHR241). On day 60, T cell frequencies were determined by Elispot. The standard deviations from 15 individual mice per group are indicated. The P value from mice of group A and group B indicates no difference ($P > 0.1$).

Thus, the application of a heterologous prime-boost immunization protocol using different *Salmonella* serovars augments antigen-specific T-cell induction. This phenomenon is probably due to longer colonization and persistence of the heterologous *Salmonella* vaccine strain used for boost immunizations.

3.6. Induction of LLO-specific CD8 T cells after heterologous prime-boost immunization

As shown above, serovar-specific immunity against a *Salmonella* vaccine carrier could be partially overcome by employing different *Salmonella* serovars for prime and boost immunizations.

To further assess the reuse of *Salmonella*-based vaccines in a prime-boost strategy, we investigated, whether a heterologous boost immunization could lead to antigen-specific CD8 T-cell response against a heterologous antigen different from the one expressed during the first immunization.

For this experiment, plasmid pHR231 was employed. This plasmid encodes the genetic information for the YopE-specific chaperone, SycE, the translocated *Yersinia* outer protein ($YopE_{1-138}$), the listeriolysin O of *L. monocytogenes* (LLO), and the M45-epitope-tag. The N-terminal 50 and the C-terminal 165 amino acids of LLO were deleted to prevent pore-forming activity in the phagosome (217). LLO_{51-363} bears residues 91-99, which are presented by H2-K^d MHC class I molecules. The transcription of the hybrid *yopE* gene fusion is controlled under the *lac* promoter which is constitutively active in *Salmonella*.

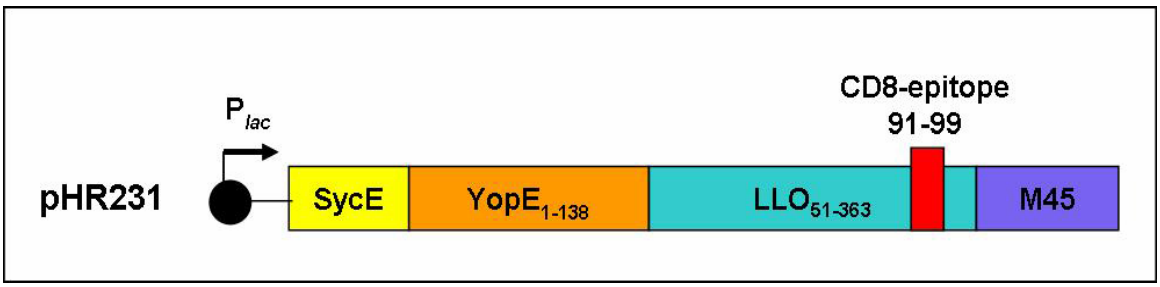

Fig. 22. Scheme of plasmid pHR231. Plasmid pHR231 bears the genetic information for the specific chaperone of YopE, SycE, the translocated chimeric $YopE_{1-138}$ fused to LLO_{51-363} and the epitope-tag M45. Transcription of gene fusion is achieved under the control of the *lac* promoter, which is constitutively active in *Salmonella*.

Two groups of BALB/c mice were orally immunized on day 0 with either *S. dublin* BRD620 (pHR241) (see Fig. 23, group A) or with *S. typhimurium* SB824 (pHR241) (see Fig. 23, group B), expressing translocated YopE/p60 (see Fig. 7). Thirty days later, both groups of mice received a second dose of *S. typhimurium* SB824 (pHR231), expressing translocated YopE/LLO (see Fig. 22). By day 60, an Elispot assay was performed in order to determine the magnitude of LLO_{91-99}-specific CD8 T cells.

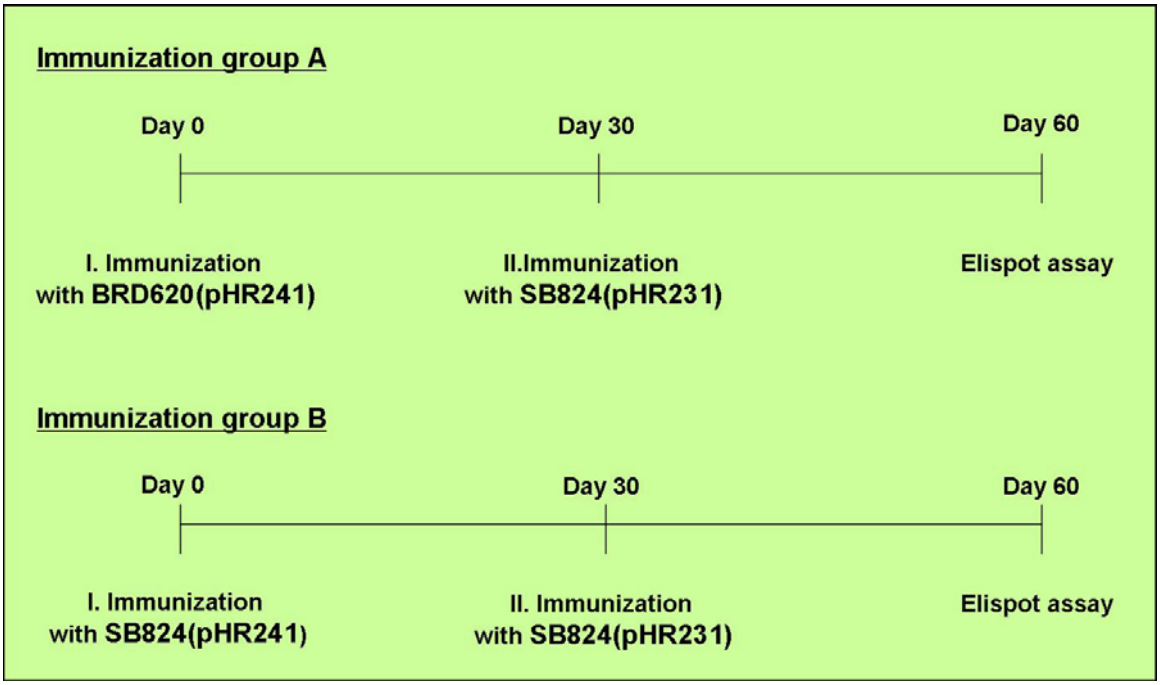

Fig. 23. Immunization, sampling and Elispot schedule. Two groups of BALB/c mice were orally immunized on day 0 with either BRD620 (pHR241) (group A) or with SB824 (pHR241) (group B). On day 30, both groups of mice were boost-immunized with SB824 (pHR231). On day 60, the frequencies of LLO_{91-99}-specific CD8 T cells were determined by Elispot assay in both groups.

Our results clearly showed that mice of immunization group A (14-days colonization) were able to elicit a prominent LLO-specific CD8 T-cell response (Fig. 24, hatched bar). In contrast, mice immunized twice with SB824 (pHR241) (immunization group B) revealed significantly lower numbers of IFN-γ-producing cells reactive with LLO_{91-99} (Fig. 24, hatched bar).

In conclusion, these results confirm that a short-term colonization (6-days colonization) results in a weak antigen-specific CD8 T-cell response. Efficient induction of antigen-specific CD8 T cells against a second heterologous antigen administered during boost immunization was only possible when using a heterologous prime-boost immunization strategy.

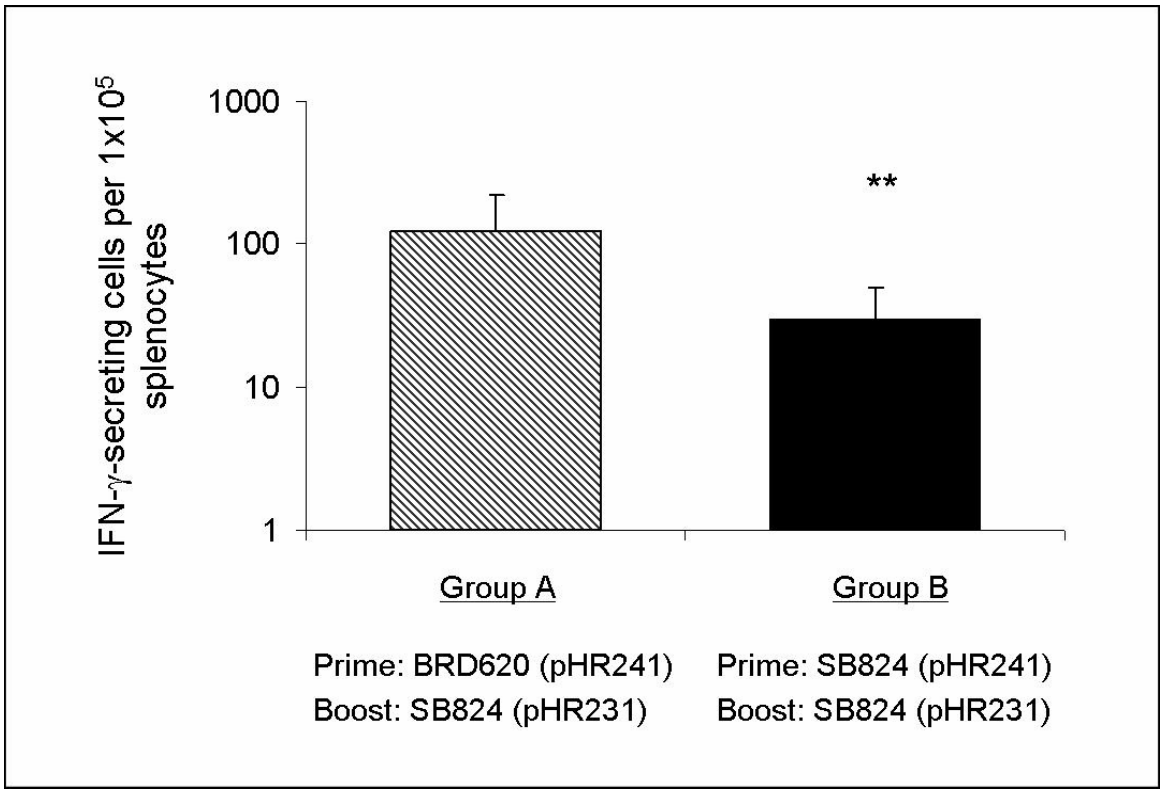

Fig. 24. CD8 T-cell responses after heterologous and homologous immunizations with *Salmonella* expressing translocated YopE/LLO. Two groups of BALB/c mice were prime immunized on day 0 with either *S .dublin* BRD620 (pHR241) (hatched bar) and *S. typhimurium* SB824 (pHR241) (filled bar). On day 30, both groups were boost-immunized with *S. typhimurium* SB824 (pHR231). By day 60, an Elispot assay was performed to determine the frequency of LLO_{91-99}-specific CD8 T cells. The standard deviations from 15 mice per group are indicated. Asterisks indicate values that differ significantly ($P < 0.05$) from that of mice prime immunized with *S. dublin* and boost-immunized with *S. typhimurium.*

E. DISCUSSION

In recent years, several attenuated pathogens and non-pathogenic commensal microorganisms have constituted a solid platform for the design of new live vaccines against both homologous and heterologous antigens (41, 114, 124). Bacteria-based vaccines seem especially attractive because they confer an effective and long-lasting immunity that involve not only the typical humoral response elicited by almost all licensed vaccines but also a cellular-mediated immunity (31, 123). *Salmonella* spp. have received particular interest for their potential as homologous and heterologous antigen delivery systems for oral immunizations (21, 39, 79, 103).

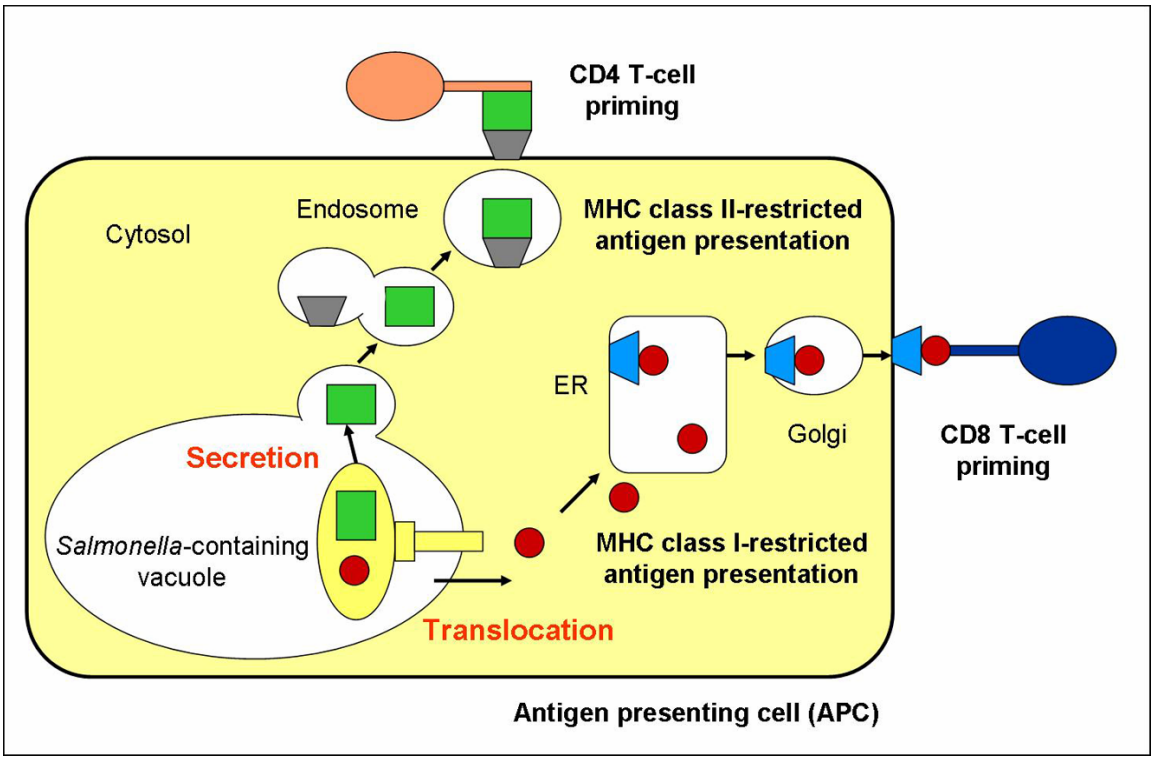

Fig. 25. Scheme of T3SS-mediated secretion and translocation of heterologous antigens by *Salmonella*. Cytosolic delivery of hybrid proteins from the macropinosome or *Salmonella*-containing vacuole (SCV) results in MHC class-I restricted antigen presentation and antigen-specific cytotoxic CD8 T-cell priming. In contrast, secretion of heterologous antigens into the macropinosome or SCVs leads to MHC class II-restricted antigen pathway and antigen-specific CD4 T-cell priming.

It is well known that *Salmonella* resides within the *Salmonella*-containing vacuole (SCV) after gaining access into eukaryotic host cells. This confinement allows *Salmonella* to

secrete proteins into the macropinosomal compartment, therefore resulting in MHC class II-restricted antigen presentation and peptide-specific CD4 T-cell priming (211). In contrast, delivery of foreign antigens into the cytosol remains limited, thus avoiding a MHC class I-restricted antigen presentation and consequently, complicating the antigen-specific CD8 T-cell priming. The CD8 T-cell response is of relevant importance against a wide range of intracellular pathogens, because it provides resistance and effective protection against the infection. In this context, our laboratory has focused its research on the development of a new vaccination strategy by using the type III secretion system (T3SS) of *S. typhimurium* as a delivery system for targeting antigens of viral (169) and bacterial origin (86, 151, 168) into the cytosol of antigen-presenting cells (APC) (see Fig. 25). This results in an efficient CD8 T-cell response (169).

It has been demonstrated by our laboratory that the single oral application of a *S. typhimurium aroA* mutant strain translocating either the chimeric protein YopE/p60 or YopE/LLO in a T3SS-mediated fashion triggered an efficient p60- or LLO-specific CD8 T-cell induction, respectively (86,168). However, the reuse of the same *Salmonella* serovar for boost immunizations may lead to reduced immunogenicity due to the induction of specific immune responses directed against the carrier itself. Furthermore, it has been shown that oral application of attenuated *Salmonella* strains harboring mutations in the prechorismate pathway induces anti-lipopolysaccharide serum IgG and intestinal IgA antibody titers (203). This finding contradicted the original work of Bao and Clements, who reported that prior priming with an *aroA S. dublin* strain potentiates the subsequent antibody response against the *E. coli* LT-B toxin subunit delivered by the same vector (7). Likewise, Whittle and Verma found that responses to a MVE (Murray-Valley-encephalitis virus) B cell epitope located in the flagellar subunits of an *aroA S. dublin* strain were enhanced in mice previously exposed to the bacterial carrier, although in this case the intraperitoneal route of immunization was used (208). In contrast, several other studies reported reductions in serum responses in orally immunized mice as a consequence of previous exposure to the *Salmonella* carrier (5, 102, 162, 203). For instance, Roberts *et al.* found that prior exposure to strains of homologous and heterologous serovar reduced the immunogenicity and protective efficacy of recombinant *Salmonella* expressing the fragment C of tetanus toxin (162). Consistent with this result is the finding of Kohler *et al.* who reported reduced serum IgG and salivary IgA responses to the hemagglutinin of *Porphyromonas gingivalis* presented by *Salmonella* in vector-primed mice (102).

In addition to these controversial publications, which focused their work on the antibody response, no information about the influence of the repeated use of *Salmonella* vaccine carriers on CD8 T-cell responses was available in the literature. A favored approach for generating protective CD8 T-cell responses against a number of diseases including AIDS, malaria and cancer involves primary vaccination with a DNA vaccine followed by boosting with, for example, a recombinant poxvirus (modified vaccinia Ankara (MVA)) or adenovirus vector encoding the same immunogen (3, 152, 161, 176). Similar to *Salmonella* as a vaccine carrier, antivirus neutralizing antibody responses generated by priming immunizations were able to inhibit effective boosting by the same viral vector (180). However, in other studies it was found that co-induction of anti-vector responses during priming did not appear to significantly influence the generation of epitope-specific CD8 T-cell responses following priming or after prime-boost immunizations (61, 215).

The first goal of our study was to investigate whether antigen-specific cytotoxic T lymphocytes induced by the *Salmonella* prime immunization contribute to a more rapid clearance of the vaccine carrier after subsequent boost immunizations and whether oral boost immunizations lead to an augmented p60-specific CD8 T-cell response.
First we showed that the *aroA S. typhimurium* SB824 (pHR241) strain expressing translocated YopE/p60 was able to colonize and persist in BALB/c mice at least for 21 days after the oral prime immunization (see Fig. 8). This result is in line with observations published by Dunstan *et al.* (43) who demonstrated that an *aroA S. typhimurium* mutant was also detectable in Peyer's patches and spleens for 21 days. In this report, bacteria reached a maximum burden of approximately 10^3 CFU in spleens on day 14 post immunization. Furthermore, on day 21 after inoculation the bacterial load in spleens declined to approximately 10^2 CFU. These results showed that the number of viable bacteria recovered in these organs were 10-fold higher as compared with our findings. This difference in bacterial load might be attributed to the administration doses employed ($3x10^{10}$ CFU versus $5x10^8$ CFU).

Recently, consideration has been given to the possibility that prior exposure to a heterologous antigen might compromise the immunogenicity of a multi-valent *Salmonella*-based vaccine. It is known that *Salmonella* invades both major types of APC, macrophages and dendritic cells (84, 211). After boost immunizations, existing p60-specific cytotoxic T

cells could potentially recognize and subsequently eliminate these cell types infected with recombinant *Salmonella* expressing YopE/p60. Indeed, we could observe a rapid elimination of the *aroA* mutant strain within 7 days after the second and within 4 days after the third oral application (see Fig. 10B and 10C). However, this was not attributed to the presence of *Listeria* antigen-specific CD8 T cells because SB824 was cleared as fast as SB824 (pHR241).

The observed rapid clearance of the *Salmonella* carrier after the first and second boost immunization could be due to the already preformed immune response against the carrier itself. *Salmonella* infection induces the generation of specific CD4 and CD8 T cells. Both T cell populations are important for the control of the primary infection and for the protection against secondary infections (18, 136). Infection of mice with *S. typhimurium* also results in a profound antibody response (73, 135). Antibodies, particularly IgM and IgA, can block penetration of *Salmonella* into deeper tissues. Indeed, the injection of a B cell hybridoma producing *Salmonella*-specific IgA has been shown to prevent oral infection of mice (130). Further analysis indicated that this protection was probably mediated by the inhibition of bacterial adhesion and infection of epithelial cells and M cells (131). Our results suggest the presence of a strong antibody-mediated immunity induced by two immunizations, because after the third oral administration, bacteria were only able to colonize the intestinal lumen. Our data indicate no penetration and systemic spreading of bacteria in mice at this time point (see Fig. 10C).

In addition, Fig. 10A and 10B clearly show that the *Salmonella* vaccine strain revealed a more rapid invasion and systemic dissemination after the first boost immunization. A possible explanation for this phenomenon might be the recruitment of chemokine receptor CX3CR1-positive lamina propria dendritic cells after the first oral application. This cell type has been shown to sample luminal antigens as well as entero-invasive pathogens like *Salmonella* via a mechanism distinct from the uptake by M cells, thus contributing not only to a faster dissemination but also to a more rapid elimination of bacteria (138). Another hypothesis for the rapid bacterial spread from intestine to systemic sites might lie in the employment of CD18+ macrophages and dendritic cells by *Salmonella.* These cells have been reported to act as Trojan horses of the host defence (111)

To date, the possibility to induce enhanced levels of antigen-specific CD8 T cells by oral boost immunizations with recombinant *Salmonella* expressing a chimeric type III protein

has not been explored. Our data demonstrate that the frequencies of p60-specific CD8 T cells could not be increased after the second or the third oral application (see Fig. 11). Most likely, the rapid clearance of the vaccine carrier prevented a significant elevation of T lymphocytes. A second possible explanation, as shown by Kursar *et al.,* could be that the host immune system controls the magnitude of the antigen-specific immune response through regulatory or suppressor CD4+CD25+ T cells (106).

In a next set of experiments, we took advantage of the fact that the anti-vector immunity induced after prime immunization led to the elimination of the *Salmonella* vaccine strain 6 days after the second immunization (see Fig. 13). We wanted to answer the question whether this short-term colonization is sufficient to induce a protective antigen-specific CD8 T-cell response. Therefore, mice were exposed to SB824 and subsequently boosted with SB824 (pHR241) expressing translocated hybrid YopE/p60. This brief p60 display resulted in significantly lower numbers of IFN-γ-producing cells reactive with $p60_{217-225}$ in comparison to mice colonized by the same strain for 21 days (see Fig. 14). Moreover, the amounts of antigen-specific CD8 T cells induced after short-term colonization was not able to confer protection against a lethal challenge with *L. monocytogenes*.

In many acute infection systems, the onset of the T-cell contraction phase correlates with pathogen clearance (2,14), suggesting that the duration of infection determines when antigen-specific CD8 T-cell transition between the expansion and contraction phases of the immune response occurs. However, the assumption that the magnitude of T-cell responses is determined by the amount of antigen and the duration of its presentation was questioned when studies with *L. monocytogenes* demonstrated that a brief exposure of the bacteria resulted in a normal expansion of effector CD8 T cells (214) and has a minimal impact on the expansion of CD4 T cells (32). Indeed, it was shown that the antigen-specific T-cell responses were triggered during the first 24-48 hours of *Listeria* infection (127). This brief exposure diminished the development of effector memory CD8 T cells but such cells were still capable of generating a protective recall response (120, 214). According to this model, antigen-presentation occurs within, but not after, the first days of infection (93, 127, 199), resulting in the generation of a potent CD8 T-cell response, which peaks at approximately 7 days post infection. This is followed by an equally rapid and massive attrition of the primed cells (>90%) which is complete by 2-3 weeks (94). In contrast, this paradigm is not

followed during *Salmonella* infection. In this case, CD8 T cells undergo delayed expansion, which peaks around day 21, and ensues by a protracted contraction (120).
An explanation for the priming delay of *Salmonella* could rely on the intracellular habitat. The *Salmonella* phagosomal lifestyle may allow escape from host CD8 T-cell recognition, conferring a survival advantage. In agreement with this theory, it was reported that *Mycobacterium bovis* (bacillus Calmette-Guerin), which also resides within phagosomes, induces delayed CD8 T-cell priming (42). However, with BCG the delay in CD8 T-cell priming may be attributed to the very low replication rate of the bacterium (doubling time > 24h) in contrast to *Salmonella* (doubling time 26 min). As *S. typhimurium* replicates faster than *L. monocytogenes*, the delay in CD8 T-cell priming could not be due to the replication rate of the bacterium. It might be governed by pathogen-specific interactions with the host, e.g. particular virulence mechanisms or the nature of inflammation.

Pre-exisiting anti-vector immunity has been proven to be an obstacle for the use of *Salmonella*-based vaccines by leading to a curtailment of bacterial colonization and therefore reducing immune responses against heterologous antigens. The LPS O-antigens are generally highly immunogenic and immune responses targeted against these determinants might be largely responsible for any hypo-responsiveness subsequently observed upon administration with the same *Salmonella* strain. *Salmonella* spp. are divided into more than 2400 different serovars on basis of O-, H and Vi-antigens (15). As immunity to *Salmonella* is serovar-specific (80-82, 115, 179) might be possible to circumvent the anti-vector immunity problem by delivering the heterologous antigen of interest using a *Salmonella* carrier whose serovar differs from that used for prime immunization (5,40).
As demonstrated above, two vaccine strains from different serological groups were employed for analyzing a heterologous boost immunization. One attenuated strain belonged to serogroup B, *S. typhimurium* SB824 (pHR241) and the other belonged to serogroup D, *S. dublin* BRD620 (pHR241).

Mice organs were colonized for 21 days after a single oral immunization with *S. dublin* BRD620 (pHR241). Thus, this strain exhibited a similar colonization profile as previously described for SB824 (pHR241) (see Fig. 17). Further studies revealed that BRD620 expressing the YopE/p60 hybrid protein elicited a p60-specific CD8 T-cell response. However, the frequency of p60-specific CD8 T cells induced by the *S. dublin* mutant strain

was significantly lower as compared to the number of T cells elicited by *S. typhimurium* (see Fig. 18).

When *S. dublin* BRD620 (pHR241) was used for prime immunization and *S. typhimurium* SB824 (pHR241) for boost immunization, the latter strain was able to colonize and persist for 14 days in mice (see Fig. 20). Thus, the change of the vaccine carrier serovar resulted in an extended colonization of mice organs, therefore prolonging antigenic display. As a consequence, we hypothesized this would lead to a boost-effect. Indeed, after the heterologous prime-boost immunization, we observed an enhanced response of p60-specific CD8 T cells (see Fig. 21) as compared to the single immunization with BRD620 (pHR241) (see Fig. 18). However, this immunization scheme did not lead to higher numbers of p60-specific T cells as compared to mice which received a single dose of SB824 (pHR241). An explanation for this phenomenon could be that the p60-specific CD8 T cells had reached the maximum magnitude after the single immunization with SB824 (pHR241) (see Fig. 11). No higher frequencies of $p60_{217-225}$-specific CD8 T cells could be observed after either homologous or heterologous boost immunizations.

Delivery of more than one antigen (e.g. from different infectious agents) by *Salmonella* is a very desirable feature of a live attenuated vaccine carrier. It has been demonstrated by our group that *Salmonella* can target two different listerial antigens (LLO and p60) into the cytosol of APC at the same time. This resulted in a superior protection against a *Listeria* infection (86). In the current study, we were able to demonstrate that only a heterologous prime-boost immunization strategy (priming with *S. dublin* expressing YopE/p60; boosting with *S. typhimurium* expressing YopE/LLO) led to the induction of high frequencies of LLO-specific CD8 T cells (see Fig. 24). Thus, changing of *Salmonella* serovars for consecutive immunizations is a prerequisite for efficient CD8 T-cell responses against a second heterologous antigen. An attractive alternative to the use of a different *Salmonella* serovar for boost immunization is the employment of attenuated *Yersinia* strains. Our laboratory has demonstrated that *Y. pseudotuberculosis* can be used to target heterologous antigens into the cytosol of APC via the *Yersinia*-T3SS (167). As for *Salmonella*, single oral immunization of mice with an attenuated *Y. pseudotuberculosis* strain expressing translocated YopE/LLO resulted in efficient LLO-specific CD8 T-cell priming. Thus, a prime-boost immunization strategy using *Salmonella* and *Yersinia* as carrier vaccines would definitely overcome anti-vector immunity.

Taken together, this is the first report demonstrating that antigen-specific CD8 T-cell responses induced by recombinant *Salmonella* expressing translocated heterologous proteins cannot be augmented by homologous boost immunizations. Furthermore, it was shown that a short-term colonization over 6 days of the vaccine strain is not sufficient to mount a protective CD8 T-cell response against listeriosis. However, the use of different *Salmonella* serovars in a heterologous prime-boost setting can circumvent anti-vector immunity leading to prolonged colonization and augmented CD8 T-cell responses.

F. SUMMARY

The type III secretion system of *Salmonella* can be used to target heterologous antigens directly into the cytosol of antigen-presenting cells. The single oral immunization of mice with an attenuated recombinant *Salmonella typhimurium* strain expressing the translocated *Yersinia* outer protein E (YopE) fused to the immunodominant antigen p60 or LLO from *Listeria monocytogenes* results in efficient induction of p60- or LLO-specific CD8 T cells and confers protection against a *Listeria* challenge infection.

There are conflicting reports concerning the impact of prior vector priming on the immunogenicity of recombinant *Salmonella*-based vaccines. In this context, we investigated whether p60-specific cytotoxic T lymphocytes induced by the prime immunization contribute to a more rapid clearance of the vaccine carrier after subsequent boost immunizations and whether oral boost immunizations lead to an augmented p60-specific CD8 T-cell response. We found that the ability of recombinant *Salmonella typhimurium* strains to colonize the intestine, mesenteric lymph nodes, and spleens was markedly impaired after homologous boost immunizations, but that this effect was independent of existing CD8 T cells reactive with $p60_{217-225}$. A significant elevation of antigen-specific CD8 T cells could not be detected after the second or the third oral immunization, possibly due to the rapid clearance of the bacterial vaccine carrier from lymphatic organs.

In further experiments, we demonstrated that, in contrast to a long-term colonization over 21 days, a short-term colonization of orally vaccinated mice over 6 days with recombinant *Salmonella typhimurium* is not sufficient to elicit a protective CD8 T-cell response against listeriosis. In order to overcome pre-existing anti-vector immunity resulting in a curtailment of colonization after homologous boost immunization, two different *Salmonella* serovars (*S. dublin* and *S. typhimurium*) were used for a heterologous prime-boost protocol. This strategy led to prolonged colonization over 14 days after boost immunization and augmented antigen-specific CD8 T-cell responses.

ZUSAMMENFASSUNG

Salmonellen verfügen über ein sogenanntes Typ III-Sekretionssystem (T3SS), mit dessen Hilfe Effektormoleküle mit zellmodulatorischer Funktion durch die Wirtszellmembran direkt in das Zytosol von Makrophagen, dendritischen Zellen oder Epithelzellen transloziert werden. Das *Salmonella*-T3SS kann verwendet werden, um heterologe Proteine in den endogenen MHC Klasse I-Antigenpräsentationsweg von eukaryontischen Zellen einzuschleusen. Als T3SS-Trägerprotein hat sich das „*Yersinia* outer protein E" (YopE) bewährt. Als Modellantigene dienten die immundominanten Proteine Listeriolysin O (LLO) und p60 des intrazellulären Bakteriums *Listeria monocytogenes*. Am Beispiel der murinen Listeriose konnte im Tiermodell demonstriert werden, dass die einmalige orale Immunisierung mit einem rekombinanten *S. typhimurium*-Impfstamm, der entweder chimäres YopE/LLO oder YopE/p60 exprimiert und transloziert, zu einer effektiven und protektiven CD8 T-Zellantwort führt.

In der vorliegenden Arbeit wurde erstmals der Einfluss von Booster-Immunisierungen auf die Induktion antigenspezifischer CD8 T-Zellantworten im oralen Mausmodell untersucht. Es konnte zunächst gezeigt werden, dass der rekombinante *S. typhimurium*-Impfstamm nach homologen Booster-Immunisierungen zeitlich in seiner Fähigkeit beeinträchtigt war, Darm, mesenteriale Lymphknoten und die Milz geimpfter Mäuse zu kolonisieren. Die schnelle Eliminierung der Salmonellen nach wiederholter oraler Gabe beruhte dabei nicht auf $p60_{217\text{-}225}$-spezifischen zytotoxischen CD8 T-Zellen, die durch die primäre Immunisierung induziert worden waren, sondern auf einer gegen den Impfstamm gerichteten Immunantwort, der sogenannten anti-Vektor-Immunität. Die herabgesetzte Kolonisierungsdauer nach Boost-Immunisierungen verhinderte dabei auch eine verstärkte antigenspezifische CD8 T-Zellantwort.

In weiteren Experimenten konnte gezeigt werden, dass im Gegensatz zu einer Langzeit-Kolonisierung über einen Zeitraum von 21 Tagen, eine Kurzzeit-Kolonisierung oral immunisierter Mäuse über 6 Tage nicht ausreicht, um eine schützende p60-spezifische CD8 T-Zellantwort gegen Listeriose zu induzieren. Durch die Verwendung von zwei verschiedenen *Salmonella*-Serovaren (*S. dublin* und *S. typhimurium*) im Rahmen einer heterologen „prime-boost"-Immunisierung konnte die *Salmonella*-spezifische anti-Vektor-Immunität umgangen werden. Diese Strategie führte zu einer verlängerten

Kolonisierungsdauer von über 14 Tagen und zu einer verstärkten antigenspezifischen CD8 T-Zellantwort nach Booster-Immunisierungen.

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