VACCINE PLATFORM FOR INFECTION OR AUTOIMMUNE DISEASES USING
AN ETEC FIMBRIAL SCAFFOLD

by

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ABBREVIATIONS

- *Salmonella* Pathogenesis Islands
- TTSS: type III secretion system
- GEFs: guanine nucleotide exchange factor
- MAPK: MAP kinase
- GAPs: GTPase-activating factor
- PMN: polymorphonuclear cell
- LPS: lipopolysaccharide
- CAMPs: cationic anti-microbial peptides
- TNF-α: tumor necrosis factor alpha
- SCV: *Salmonella*-containing vesicle
- NK: natural killer
- DC: dendritic cell
- iNOS: inducible nitric oxide synthase
- mAb: monoclonal antibody
- TGN: glyceryl trinitrite
- CGD: chronic granulomatous disease
- Nramp 1: natural resistance-associated macrophage protein 1
- TRLs: toll-like receptors
- TAP: transporter-associated with antigen processing
ABBREVIATIONS – CONTINUED

- DTH: delayed-type hypersensitivity
- CTL: cytotoxic T lymphocyte
- PABA:  \( \rho \)-aminobenzoic acid
- DHB: 2, 3-dihydroxybenzoate
- MALT: mucosa-associated lymphoid tissue
- ETEC: enterotoxigenic \textit{E. coli}
- CFA/I: colonization factor antigen I
- EAE: experimental autoimmune encephalomyelitis
- PLP: proteolipid protein
- CFU: colony forming unit
- PT: pertussis toxin
- NBF: natural buffered formalin
- H&E: hematoxylin and eosin
- LFB: luxol fast blue
- IHC: immunohistochemistry
- RT: room temperature
- DPBS: Dulbecco’s PBS
- NSB: normal serum block
- PP: Peyer’s patches
ABBREVIATIONS – CONTINUED

- CLN: cervical lymph node
- ANOVA: analysis of variance
- EHEC: enterohemorrhagic *E. coli*
- ELISA: enzyme-linked immunosorbent assay
- GI: gastro intestinal
- ELISPOT: enzyme-linked immunospot
- CNS: central nervous system
- MS: multiple sclerosis
- MIP-2: macrophage inflammatory protein-2
- TCA-3: T cell activation gene-3
- CFA: complete Freunds’ adjuvant
- APL: altered peptide ligand
- MBP: myelin basic protein
- KLH: keyhole limpet hemocyanin
- BCG: *Mycobacterium bovis* strain Calmette-Guèrin
- ST: heat stable toxin
- LT: heat labile toxin
- cAMP: cyclic AMP
- cGMP: cyclic GMP
- NALT: nasal-associated lymphoid tissue
**ABBREVIATIONS – CONTINUED**

- i.n: intranasal
- VT: verocell cytotoxin
- HUS: hemolytic uremic syndrome
- SLTs: “Shiga-like” toxins
- A/E: attaching and effacing
- M6PR: mannose 6-phosphate receptor.
ABSTRACT

The expression of enterotoxigenic *Escherichia coli* (ETEC) fimbriae (colonization factor antigen I (CFA/I) or K99) on the surface of a *Salmonella* vaccine vector confers protection against ETEC challenge. Application of such fimbriae as a treatment for the proinflammatory disease, experimental autoimmune encephalomyelitis (EAE), or as a molecular scaffold for heterologous antigen expression by cloning enterohemorrhagic *E. coli* (EHEC) LPS peptide mimetics into the K99 fimbriae to produce a dual vaccine for ETEC/EHEC was investigated.

The expression of CFA/I fimbriae by a *Salmonella* vaccine vector stimulates a biphasic T helper (Th) cell response and suppresses proinflammatory responses suggesting that CFA/I fimbriae may be protective against proinflammatory diseases. To test this hypothesis, SJL/J mice were vaccinated with *Salmonella*-CFA/I vaccine 1 or 4 wks prior to induction of EAE induced with encephalitogenic proteolipid protein (PLP) peptide, PLP[139-151]. Mice receiving *Salmonella*-CFA/I vaccine recovered completely from the mild acute clinical disease and showed only mild inflammatory infiltrates in the spinal cord. This protective effect was accompanied by a loss of encephalitogenic IFN-γ secreting Th1 cells and replaced with increases in IL-4-, IL-10-, and IL-13-producing Th2 cells. These data suggest that *Salmonella*-CFA/I is an anti-inflammatory vaccine capable of suppressing proinflammatory cells to protect against EAE via immune deviation.

To obtain an effective nasal vaccine for ETEC, the *fanC* gene of ETEC K99 major structural gene was cloned onto the reovirus adhesin, protein σ1, which has been shown as an M cell targeting molecule. Although FanC/protein σ1 fusion protein was successfully expressed, this vaccine failed to elicit immune responses against native FanC protein, presumably because of improper protein folding.

Using K99 fimbriae as a molecular scaffold, a LPS peptide mimetic for EHEC was cloned into the *fanH* gene of K99 fimbriae minor structural gene to enable multiple antigenic peptide expression, resulting in an ETEC/EHEC dual vaccine. Insertion of peptide mimetic into *fanH* gene had no adverse effect on the formation of polymerized K99 fimbriae. However, various oral immunization regimens failed to induce the protective secretory IgA responses against the LPS mimetic peptide, although serum IgG antibodies were induced.
CHAPTER ONE

SALMONELLA: PATHOGENESIS, HOST IMMUNE RESPONSES, AND ITS ROLE AS A HETEROLOGOUS VACCINE CARRIER

Introduction

The genus Salmonella is a member of the family Enterobacteriaceae. This genus is composed of bacteria related to each other both phenotypically and genotypically. The bacteria of the genus are also related to each other by DNA sequence (1). Before the DNA based taxonomy (2), Salmonella classification was based on clinical manifestations (Salmonella typhi, Salmonella cholerae-suis, and Salmonella abortus-ovis), serological analysis, the geographical origin of the first isolated strain of the newly discovered serovars (S. London, S. panama, S. stanleyville), or the host specificity (S. typhimurium). Using these traditional methods, newly identified Salmonella species were each considered as a new species (3). Currently, to reduce the increasing numbers of proposed species, it is accepted that all Salmonella serovars form a single DNA hybridization group, i.e., a single species composed of seven subspecies. Therefore, a genus Salmonella consists of single species (Salmonella enterica) with seven subspecies (enterica for subspecies I, salamae II, arizonae IIIa, diarizonae IIIb, houtenae IV, bongori V, and indica VI) (3). For the convenience of physicians and epidemiologist, the common serovars names are kept only for the subspecies I strains, which consist of more than 99.5% of Salmonella strains isolated from humans and other warm-blooded animals (1, 3). According to this taxonomic rule, Salmonella typhimurium designated as Salmonella enterica subspe. Enterica serovar Typhimurium, in practice, Salmonella ser. Typhimurium or S. Typhimurium.
The habitat for *Salmonella* is confined to the digestive tracts of humans and animals, and some *Salmonella* species show strict host specificity. *S. Typhi*, Paratyphi A, and Sendai are strict human serovars, but Typhimurium resides both in humans and mice (4-7).

The ingestion of *Salmonella* from contaminated foods or water initiates infection. The major clinical syndromes associated with *Salmonella* infections in human are enteric (typhoid) fever and gastroenteritis (8). *S. Typhi* is the causative agent of enteric fever. In contrast to self-limited gastroenteritis by *S. Typhimurium*, *S. Typhi* spreads through the reticulo-endothelial system, including the intestinal Peyer’s patch, mesenteric lymph node, spleen, and bone marrow, by the migration of the infected macrophage via lymphatics and blood (9). The release of bacteria and endotoxin at the mesenteric lymph node initiates the septicemic phase of enteric fever and cardiovascular “colapsus tephos”, which is the origin of the name of typhoid (10).

Typhoid fever causes severe health problem around the world, especially in many developing countries, with an approximate 33 million incidences and one-million deaths a year (11, 12). Consequently, in an effort to prevent typhoid fever, two different types of vaccines are available: an oral vaccine and a parental vaccine (12, 13).

There are two kinds of parental vaccines. One consists of inactivated *S. Typhi* virulent strain and the other is purified *S. Typhi* virulence factor, Vi polysaccharide (13, 14). Parental vaccination using inactivated bacteria is effective but is not recommended due to the co-administration of O antigen (endotoxin) that resulted in unwanted systemic and local reactions (13). Parental vaccination with Vi polysaccharide antigen showed efficacy of 72% at 17 months and 64% at 24
months from the pilot scale field studies in Nepal and South Africa (14). It was licensed in U.S in 1994 (13, 14).

Licensed in 1991 (12), the oral typhoid fever vaccine Ty21a was created by the introduction of a stable mutation to the pathogenic S. Typhi strain, resulting in a lack of the enzyme UDP-galactose-4-epimerase and Vi capsular polysaccharide (12). The Ty21a vaccine showed 96% efficacy against confirmed typhoid fever (14). The loss of UDP-galactose-4-epimerase was considered the major reason for this attenuation, but in later experiments performed with \textit{galE}^{+} complementation and \textit{galE} mutation failed to show the recovery of virulence or attenuation, respectively, and suggested an unknown cause of Ty21a’s attenuation (15, 16). To develop better typhoid vaccines and adapt them as heterologous antigen carriers, genetically defined attenuations of \textit{Salmonella} was adopted. To implement these attenuations, a better understanding of \textit{Salmonella} pathogenesis and host resistance mechanisms would be required to obtain highly attenuated \textit{Salmonella} strains while retaining its immunogenicity. The following section presents a brief summary of \textit{Salmonella} pathogenesis and host immune response.

\textit{Salmonella Pathogenesis}

\textbf{Enteritis}

\textit{Salmonella} pathogenesis can be divided according to two different disease syndrome features, enteritis and enteric fever (8, 17). The enteritis is caused by the entry of \textit{Salmonella} into the intestinal epithelial cell followed by the induction of fluid secretion and inflammation (8). However, the enteric fever is initiated by the invasion of \textit{Salmonella} into the macrophage and reproduction in the macrophage phagosome (17). The \textit{Salmonella} genes that govern the invasion into host epithelial
cell and survival in phagocytic cells are clustered in the *Salmonella* chromosome, called *Salmonella* pathogenesis islands (SPIs) (18-21). The genes of different SPIs have unique functions at different stages of pathogenesis. For example, SPI-1 encodes genes responsible for the entry of *Salmonella* into intestinal epithelium and effector proteins, but SPI-2 encodes genes necessary for the survival in the macrophage phagosome and systemic infections (21, 22). Figure 1.1 shows the locations of SPIs in the *Salmonella* chromosome (21).

The entry of *Salmonella* into intestinal epithelium can be divided into five distinct steps: 1) delivery of effector molecules via the type III secretion system (TTSS) into intestinal epithelium, with both effector molecules and TTSS encoded in SPI-1; 2) stimulation of host signal transduction pathways; 3) initiation of actin cytoskeleton rearrangement; 4) localized ruffling of intestinal epithelial cells’ membrane; 5) and shutting off of the signal transduction pathway following the entry of *Salmonella* into the intestinal epithelial cell (24-30).

Figure 1.1: *Salmonella* pathogenesis islands (SPIs). Schematic representation of *S. Typhimurium* chromosome, which is divided into 100 cs. Known SPIs are shown on the outside of the chromosomal circle. Modified from Marcus, et. al., (21).
Table 1.1: SPI-1-encoded effector proteins (24).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Biochemical activity</th>
<th>In vivo function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvrA</td>
<td>unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>SipA</td>
<td>stabilization of F-actin, reduction of the critical concentration for actin polymerization</td>
<td>actin polymerization and reorganization</td>
</tr>
<tr>
<td>SipB</td>
<td>binding of caspase-1</td>
<td>translocation of SPI-1 effectors and induction of apoptosis</td>
</tr>
<tr>
<td>SipC</td>
<td>actin nucleation and bundling</td>
<td>translocation of SPI-1 effectors and actin polymerization</td>
</tr>
<tr>
<td>SipD</td>
<td>unknown</td>
<td>translocation of SIP-1 effectors.</td>
</tr>
<tr>
<td>SlrP</td>
<td>unknown</td>
<td>putative host adaptation factor</td>
</tr>
<tr>
<td>SopA</td>
<td>unknown</td>
<td>induction of enteritis</td>
</tr>
<tr>
<td>SopB</td>
<td>inositol phosphatase</td>
<td>chloride secretion and actin polymerization</td>
</tr>
<tr>
<td>SopD</td>
<td>unknown</td>
<td>induction of enteritis</td>
</tr>
<tr>
<td>SopE</td>
<td>small G protein GEF</td>
<td>actin polymerization</td>
</tr>
<tr>
<td>SopE2</td>
<td>small G protein GEF</td>
<td>actin polymerization</td>
</tr>
<tr>
<td>SspH1</td>
<td>unknown</td>
<td>lethal infection in calves</td>
</tr>
<tr>
<td>SptP</td>
<td>small G protein GAP and tyrosine phosphatase</td>
<td>recovery of actin cytoskeleton rearrangement</td>
</tr>
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The TTSS of SPI-1 transfers *Salmonella* proteins, which are encoded within the SPI-1 or other segments of *Salmonella* chromosome, into the intestinal epithelial cell. The TTSS consists of several proteins encoded in SPI-1; for example, the base of the TTSS structure is composed of InvG, PrgH and PrgK, and the main component is PrgI (31). Together with the base proteins and the main needle protein, the characteristic TTSS needle-like structure is assembled (31). Through the TTSS, the effector proteins are injected into the host and trigger signal transduction pathways that lead to a variety of host cellular responses (24). The functions of effector proteins are summarized in Table 1.1 (24).

It has been known that the Rho family of GTP binding proteins (Rho, CDC42, and Rac) regulates signal transduction in response to extracellular signals and results in actin cytoskeleton
rearrangement by cycling between a biologically inactive GDP-bound and an active GTP-bound conformation (32, 33). Among the SPI-1 TTSS delivered proteins, SopE and SopE2 activate directly both CDC42 and Rac by acting as a phosphate exchange factor (34, 35). The SopE proteins exert their activity by binding to target Rho GTPases and inhibiting their GTP hydrolyzing activity, resulting in permanent activation of downstream signal transduction (34). However, the Rho GTPase regulation by SopE is different from the other bacterial toxins with noncovalent modification of its target, and this noncovalent modification of the target closely resembles eukaryotic guanine nucleotide exchange factors (GEFs) (34). Unlike the SopE, SopE2 is present in every Typhimurium and has a phosphate exchange factor activity for Rho family GTP-binding proteins (35). Mutation and complementation of SopE2 demonstrates its role in membrane rearrangement and host cell invasion (35). Activated CDC42 induces the formation of filopodia (36). Rac1 promotes the subsequent formation of lamellopodia and membrane ruffling (37). In addition, activation of CDC42 results in the activation of downstream MAP kinase (MAPK) mediated by the recruitment of Ras-GRF to the membrane, a GEF for the Ras family of small GTP-binding proteins. However, there is no evidence of direct interaction between CDC42 and Ras-GRF (38). Activation of MAPK, JNK, p38-MAPK, and IκBα kinase is followed by the proteolysis of IκBα in proteosome and results in release of NFκB. The translocation of NFκB to the nucleus stimulates the production and secretion of IL-8, which directs the neutrophil to the site of Salmonella infection and causes the distinctive inflammatory diarrhea of Typhimurium infection (39, 40).

Following the induction of cytoskeleton rearrangement and initiation of membrane ruffling by the activation of CDC42 and Rac, spatial localization and more extended ruffles are needed to
internalize *Salmonella*. Another SPI-1 TTSS-transferred protein, SipA, seems to responsible for the localization and enhancement of cytoskeleton rearrangement by decreasing the critical concentration of actin polymerization (27, 28, 41). The decreased critical concentration of actin polymerization by binding of the SipA to actin results in inhibition of actin filament depolymerization and further initiates *Salmonella* induced actin polymerization (28, 41). The null SipA mutant *Salmonella* infected cell shows a reduced amount of F actin and suggests that the function of SipA is to stabilize actin filaments during *Salmonella* infection (28). SipA mutant strain of *Salmonella* is less effective in inducing cytoskeleton rearrangement and the rearrangement is scattered rather than localized (41). In addition, SipA mutant shows impaired entry in cell a culture system (41).

As a final step of *Salmonella* invasion into intestinal epithelial cells, the recovery of host cytoskeleton structure is required. Another SPI-1 TTSS-delivered effector, SptP, is responsible for the cytoskeleton recovery of the host by acting as a specific antagonist of CDC42 and Rac-1 (29, 30, 42, and 43). As a member of GTPase-activating proteins (GAPs), SptP preferentially binds to the active form of CDC42 (GTP-bound) and Rac, and activates their intrinsic GTPase activity (42). The specific SptP binding activity to its cognate G protein was confirmed by the experiment with a constitutively active GST fusion form of GST-Rac1 (Rac1 (L61)). The SptP only binds to Rac1 (L61), but does not bind to GST-Rac1 or GST alone (42). In addition to GAP activity, SptP carboxyl-terminal tyrosine phosphatase activity is involved in reversing MAPK activity (30). Activation of GTPase activity of CDC42 and Rac1, and with its tyrosine phosphatase activity, SptP downregulates the cytoskeleton rearrangement induced by SopE, as well as a pro-inflammatory response caused by the induction of the MAP-kinase pathway (30, 42).
The whole concerted process of manipulating the host signal transduction process by the SPI-1-encoded TTSS and effector proteins explains the *Salmonella* strategy for invasion and survival in the host intestinal epithelial cell. The invasion of *Salmonella* into intestinal epithelial cells causes enteritis characterized by the severe loss of chloride ion and polymorphonuclear cell (PMN) accumulation at the intestinal lumen (44-46). The loss of chloride ion is the result of blocking the inositol phosphate signaling pathway by SopB with its inositol phosphate phosphatase activity. SopB hydrolyzes phosphatidylinositol 3, 4, 5-triphosphate, an inhibitor of Ca$^{2+}$-dependent chloride secretion, resulting in severe loss of chloride ion (44).

The transepithelial PMN accumulation is thought to be induced by the secretion of IL-8 as a result of the nuclear response triggered by TTSS effector protein (39, 40, 46). However, IL-8 neutralization, transfer, and induction experiments suggest the involvement of other factors besides IL-8 which are necessary for the PMN transepithelial migration (45).

**Enteric Fever**

The survival and replication of *Salmonella* in the host macrophage phagosome causes systemic infection and results in enteric fever. To survive and replicate in the macrophage phagosome, *Salmonella* is endowed with several resistance mechanisms, including the PhoP/PhoQ two component regulatory system and SPI-2 encoded TTSS and effectors (47-49).

In response to macrophage phagosomal microenvironment, especially low concentration of Mg$^{++}$ ion, *Salmonella* regulates its gene transcription through PhoP/PhoQ two component regulatory systems in which phosphorylation and dephosphorylation are involved (51). The PhoP/PhoQ system consists of phospho- transmitter (PhoQ) and phospho-receiver (PhoP), also called sensor kinase and effector protein, respectively (47). In addition to regulating downstream
expression, PhoP/PhoQ expression is also regulated in a transcriptionally auto-regulated fashion with two different \textit{phoP/phoQ} promoters: PhoP/PhoQ-independent and PhoP/PhoQ-dependent (52). For example, the PhoP protein acts as a transcriptional activator of \textit{phoP/phoQ} when it is phosphorylated by PhoQ in response to microenvironmental signals. Then, the phosphorylated PhoP (PhoP-p) activates the PhoP/PhoQ-dependent promoter of \textit{phoP/phoQ} regulon, resulting in increased concentration of PhoP-p and further increase of PhoP-p downstream activation (51, 52). However, the PhoP/PhoQ independent promoter of the \textit{phoP/phoQ} regulon activates very low basal level of PhoP expression (52). One of the well-known PhoP/PhoQ system-dependent event is modification of the lipid A structure of \textit{Salmonella} lipopolysaccharide (LPS), a host signaling moiety of LPS, by PagP protein, with the transferring palmitate to 2-hydroxy myristate on lipid A (53, 54). The addition of palmitate and the resulting charge distribution change on LPS hinders the interaction between LPS and cationic anti-microbial peptides (CAMPs) and further confers the \textit{Salmonella} resistance against membrane damage (54). In addition, the PhoP/PhoQ system also activates other two component system, PmrA/PmrB (50). The activation of PmrA/PmrB two-component system by \textit{phoP/phoQ} modifies lipid A with the addition of aminoarabinose mediated by PmrE and PmrF and confers \textit{Salmonella} resistance to the anti-microbial peptide, polymyxin (55). The modification of lipid A structure also alters LPS-mediated host responses. For example, reduced expression of E-selectin and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) was observed in the host infected with \textit{E. coli} which had modified lipid A without myristic acid (56). In \textit{Salmonella}, PhoP constitutive phenotype (\textit{PhoP}\textsuperscript{c}), which constitutively activates downstream genes, modifies the lipid A structure by the addition of aminoarabinose and 2-hydroxymyristate (57). The PhoP\textsuperscript{c} infected endothelial cell (HUVEC) and adherent mononuclear cell show reduced induction of E-selectin and
TNF-α, respectively (57). This reduced expression of the adhesion molecule and proinflammatory cytokine by the host implicates a reduced migration of macrophages to the site of inflammation and killing activity of phagocytes, but increases viability of Salmonella in the host (56, 57). Taken together, the membrane remodeling by the PhoP/PhoQ two-component system plays an important role in resisting the host innate immune system by providing Salmonella resistance to CAMPs and modifying host cell adhesion molecule and effector expression (54, 55, 57-60). Figure 1.2 shows the illustrated summary of the PhoP/PhoQ regulation cascade (50).

The phagosomal condition induces another TTSS and effector expression encoded by SPI-2 (21, 22, 49). The SPI-2 TTSS and effectors are required for Salmonella virulence and proliferation in macrophages (49). Among the SPI-2 TTSS effectors, the role of SpiC protein in pathogenesis has been well-characterized (61, 62). SpiC has no homologues in the sequence database and motifs that predict function (62). However, animal studies with spiC mutant Salmonella show the requirement of spiC for virulence, with an increase of LD$_{50}$ more than $3.6 \times 10^5$ times and defective intramacrophage survival (62). Complementation of the spiC mutant with spiC gene containing plasmid shows the recovery of intramacrophage survival (62). The functional SpiC protein exerts its activity by inhibiting phagosome-lysosome fusion and interfering with normal vesicle trafficking devoid of Salmonella (62). In addition to SpiC protein, other SPI-2 encoded genes, such as ssrA, ssaJ, sssV, and sseB, are required for virulence (63). The functions of the above genes were confirmed by the experiments with gp91phox phagocyte NADPH oxidase knockout mice (64). They antagonize the NADPH phagocyte oxidase, the most potent antimicrobial tool of phagocytic cells, by interfering with trafficking of oxidase-containing vesicles to the Salmonella containing
vesicle (SCV) (49, 63, and 64). Taken together, it is suggested that the SpiC and other SPI-2 effector proteins block fusion of vesicles harboring NADPH oxidase with SCV.

Figure 1.2: phoP/phoQ regulon involved regulatory cascade. Diagram of the regulatory cascade involving PhoP/PhoQ that results in PhoP-activated (pag) or PhoQ-repressed (prg) gene expression. Upon entry of bacteria into macrophage phagosome, environmental signals are sensed by PhoQ, which activates PhoQ through a phosphor-relay system. Revised from Ernst, et. al., (50).
Host Resistance against *Salmonella* Infection

Understanding host defense mechanisms against *Salmonella* infection, including innate and acquired immunity is important for the design of a *Salmonella* vaccine strain, as well as to exploit *Salmonella* as a carrier for heterologous antigen. The host responses can be divided into two parts; innate and acquired immune response. The following sections summarize host defense mechanisms against *Salmonella* infection.

**Innate Immunity**

The macrophage is the central controlling cell in infection with *Salmonella*, as well as with other intracellular bacteria (65). The early innate immunity by macrophages and natural killer (NK) cells is important for the successful clearance of *Salmonella* at initial stages of invasion. Upon response to *Salmonella*, macrophages and dendritic cells (DCs) produce IL-12 and IL-18 (66-72). In response to IL-12 and IL-18 stimulation, NK cells produce IFN-γ and activate macrophages (66, 67, 73, and 74). Furthermore, macrophages also produce IFN-γ in response to their own IL-12 and IL-18, which sustains increased levels of IL-12 in the surrounding microenvironment to ensure elevated levels of IFN-γ (67). At early stages of *Salmonella* infection, IFN-γ confers restriction of intracellular bacterial replication (75). The importance of IFN-γ for bacteriostatic activity at initial stages of infection was confirmed with experiments of IFN-γ depletion with specific monoclonal antibody (mAb) treatment, IFN-γ knockout mice (IFN-γ−/−), and administration of recombinant IFN-γ (76-80). However, this bacteriostatic action of IFN-γ failed to provide bacterial clearance in the later elimination stage of attenuated *Salmonella* (ΔaroA) infection (79, 80),
suggesting the bacteriostatic role of IFN-γ in the initial infection stages rather than bacteriocidal activity in the later clearance stages. IFN-γ−/− mice with oral Salmonella challenge resulted in disseminated septicemia 2 weeks later with a 100-fold increase of specific systemic and local antibody titers (78). In contrast to aroA−-Salmonella infection to the IFN-γ−/− mice, phoP−-Salmonella was eliminated from the IFN-γ−/− mice. This suggests the differential role of IFN-γ against Salmonella infection according to Salmonella virulence gene attenuation. IFN-γ exerts its antimicrobial activity through the induction iNOS and phagocyte NADPH oxidase (81, 82). iNOS synthesizes antimicrobial reactive nitric oxide radical (NO·), which acts as an oxidizing agent and further forms toxic peroxynitrite (ONOO−) through its interaction with superoxide (O2·−). These iNOS products have different anti-Salmonella activity according to their redox state. For example, co-culture of Salmonella with NO· donor, diethylenetriamine-nitric oxide, does not exhibit antibacterial activity, but Salmonella culture with the OONO− producer, 3-morpholinosydnonimine hydrochloride shows oxygen-dependent Salmonella killing (83). It seems that DNA is an important target for reactive nitrogen derivatives (81). For example, treatment of DNA repair-deficient Salmonella (His−) with NO donor, glyceryl trinitrite (TGN), shows a significant increase in His+ Salmonella revertants compared to normal His− Salmonella by the transition of DNA base from C to T at the HisG46 target codon CCC (84). Reactive oxygen species are produced by the NADPH phagocyte oxidase, such as, superoxide (O2·−), hydrogen peroxide (H2O2) and hypochlorous acid (HOCl). The phagocyte NADPH oxidase consists of several subunits, including membrane-bound (gp91PHOX and p22PHOX), cytosolic (p67PHOX, p47PHOX and p40PHOX) and a low-molecular-weight G protein (rac2 or rac1) (85). Assembly of the functional NADPH oxidase initiates with the phosphorylation of a cytosolic component, p47PHOX. The activated p47PHOX is translocated with
other cytosolic subunits to membrane-bound components and resulting in a functional NADPH oxidase assembly (85, 86). Regarding the cells from X-linked (gp91\textsuperscript{PHOX} mutation) chronic granulomatous disease (CGD) patients, the translocation of activated p47\textsuperscript{PHOX} and other cytosolic components to the membrane is normal. However, due to the lack of gp91\textsuperscript{PHOX}, stable membrane association of p47\textsuperscript{PHOX} and p67\textsuperscript{PHOX} can not occur, and therefore functional NADPH phagocyte oxidase assembly is hindered (86). This indicates that the requirements of gp91\textsuperscript{PHOX} and p22\textsuperscript{PHOX} (flavocytochrome b) for the stable association of cytosolic p47\textsuperscript{PHOX} and p67\textsuperscript{PHOX} to the membrane and further formation of functional NADPH oxidase (86). The Rac protein is also translocated with cytosolic components of NADPH oxidase, and Rac translocation is associated with NADPH phagocyte activation (87). The function of another NADPH oxidase cytosolic component, p67\textsuperscript{PHOX}, is not clear, but appears to be involved in electron transfer from NADPH to oxygen by regulating electron flow from NADPH to flavin in flavocytochrome b (85, 88). Both the NADPH oxidase and iNOS are required for Salmonella killing in macrophages, but they seem to have different Salmonella killing kinetics according to the Salmonella infection stage (89, 90). Experiments with macrophages from the gp91\textsuperscript{phox}\textsuperscript{−/−}, iNOS\textsuperscript{−/−}, and gp91\textsuperscript{phox}\textsuperscript{−/−}/iNOS\textsuperscript{−/−} mice show the contribution of NADPH oxidase and iNOS for Salmonella killing at different stage of infection (90). Macrophages from gp91\textsuperscript{phox}\textsuperscript{−/−} mice show impaired Salmonella killing, as indicated by almost 100% survival from the initial times of infection and a prolonged Salmonella burden at the later stage (89). However, iNOS\textsuperscript{−/−} macrophages show the initial percentage decrease in survival rate of Salmonella, but uncontrolled Salmonella replication at later times of infection (89). Macrophages from gp91\textsuperscript{phox}\textsuperscript{−/−}/iNOS\textsuperscript{−/−} mice show total failure in controlling Salmonella replication (82). Mice challenged with Salmonella also show different susceptibility according to their genetic background,
gp91phox−/− or iNOS−/−. The bacterial counts in the spleen and liver of gp91phox−/− were greatly increased, as early as 1 day of infection and resulted in death in 5 days. In contrast, iNOS−/− mice had a bacterial count increase after 1 week of infection (90). Taken together, the suggestion is that concerted NADPH oxidase and iNOS activity is required for controlling *Salmonella* replication at different stages of infection (81, 89, 90). Figure 1.3 represents the potential interaction between NADPH oxidase derived reactive oxygens species and iNOS nitrogen intermediates (81).

Along with reactive oxygen species, natural resistance-associated macrophage protein 1 (Nramp1), previously known as the *Bcg/Lsh/Ity* genes plays an important role in innate host resistance against *Salmonella* infection, as well as other intracellular bacteria (91, 92). Nramp1 protein contains a divalent metal binding motif (Fe++ and Mn++) and is supposed to function as a divalent metal efflux pump (91, 92).
Figure 1.3: Schematic diagram of concerted action of NADPH phagocyte oxidase, myeloperoxidase, and iNOS. Potential interactions between phagocyte-derived reactive oxygen and nitrogen intermediates. Some possible reactions of products originating from NO synthase, phagocyte oxidase, and myeloperoxidase are shown in relation to hypothetical microbes situated within a phagolysosome. “Extracellular” refers to the phagolysosomal compartment, and “intracellular” refers to the microbial cytosol. Adapted from Fang (81).
Functional analysis of Nramp1 with murine macrophage cell line RAW264.7, which contains a homozygous mutation in *Nramp1*, revealed that when intact *Nramp1* was used to transfect these cells, *Salmonella* replication was inhibited in contrast to untransfected RAW264.7 cells (93). Further, Nramp1 phagosomal recruitment was confirmed (93, 94). RAW264.7 (*Nramp1*−/−) cells transfected with plasmid expressing Nramp1-e-myc tag showed the phagosomal localization of Nramp1 (93). Phagosomal localization of Nramp1 was also assessed in peritoneal macrophages from 129/sv mice (*Nramp1*+/+) and wild-type mice, 129/sv. Macrophages from 129/sv wild-type mice showed the phagosomal co-localization of Nramp1 with fluorescent coated latex bead, in contrast to the 129/sv (*Nramp1*−/−) (94). Taken together, this suggests that Nramp1 is recruited to *Salmonella*-containing phagosomal membranes, then deprived of phagosomal cations, which are required as cofactors for the *Salmonella* catalase and superoxide dismutase, resulting in control of *Salmonella* replication (91-94). In addition, Nramp1’s role in regulation of SCV maturation is known (95). The acquisition of mannose 6-phosphate receptor (M6PR), which cycles between the trans-Golgi network and the prelysosomal compartment of the endocytic pathway, and externally supplied labeled dextran by SCV was remarkably enhanced in a *Nramp*-transfected *Nramp1*+/− macrophage cell line and in macrophages from *Nramp1*+/+ mice, in contrast to the *Nramp1*−/− macrophage line and macrophages from Nramp1 null (*Nramp1*−/−) mice (95). This indicates that Nramp1 regulates SCV maturation by modulating endocytic vesicular trafficking (95).

Some Toll-like receptors (TRLs), TLR4 and TLR5, host LPS sensing factor and flagellin receptor, respectively, also influence *Salmonella* susceptibility (96-98). TLRs were originally characterized as *Drosophila* factors involved in dorso-ventral polarization of embryos and in resistance to fungal infection (99, 100). Mice having point mutations in the TLR4 gene (C3H/HeJ)
or homozygous null mutation of TRL4 (C57BL/10ScCr) were resistant to LPS-induced shock and death (96, 101), implying that the impaired LPS signaling in these mutant strains of mice was due to altered TLR4 function. However, the TLR4 mutant strain was highly susceptible to *Salmonella* infection despite its resistance to LPS-induced shock (102). TLR5 is a Toll-like molecule that recognizes flagellin from both *Gram(-)* and *Gram(+) bacteria* (97, 98). Activation of TLR5 mobilizes NF-kB and stimulates TNF-α (96, 102). Murine TLR5 lies within a locus that is associated with susceptibility to *Salmonella* (104). The expression level of TLR5 between *Salmonella*-susceptible MOLF/Ei mice and *Salmonella*-resistant 129/Sv mice is remarkably different in response to *Salmonella* infection and suggests the role of TLR5 in response to *Salmonella* or *Gram(-)* bacterial infection (104).

**Acquired Immunity**

Two types of phagocytic cells (macrophages and immature DCs) are critical in the interface between innate and adaptive immunity. DCs are especially involved in the initiation of adaptive immune responses by priming naïve T cells.

Both macrophages and DCs process *Salmonella* antigen for peptide presentation on MHC-I, as well as MHC-II (226, 227). For MHC-I presentation of antigen, macrophage takes alternative antigen presentation routes without involving the classical (cytosolic) MHC-I component, but using post-Golgi MHC-I molecules, e.g. functional transporter associated with antigen processing (TAP)-independent (228, 229). However, DCs use a cytosolic pathway for *Salmonella*-encoded antigen presentation on MHC-I (230-232). In addition to direct *Salmonella* antigen presentation by macrophages and DCs, bystander *Salmonella* antigen presentation also occurred by bystander DCs, with uptake of *Salmonella*-induced apoptotic or necrotic debris of macrophages or DCs (233).
Although, bystander macrophages can internalize apoptotic debris of *Salmonella*, the peptides are not presented (233). The DC encounter with *Salmonella* also induces DC maturation, as evidenced by increased surface expression of MHC-I, MHC-II, CD40, CD 54, CD80, CD86, and TNF-α (230, 234, 235). DCs show reduced *Salmonella* phagocytosis and antigen presentation (230). The coupled high expression of signaling molecules (230) and TNF-α (234, 235) and lowered subsequent *Salmonella* antigen presentation (230) suggest the migration of mature DC to secondary lymphoid organs and the optimal capacity to stimulate naïve T cells for the initiation of immune responses. DC migration seems to be mediated by the alteration of chemokine and chemokine receptor production (236). The critical role of DC for T cell priming was suggested by an experiment in which DC loaded with heat-killed or viable *Salmonella* can prime both CD4+ and CD8+ *Salmonella*-specific T cells on transfer into naïve mice (237).

T cell-mediated specific immune responses play a critical role in controlling *Salmonella* infection through the induction of cell-mediated or humoral immune responses. CD28-dependent activation of CD4 is critical for the clearance of bacteria (238). The role of TCRγδ T cells in *Salmonella* infection is controversial (112, 113). However, γδ T cells seem to confer resistance to *Salmonella* in *ity* (Nramp1*) mice (112, 114,115). The major CD4+ T cell subset required for protection against *Salmonella* infection is Th1 cells, as evidenced by delayed-type hypersensitivity (DTH) responses (239) and the dominant production of IL-2 and IFN-γ (105, 106, 238, 240). *Salmonella* antigen-specific CD4+ Th1 cells exert their regulatory role through IFN-γ (105, 106, 238). IFN-γ activates macrophages and cytotoxic T lymphocytes (CTLs), and is a B cell switch factor stimulating the production of murine IgG2a and IgG2b, the potent opsonizing antibodies, for clearing *Salmonella* from infected tissues (105-110). The innate immune cytokine network is also
an important factor for stimulating and sustaining adaptive Th1 responses (66, 67, 73, 74). IL-12 exerts its regulatory role through IL-12 receptors on Th1 cells, resulting in the upregulation of IFN-γ production (67). By contrast, administration of heat-killed *Salmonella* or purified antigen induced Th2-type responses with predominant production of IL-4 and elevated levels of antigen specific IgG1 antibodies, resulting in lower DTH responses (241, 242). This suggests that the involvement of different subsets of DCs for live and dead *Salmonella*, e.g. CD11c⁺CD8α⁺MHC-II⁺ for Th1 vs. CD11c⁺CD8α⁻MHC-II⁺ for Th2 (243). CD8⁺ T cell-mediated CTL is also a very important acquired immune response. It requires MHC1-mediated *Salmonella* antigen presentation and specific CD8⁺ T cell stimulation (111). CD8⁺ T cell functions via the secretion of perforin, granzyme, and IFN-γ and results in lysis of *Salmonella*-infected cells (107, 111).

B cells also play a role in resistance to *Salmonella* infection (110, 116, 117), cooperating with T cells which modulate humoral responses during *Salmonella* infection. For example, *mu/mu* (T cell-deficient) and CD28⁻/⁻ (impaired T cell activation and reduced T- and B cell signaling) mice elicited minimal to no *Salmonella*-specific IgG subclass antibodies, and only low IgM and IgG3 levels (244, 245). The protection against wild-type *Salmonella* challenge after vaccination with attenuated *Salmonella* is B cell dependent (116, 117). B cell-deficient mice immunized with attenuated *Salmonella* fail to survive with wild-type *Salmonella* challenge (116, 117). In addition, CD4⁺ T cells from the B cell-deficient mice immunized with attenuated *Salmonella* showed diminished production of IL-2 and IFN-γ after in vitro stimulation (116). However, in contrast to the critical role of B cells for the vaccine-induced clearance of *Salmonella*, resolution of attenuated *Salmonella* only depends on CD4⁺ CD28⁺ T cells (309). Taken together, with the major role of T cells in controlling *Salmonella* infection, B cells also have an important part in controlling
Salmonella secondary infection after immunization, but not in initial clearance of attenuated Salmonella.

Attenuated Salmonella Vaccine and Its Role as a Heterologous Antigen Carrier

Live Attenuated Salmonella Vaccine

The vaccination strategy based on live attenuated microorganisms has higher efficacy over subunit and DNA vaccines. During the course of vaccination, live attenuated microorganisms induce immune responses similar to the natural infection because they have antigenic diversity, as seen in wild-type microorganisms and take a natural antigen processing and presentation pathway. In addition, with viability, they could supply antigens for an extended period of time. Therefore, in many cases, a single immunization would be enough for inducing protective immunity (118).

Salmonella is one of the most extensively studied microorganisms as a live attenuated vaccine and recently as a heterologous antigen carrier. For use as a vaccine or a foreign antigen carrier, attenuation of Salmonella is crucial. Attenuation can be divided in two different categories based upon the method of mutagenesis. Production of Salmonella mutants can be accomplished in an undefined method such as chemical treatment or UV irradiation (12). In contrast, genetic recombination can be used to mutate metabolic or virulence genes (119-133). In the discussion below, a summary of the development of live attenuated Salmonella-based typhoid fever vaccine is described. Table 1.2 presents the characteristics of live vaccines developed against typhoid fever.

The first generation of typhoid fever vaccine, S. typhi Ty21a, was obtained by random mutagenesis with nitrosoguanidine (NTG) treatment (12). Ty21a shows galE− phenotype, the loss
of galactose-4-epimerase activity (15, 16). It was assumed that the loss of this enzyme activity was the major cause of Ty21a attenuation. However, in subsequent \textit{galE}^{+} complementation and \textit{galE} mutation experiments, these failed to recover the virulence or attenuation, respectively (15, 16).

Since this failed to confirm the specific mutation, the impetus for adapting genetic attenuation for \textit{Salmonella} was significantly increased.

Table 1.2: Summary of typhoid fever vaccines and their characteristics (134).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mutation</th>
<th>Safety</th>
<th>Immunogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ty21a</td>
<td>undefined</td>
<td>safe</td>
<td>IgG, IgA, IgA ASC, α4β7 ASC</td>
</tr>
<tr>
<td>541Ty, 543Ty</td>
<td>\textit{aroA purA Vi}</td>
<td>safe</td>
<td>IgG, IgA ASC (&lt;Ty21a)</td>
</tr>
<tr>
<td>CVD906 or CVD908</td>
<td>\textit{aroC aroD}</td>
<td>Bacteremia and fever</td>
<td>IgG, IgA, IgA ASC T</td>
</tr>
<tr>
<td>CVD906-\textit{htrA}, CVD908-\textit{htrA}</td>
<td>\textit{aroC aroD htrA}</td>
<td>diarrhea and fever</td>
<td>IgG IgA ASC, T</td>
</tr>
<tr>
<td>Ty455</td>
<td>\textit{aroA phoP/phoQ}</td>
<td>safe</td>
<td>Non immunogenic</td>
</tr>
<tr>
<td>Chi3927</td>
<td>\textit{cya crp}</td>
<td>bacteremia</td>
<td>IgG IgA ASC</td>
</tr>
<tr>
<td>Ty800</td>
<td>\textit{phoP/phoQ}</td>
<td>safe</td>
<td>IgG IgA, IgA ASC</td>
</tr>
<tr>
<td>Chi4073</td>
<td>\textit{cya crp cdt}</td>
<td>safe</td>
<td>IgG IgA ASC</td>
</tr>
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</table>

ACS; antibody secreting cell, T; proliferative responses of peripheral blood lymphocyte

The \textit{Salmonella} strain with metabolic gene mutation was developed on the basis of an \textit{aroA} transposon-induced deletion mutant. This \textit{Salmonella (ΔaroA)} depended on aromatic compounds, such as \textit{ρ}-aminobenzoic acid (PABA) or 2, 3-dihydroxybenzoate (DHB), for its growth, and showed higher attenuation (10^{6}-fold increase of LD_{50}) (120-125). In addition, \textit{Salmonella (ΔaroA)} was highly immunogenic and protective against lethal challenge (120, 121).

The first \textit{Salmonella (ΔaroA)} strains were Ty-2 and CDC10-80 (121, 135). Further, to guarantee the safety and to protect against reverse mutation, the purine biosynthesis-controlling \textit{purA} gene was deleted at the same time. The resulting \textit{Salmonella} strains were referred to as 541Ty and 543Ty (Vi
antigen mutant of 541Ty) (135). The clinical study with these strains showed high attenuation, but less immunogenicity than Ty2 (135). In addition, the poor immunogenicity was linked to purA mutation (135). This suggested that the double mutation in two different biosynthetic pathways was not desirable in inducing high immunogenicity. In an effort to develop a Salmonella strain-with a nonreverting single biosynthetic pathway mutant, ΔaroA and ΔaroD, containing CVD906 and CVD908, were generated from the Salmonella strains of ISP1820 and Ty2 (125, 136), respectively. In clinical trials, both CVD906 and CVD908 were highly immunogenic with a single oral immunization, but these were also reactogenic, with significantly different severity of bacteremia and adverse febrile reaction (310, 311). The differences in reactogenecity between CVD906 and CVD908 seemed to originate from the parental strains. In fact, in CVD908, which has been shown to produce minor bacteremia without adverse febrile reaction (311), an extra-aro mutation was found (137). The extra-aro mutation in CVD908 was identified at rpoS, the alternative sigma factor σS, involved in general stress resistance, survival under stress conditions, and virulence in mice (137). The rpoS mutation in CVD908 is identical to that of Ty21a (137), a live oral typhoid vaccine derived from Ty2. Thus, it is suggested that the rpoS mutation had occurred before the Ty21a development during the long time of laboratory transfer and adaptation since its isolation in early 20th century. With an introduction of htrA gene mutation, which encodes for a heat shock protein (138), further derivatives of CVD906 and CVD908, called CVD906-htrA and CVD908-htrA, respectively, were developed (139). In a clinical study, both of these were highly attenuated, but less immunogenic than their parental strains (139). It seems that the htrA mutation conferred increased attenuation of CVD906-htrA and CVD908-htrA due to its influence of htrA on Salmonella
pathogenesis. In fact, it was shown that the loss of htrA protease activity greatly reduced Salmonella survival in in vitro and in vivo experiments (138).

The attenuated Salmonella strains were also obtained by induction of the mutation in virulence genes. In contrast to metabolism-related gene mutations, virulence gene mutation did not affect bacterial growth in vivo. Ty800 Typhi vaccine strain was produced by the induction of mutation in the phoP/phoQ-two component regulatory systems that modifies lipid A structure of LPS in response to phagosomal microenvironments (47, 53, 54, 129, 140). In a clinical trial, Ty800 was safe and highly immunogenic, with the induction of Salmonella-specific IgA-producing B cells in peripheral blood 7 days after vaccination (140). However, in contrast to the licensed Ty21a, the S-IgA antibody in the mucosal sites was rarely induced (140). Using a different approach, the Ty445 strain was developed which has mutations both in metabolic (∆aroA) and virulence (∆phoP/phoQ) genes. It was highly attenuated, but poorly immunogenic, possibly due to its over-attenuation (141). In the same vein, the Chi4037 strain contained both metabolic and virulence gene mutations together; ∆cyt, which is responsible for the biosynthesis of adenylate cyclase, ∆crp, which is cAMP receptor; and ∆cdt, which is responsible for the colonization of Salmonella in deep tissues in the mucosa-associated lymphoid tissue (MALT) (127, 142). The Chi4037 showed similar responses to Ty800 and both of them are in the field trials.

Live Attenuated Salmonella as a Carrier for Heterologous Antigens

Previous research has shown that live attenuated Salmonella vaccines are effective in protecting against wild-type Salmonella challenge (S. Typhi and S. Typhimurium) in human
volunteer trials and animal studies (134). In addition to using live attenuated *Salmonella* as a vaccine, it has received attention as a heterologous antigen carrier, along with its innate character of inducing strong and sustained humoral and cellular responses in both mucosal and systemic compartments after oral administration. In fact, attenuated *S. Typhimurium*-based studies for foreign antigen carrier have been extensively performed as a model system instead of the *S. Typhi*-based one (143). In fact, an extensive number of bacterial (e.g., *Yersinia pestis*, *Bordetella pertussis*, *Helicobacter pyroli*), viral (e.g., HIV-1, HSV, influenza, HBV), and parasitic antigens (e.g., *P. palcifarum, L. major, S. mansoni*) have been expressed in attenuated *S. Typhimurium*. Table 1.3 summarizes the attenuated *Salmonella*-based foreign antigen delivery studies.

A single oral dose of *S. Typhimurium* (*ΔaroA, ΔaroD*) vaccine strain BRD509 expressing *B. pertussis* antigen, pertactin, induced strong antigen specific Th1 cell responses and conferred a significant level of protection (144, 145). *S. Typhimurium* with gp63, a major leishmanial antigen, elicited antigen-specific Th1 cell responses, as well as protective immunity against *Leishmania* challenge in a Nrramp1-dependent manner (146-148). MHC-1 mediated CD8+ T cell response (CTL) was also induced subsequent to *S. Typhimurium* vaccination with bacterial, viral, and parasite antigens (143). In addition, *S. Typhimurium* vectors appear to be able to induce immunological memory. *S. Typhimurium* expressing the *hagB* gene of *P. gingivalis* induced IgA and IgG responses after 52 weeks of single oral immunization (149). Taken together, with an induction of mucosal and systemic immune responses including humoral, CTL, and long-term memory, live attenuated *Salmonella*-based heterologous antigen delivery has multiple advantages over nonviable subunit vaccines. In the next chapter, influences of passenger antigen on host
immune responses are described with emphasis on *Salmonella* surface expression of the enterotoxigenic *E.coli* (ETEC) fimbrial antigen, colonization factor antigen I (CFA/1). In addition, its application to protection against inflammatory autoimmune disease, experimental autoimmune encephalomyelitis (EAE), is suggested.

Table 1.3: Animal based foreign antigen delivery study with *Salmonella* (143). A. Viral antigens (143).

<table>
<thead>
<tr>
<th>Strains and Mutation</th>
<th>Passenger Antigen</th>
<th>Immunogenicity</th>
<th>Assay</th>
</tr>
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<tbody>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>HA, NP (Influenza)</td>
<td>H, C (Th1)</td>
<td>CTLs</td>
</tr>
<tr>
<td>Typhimurium or Typhi; <em>cyo/crp, phoP</em> or <em>aroA</em></td>
<td>pre-S core and peptide antigen (HBV)</td>
<td>IgG, IgA (M, S)</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium or Dublin; <em>aroA</em></td>
<td>gp120, gp41, Gag (HIV-1, HIV-2)</td>
<td>IgG, IgA (M, S), C (Th1)</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium or Dublin; <em>aroA</em></td>
<td>Nef, Gag (SIV)</td>
<td>H, C</td>
<td>CTLs</td>
</tr>
<tr>
<td>Typhimurium; <em>cyo/crp</em></td>
<td>S protein (Gastroenteritis virus)</td>
<td>IgG, IgA (M, S)</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>VP1 (Poliovirus)</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>HPV type16</td>
<td>H, C</td>
<td>Virus N</td>
</tr>
<tr>
<td>Typhimurium; <em>htrA</em></td>
<td>gpD (HSV)</td>
<td>H</td>
<td>Virus N, P</td>
</tr>
</tbody>
</table>

A. Viral antigens

<table>
<thead>
<tr>
<th>Strain and Mutation</th>
<th>Passenger Antigen</th>
<th>Immunogenicity</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhi, Typhimurium, Enteritidis and Dublin; <em>galE, aroA</em>, or <em>aroA/purA</em></td>
<td>ETEC LTB</td>
<td>IgG and IgA (M,S), C</td>
<td>toxin N</td>
</tr>
<tr>
<td>Typhimurium, Enteritidis or Typhi; <em>aroA</em> or <em>galE</em></td>
<td>CFA/1, CS3, K88 and K99 fimbriae</td>
<td>IgG, IgA (M, S), C</td>
<td>P</td>
</tr>
<tr>
<td>Typhi; <em>galE</em></td>
<td>O antigen (<em>Shigella. spp</em>)</td>
<td>H</td>
<td>PP</td>
</tr>
<tr>
<td>Typhi; <em>galE</em></td>
<td>O antigen (<em>V. cholerae</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em> or <em>cyo/crp</em></td>
<td>β-galactosidase (<em>E.coli</em>)</td>
<td>IgA, IgG (M,S), C (DHT)</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium or Typhi; <em>aroA</em>, *aroC/<em>aroD</em></td>
<td>Tetanus toxin C</td>
<td>IgA, IgG (M,S), C (Th1; DHT)</td>
<td>P</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>Pertactin, S1 (<em>B.pertussis</em>)</td>
<td>H, C</td>
<td>P</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>F1, V antigen (<em>Y. pestis</em>)</td>
<td>H, C</td>
<td>PP</td>
</tr>
<tr>
<td>Typhimurium or Dublin; <em>aroA</em> or <em>cyo/crp</em></td>
<td>SpaA, M protein (<em>Streptococcus.spp</em>)</td>
<td>IgA, IgG (M,S), C</td>
<td>P</td>
</tr>
</tbody>
</table>

B. Bacterial antigens
Table 1.3 CONTINUED

<table>
<thead>
<tr>
<th>Strains and Mutation</th>
<th>Passenger Antigen</th>
<th>Immunogenicity</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>Alkaline phosphatase (<em>E.coli</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; cya/crp</td>
<td>HA (<em>P.gingivalis</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium or Dublin; <em>aroA</em></td>
<td>Exoprotein A, OMP1 (<em>P.aerougina</em>)</td>
<td>IgA (M)</td>
<td>P</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>p60, Hly, SOD (<em>L.monocytogenes</em>)</td>
<td>C</td>
<td>CD8⁺ CTL P</td>
</tr>
<tr>
<td>Typhimurium; <em>phoP</em></td>
<td>urease (<em>H.pyroli</em>)</td>
<td>H, C</td>
<td>P</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>MOMP (<em>C.trachomonias</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
</tbody>
</table>

C. Parasite antigens

<table>
<thead>
<tr>
<th>Strains and Mutation</th>
<th>Passenger Antigen</th>
<th>Immunogenicity</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhimurium, Dublin or Typhi; <em>aroA</em></td>
<td>CSP, MSP, RESA (<em>Plasmodium</em>)</td>
<td>H, C</td>
<td>CD8⁺ CTL P</td>
</tr>
<tr>
<td>Typhi; cya/crp</td>
<td>SREHP (<em>E.histolitica</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>Fatty acid-binding protein (<em>E.granulosus</em>)</td>
<td>H, C (Th1 and Th2)</td>
<td>ND</td>
</tr>
<tr>
<td>Typhimurium; <em>aroA</em></td>
<td>GST (<em>S.mansoni</em>)</td>
<td>H</td>
<td>ND</td>
</tr>
</tbody>
</table>

N; neutralization, P; protection, PP; partial protection, ND; non detected, M; mucosal immune response, S; systemic immune response, C; cellular immune response, H; humoral immune response.
CHAPTER TWO

MATERIALS AND METHODS

EAE Study

Animals

Female SJL/J mice 6-8 wks old were obtained from The Jackson Laboratories (Bar Harbor, ME). All mice were maintained at the Montana State University Animal Resource Center under pathogen-free conditions in individually ventilated cages under HEPA-filter barrier conditions and were fed sterile food and water ad libitum. The mice were free of bacterial and viral pathogens, as determined by antibody screening and histopathologic analysis of major organs and tissues. All experiments adhered to the “Guide for the Care and Use of Laboratory Animals,” prepared by the Committee on Care and Use of laboratory Animal Resources, National Research Council (NIH Publication No, 86-23, Revised (1985) ) and were approved by MSU’s IACUC.

Cloning of PLP139-151 into CFA/I Fimbriae and Its Expression on Salmonella Vaccine Vector

To exploit the immunomodulatory nature of Salmonella-CFA/I for protection against EAE, encephalitogenic PLP139-151 coding sequence was cloned into the 315 base position of CFA/I fimbrial structural gene, cfaB, by PCR mutagenesis. Briefly, with two different primers sets, two different cfaB fragments bearing PLP139-151 were amplified (F1: TCT AGA ATG AAA TTT AAA AAA ACT ATT GGT GCA ATG , R1: GGG TGG CCA AGC CAT TTC CCC AGG GAG TGC ACA CTG ATA GGC ATT TGA, F2: GCT TGG CCA AGC CCC GGA TAA ATT TTC ATG GGG AGG ACA AGT A, R2: CTA AGC TTT CAG GAT CCC AAA GTC ATT ACG). After Msc
I restriction digestion, these fragments were ligated, then cloned into TA vector and designated as pTACP. After Dra I and Bam H I restriction digestion of pTACP, cfaB/ PLP_{139-151} DNA fragment was subcloned into pUC19 and designated as pUCPLP1. The DNA fragment between BamH I and Sph I of pJGX15C-asd plasmid was amplified and cloned into pUCPLP1 (BamH I/Sph I) and designated as pUCPLP2. The EcoR I fragment from pJGX15C-asd (155) then replaced with EcoR I fragment, and resulting in pCFA/PLP, which encodes chimeric CFA/I with PLP_{139-151}. The *E. coli* and *Salmonella* strains expressing CFA/I-PLP_{139-151} were designated as JYL001 and JYL002, respectively. The chimeric CFA/I expression was probed with polyclonal rabbit-anti CFA/I serum.

**Oral Vaccination with *Salmonella* Vaccines and PLP_{139-151} Challenge**

The ΔaroA Δasd *S. enterica* serovar Typhimurium-CFA/I vector vaccine, strain H696, expressed functional CFA/I fimbriae on the vector’s cell surface (155). This phenotype was maintained by a plasmid bearing a functional *asd* gene to complement the lethal chromosomal *asd* mutation in the parent *Salmonella* strain, allowing stabilized fimbrial expression in the absence of antibiotic selection (162). Groups of five female SJL/J mice (five/group), pretreated with an oral 50% saturated sodium bicarbonate solution, received a single oral dose of ~5x10^9 colony-forming units (CFU) of the *Salmonella*-CFA/I construct, *Salmonella*-CFA/I-PLP_{139-151} or its isogenic control strain H647, which lacked the CFA/I operon (162). Control mice received PBS only.

The encephalitogenic proteolipid protein (PLP) peptide (PLP_{139-151}) (HSLGKWLGHPDKF) was synthesized by Global Peptide Services, LLC (Fort Collins, CO), and
HPLC-purified to > 90%. One or four weeks after oral immunization with the \textit{Salmonella} vaccines, or PBS, mice were given s.c challenge with 200 µl of 100 µg of PLP$_{139-151}$ emulsified in a modified Freund's adjuvant containing 1.5 mg/ml of dead \textit{Mycobacterium tuberculosis} strain H37RA (Difco Laboratories, Detroit, MI) per ml of incomplete Freund's adjuvant. The mice also received i.p 200 ng of \textit{B. pertussis} toxin (PT; List Biological Laboratories, Campbell, CA) on days 0 and 2, relative to the day of challenge. Mice were monitored daily for clinical signs, and clinical scores were assigned as follows (216): 0, normal; 1, a limp tail; 2, hind limb weakness; 3, hind limb paresis; 4, quadriplegia; 5, death.

**Histological and Immunohistochemistry Evaluation**

For histological evaluation of tissue pathology, spinal cords were removed, fixed with neutral buffered formalin (NBF), routinely processed, embedded in paraffin, and sectioned at 5 micron cross (transverse) sections from the spinal cord lumbar region were stained with hematoxylin & eosin (H&E) for pathological changes and inflammatory cell infiltration. Adjacent sections were stained with luxol fast blue (LFB; 217) and examined for loss of myelin. Pathological manifestations were scored separately for cell infiltration and demyelination. Each H&E section was scored from 0 to 4 (216): 0, normal; 1, cell infiltrate into meninges; 2, one to four small focal perivascular infiltration; 3, five or more small focal perivascular infiltrates and/or one or more large infiltrates invading the parenchyma; 4, extensive cell infiltrates involving 20% or more of the white matter. In each LFB stained section, demyelination was also scored from 0 to 4 (216): 0, normal; 1, one small focal area of demyelination; 2, two to three small focal areas of demyelination; 3, one to two large areas of demyelination; 4, extensive demyelination involving 20% or more of the white matter.
To identify infiltrating lymphocytes, immunohistochemistry (IHC) was performed on cryosections of spinal cord from SJL/J mice between days 11 and 12 after PLP$_{139-151}$ challenge from each immunization group. Mice were euthanized, and spinal cords were removed via saline injection into the spinal column. Lumbar regions of cords were embedded in O.C.T.$^\text{®}$ cryoembedding media (Sakura Finetek, Torrance, CA) and snap frozen with a dry ice/2-methyl butane slurry (-90°C). Lumbar regions of cords were transversely sectioned at -16°C in a cryostat and mounted on Plus Charge$^\text{®}$ (Erie Scientific, Portsmouth, NH). Frozen sections were air dried overnight at room temperature (RT) and fixed the next day in a 75% acetone/25% absolute ethanol mixture for 5 min at RT and rinsed immediately in three changes of Dulbecco’s PBS (DPBS). Appropriate rinsing was done between all IHC steps using a rinse buffer (DPBS with 0.2% goat serum and 0.05% Tween 20). Normal spleens were used as positive control for CD4, CD8, and SK208 staining; for Mac-1$^+$ (CD11b) macrophage staining, a *Salmonella* infected spleen was used. Endogenous peroxidase was blocked 10 min with Peroxidase Block (#S2001, DakoCytomation, Carpinteria, CA) followed by an endogenous biotin block (according to manufacturer’s instruction, Avidin/Biotin Blocking Kit, Vector Laboratories, Inc., Burlingame, CA) and a normal serum block (NSB, 10% goat and 2.5% mouse sera in rinse buffer) for 30 min at RT. The primary Abs were diluted in NSB, and all were incubated for 30 min at RT. Biotinylated rat anti-mouse Abs (BD Pharmingen, San Diego, CA) specific for CD4 (clone GK1.5, IgG2a) and Mac-1 (CD11b, clone M1-70, IgG2b) were used at 1.0 µg/ml. Isotype-matched biotinylated Abs were used as negative controls. To stain for neutrophils, an indirect staining procedure was used in which rat anti-mouse neutrophil (SK208; compliments of Dr. Mark Jutila, Montana State University; [218, 249]) mAb
was applied to sections as an undiluted hybridoma supernatant for 30 min at RT, followed by the secondary biotinylated F(ab')2 fragments of goat anti-rat IgG adsorbed to mouse at 2.0 µg/ml for 30 min. Rat IgG (10µg/ml; Jackson ImmunoResarch Laboratory, West Grove, PA) was used as a negative control. Biotinylated Abs were detected with 1.0 µg/ml of Strepavidin-HRP (Biosource/TA GO, Camarillo, CA) in rinse buffer for 20 min at RT. Following a DPBS/0.05% Tween 20 buffer rinse, AEC+ chromogen (DakoCytomation) was applied and color developed using microscopic monitoring. Color reaction was halted with DPBS, followed by a water rinse, a light hematoxylin counterstain, and coverslipping with an aqueous mounting media.

Ab ELISA

CFA/I fimbriae-specific endpoint titers from dilution of immune sera or fecal extract were measured by an ELISA, as previously described, using purified CFA/I fimbriae Ag (162) as a coating Ag. Specific reactivity to CFA/I fimbriae was determined using HRP conjugates of goat anti-mouse IgG-, IgA-, IgG1-, and IgG2a-specific Abs (1µg/ml; Southern Biotechnology Associates, Birmingham, AL), and ABTS (Moss, Inc., Pasadena, CA) enzyme substrates were used to develop color reaction. The absorbance was measured at 415 nm on a Kinetics Reader model EL312 (Bio-Tek Instruments, Winooski, VT). Endpoint titers were expressed as the reciprocal dilution of the last sample dilution, giving absorbance ≥ 0.1 OD units above the OD415 of negative control after one hour incubation.

Cytokine ELISA

Lymphocytes from various tissues (spleens, Peyer’s patches [PP], cervical-lymph nodes [CLN], and spinal cords) from the different immunization groups (PBS, H647, and H696)
following PLP\textsubscript{139-151} challenge were cultured at 5 x 10\(^6\)/ml in medium alone or in the presence of OVA (10 µg/ml), CFA/I fimbriae (10 µg/ml), or PLP\textsubscript{139-151} (30 µg/ml) in a total volume of 1.5 ml in a 24-well tissue culture plate. Lymphocytes were cultured for 60 hrs at 37\(^\circ\)C. The culture supernatant was collected by centrifugation and saved at -80\(^\circ\)C until assayed. IFN-\(\gamma\), IL-4, IL-10, and IL-13 were measured on a duplicate set of samples by capture ELISA, as previously described (219). For IL-13 ELISA, 2.0 µg/ml of rat anti-mouse IL-13 mAb (clone 38213) were used as the capture Ab, and 0.2 µg/ml of biotinylated goat anti-mouse IL-13 Ab was used as the detecting Ab (R & D Systems, Minneapolis, MN). The color reaction was developed using goat anti-biotin HRP conjugated Ab (Vector Lab, Inc.) and ABTS (Moss, Inc.), as previously described (219). Cytokine concentrations were extrapolated from standard curves generated by recombinant murine cytokines IFN-\(\gamma\), IL-4, IL-10 (R & D Systems), and IL-13 (Peprotec, Inc., Rocky Hills, NJ).

Statistical Analysis

The statistical significance of the differences in clinical scores between groups was assessed by a one-way ANOVA followed by with a post-hoc Tukey test when more than two groups were analyzed. The significance of cytokine production and histopathology from the control group was determined by Student’s t test. A value of \(p < 0.05\) was considered significant.

ETEC/EHEC Vaccine Study

ETEC/EHEC Dual Vaccine Construction

To take advantage of the repetitive nature of K99 fimbriae, oligonucleotide for a 10 amino acids peptide mimetic to enterohemorrhagic \textit{E. coli} (EHEC) LPS was cloned into plasmid
pMAK99-asd$^+$ (250). Briefly, $fanH$ gene of the K99 operon was amplified and subcloned into pUC18 vector using $fanH$ Sac I-forward (TAG AGC TCA TCA AAA CCT TTT GAG CGG) and $fanH$ Hind III-reverse (TAA AGC TTT GAA TT T CTT ATT CCC CTC) primers: pfanH. Subsequently, MH6083 (dam-) cell was transformed with pfanH. Bcl I restriction digestion product of pfanH was ligated with LPS peptide mimetic oligonucleotides with Bcl I sequence: pfanH/LPS. Oligonucleotides were annealed in vitro. $fanH$ gene with LPS mimic sequence was amplified (forward: ATG CAT ATC AAA ACC TTT TGA GCG G, reverse: ATG CAT TGA ATT TCT TAT TCC CCT C) and cloned into TA vector: pTA/LPS. Nsi I treated fragment (fanH/LPS) from pTA/LPS was cloned into Nsi I (5899 and 6466 of K99 operon) treated pMAK99-asd$: pFH/LPS. H681 (asd-) E.coli and H683 (asd-) Salmonella strains were transformed with pFH/LPS, resulting in JY001 and JY002, respectively.

**Restriction-, Sequencing- and Western Blot Analysis**

Comparative Nsi I restriction analysis was done with pMAK99-asd$^+$, pFH/LPS, and Nsi I treated self-ligation plasmid of pMAK99-asd$^+$ to examine the size differences of Nsi I treated fragments compatible to LPS mimic sequence cloning. Sequencing of pFH/LPS was done with facilities at University of Montana (Missoula, MT). Western blot analysis was done to examine the expression of K99 fimbriae with LPS peptide mimicetic. Proteins were transferred from the SDS-PAGE (15% polyacrylamide) gel to 0.2 µm-pore-size nitrocellulose membrane (Bio-Rad). The membrane was probed with the rabbit polyclonal K99 fimbrial antiserum and then with a goat anti-rabbit IgG conjugated to horse radish peroxidase (Southern Biotechnology Associates).
Immunization

Two groups of BALB/c mice (5 mice/group), pretreated with 50% saturated sodium bicarbonate solution, received single oral dose of $5 \times 10^9$ or $5 \times 10^{10}$ CFU of *S. Typhimurium*, JY002. Fimbrial expression was examined by an agglutination test with K99-specific antiserum.

Ab ELISA

Antibody titers in serum and fecal samples were determined by an ELISA adapted from previously described methods (250). Briefly, K99 fimbriae (1 µg/ml) or LPS peptide mimetic conjugated to OVA (EC-OVA: 10 µg/ml) in sterile PBS was used to coat Maxisorp Immunoplate II microtiter plates (Nunc) at 50 µl/well, and the plates were incubated overnight at the RT. Various dilutions of immune mouse serum or fecal extracts were diluted in ELISA buffer and incubated overnight at 4°C. Specific reactivity to K99 or peptide mimetic was determined with horseradish peroxidase conjugates of the following detecting antibodies (1 µg/ml): goat anti-mouse IgG and IgA antibodies (SBA). Following 90 min of incubation at 37°C and a washing step, the specific reactivity was determined by the addition of an enzyme substrate, ABTS (Moss, Inc.), and absorbance was measured at 415 nm. End point titers were expressed as the reciprocal log$_2$ of the last sample dilution giving an absorbance of $\geq 0.1$ optical density units above negative controls after 1 hour of incubation.

Construction of FanC/protein σ1 Expression Vector

Three FanC/protein σ1 expression constructs were made, two for *E. coli* expression, and the other for yeast expression. Briefly, single or triplicates of the *fanC* fimbrial gene was cloned in between maltose binding protein/protein σ1 fusion expression construct, using BamH I/Xba I
restriction sites for single FanC/protein σ1 (p1FS) and EcoR I/Sal I for triple FanC/protein σ1 (p3FS), for *E. coli* (251). Cloning was confirmed by restriction analysis and nucleotide sequencing.

For yeast expression, single *fanC*/σ1 nucleotides were amplified by PCR with EcoR I-forward (ATG AAT TCA TGA ATA CAG GTA CTA TTA AC) and Kpn I-reverse (TAG GTA ACC ATA TAA GTG AC) primers, and TA cloned. TA cloning was selected on ampicillin plate. The TA plasmid with *fanC*/protein σ1 was treated with EcoR I and Kpn I and cloned into yeast shuttle vector pPICZ A (pPI1FS) compatible sites. Subsequently, yeast *Pichia pastoris* was transformed with pPI1FS, and selected on zeocin plates. FanC/σ1 protein expression was determined with anti-K99 poly clonal rabbit antibody (250). FanC/σ1 fusion protein was purified using Ni^{2+}-NTA affinity column chromatography.
CHAPTER THREE

ETEC FIMBRIAL (CFA/I) EXPRESSION ON SALMONELLA: ITS IMPACT ON HOST IMMUNERESPONSES, AND PROTECTION AGAINST INFLAMMATORY AUTOIMMUNE DISEASE, EAE

Introduction

ETEC remains problematic in developing countries, particularly for young children who have not acquired immunity and for travelers to endemic areas (reviewed in 220). Enteric symptoms of *E. coli* infection are often referred to as “traveler’s diarrhea.” *E. coli* becomes enterotoxigenic upon acquisition of a plasmid or plasmids containing the heat-stable enterotoxin (221) or the cholera-like exotoxin, which is commonly termed the heat-labile enterotoxin (222, 223). Both toxins are responsible for inducing fluid loss and electrolyte imbalance. Virulence by ETEC is also partly contributed by acquisition of the plasmid for the pili or CFAs, which enhances the colonization of *E. coli* in the GI tract. The CFA pili are a heterogenous group of fimbrial adhesins and are responsible for adherence to small intestinal epithelium via their fimbriae or long, hair-like projections extending from the bacterial cell surface, to epithelial mannose-containing glycoproteins (150). Previous studies show that oral delivery of purified CFAs fails to induce significant serum IgG or S-IgA Abs (152, 153), and these anti-fimbriae Ab titers (152, 154) fail to protect human volunteers from pathogenic ETEC challenge (152). Thus, live vaccines may be necessary to retain immunogenicity of CFAs (155).

Previous work shows that oral immunization with *Salmonella* vaccine vector expressing the ETEC fimbriae, CFA/I, elicits high Ab titers, as evidenced by elevated serum IgG1 and mucosal IgA (162). These elevated Ab titers are supported by a biphasic Th cell response in which Th2 cells
are rapidly induced and precede development of Th1 cells (162). Such findings contrast to the immune responses obtained with conventional *Salmonella* vaccine vectors, which generally stimulate Th1 cell-dominating responses toward both *Salmonella* and passenger Ags (156-161). These responses are characterized by elevated IFN-γ-regulated IgG2a production and suboptimal mucosal IgA responses. It has also been observed that the *Salmonella*-CFA/I vaccine fails to elicit proinflammatory cytokines upon infection of macrophages (163). While the isogenic *Salmonella* vector strain H647 could elicit elevated levels of TNF-α, IL-1, and IL-6, the *Salmonella*-CFA/I vaccine remains, as if it were stealth, and fails to elicit these cytokines despite identical infectivity with the isogenic *Salmonella* strain.

EAE is an animal model of inflammatory, demyelinating human disease of the central nervous system (CNS), multiple sclerosis (MS) (164-166). It shares many features with MS, such as ascending clinical paralysis, T cell immunity to neuroantigen, CNS mononuclear infiltration, and epitope spreading (167-170). EAE can be induced by either the immunization against specific myelin antigens, MBP, PLP, myelin oligodendrocyte glycoprotein (MOG), or by passive transfer of activated myelin-specific CD4+ Th1 lymphocytes (171-173). Encephalitogenic T cells secrete Th1-type cytokines, IFN-γ and IL-2, and induce local macrophage and microglial activation, infiltration of inflammatory cells from peripheral lymphoid tissue, and demyelination (175-181). EAE can be suppressed by adoptive transfer of myelin-specific Th2 cells, which secrete IL-4, IL-10, and IL-13 and inhibit Th1 cell development (182-185). The protective effect of Th2-type cytokines has also been demonstrated by direct delivery into the CNS using retrovirally transduced T cells (186, 187) and experiments with IL-4 knockout mice (188, 189).
These studies clearly indicate that induction of Th2 cell-like responses is a viable strategy for treatment of diseases dominated by Th1-mediated pathophysiology. Several experimental strategies for induction of Th2 cell-dependent protection in EAE have been reported, including oral administration of myelin antigens for oral tolerance, immunization using altered peptide ligands bearing encephalitogenic T cell epitopes, and immunization with Ags that selectively induce responses (199-203). Low dose tolerance, believed to act by suppressing the induction of encephalitogenic T cells by Th2 cells, has also been adapted for MBP-specific Th2 cells (204). In addition, high dose oral tolerance, believed to result in clonal deletion/anergy of encephalitogenic T cells, has also been tested (205). It has also been observed that MBP-specific Th2 cell responses suppressed PLP-specific EAE, suggesting that the bystander suppression alters the T cell microenvironment to become Th2-type dominated (200). Immunization with an altered peptide ligand for PLP139-151 induced Th2 cells specific for the immunogen and suppressed EAE upon PLP challenge (202). Other myelin Ag-specific EAE challenges have also been suppressed, supporting a role for bystander suppression in this model. Protection to EAE could also be achieved upon immunization with non-self Ags associated with down regulation of CD4 co-receptor at the time of EAE onset and/or EAE-specific immune deviation from Th1- to Th2-type by the change in the cytokine microenvironment in which encephalitogenic T cells developed (203, 215). Collectively, these observations clearly provide precedence for developing strategies for the treatment of autoimmune diseases using non-self Ags to induce bystander Th2 cell-mediated suppression.

We questioned whether we could adapt bystander or active suppression of EAE by using our previously described diarrheal vaccine or exploiting it as a molecular scaffold for the encephalitogenic T cell epitope to induce EAE-protecting Th2 cells, respectively. (162).
hypothesized that oral immunization with *Salmonella*-CFA/I or *Salmonella*-CFA/I-PLP could protect against the development of EAE via different mechanisms, bystander suppression and active induction of epitope-specific Th2 cells.

**Rationale and Aim of Research**

As an intracellular parasite, most attenuated *Salmonella*-based vaccine induced Th1-type inflammatory responses toward both *Salmonella* vehicle and passenger antigens. However, oral immunization with *Salmonella* vaccine expressing ETEC fimbriae CFA/I rapidly induced strong Th2-type immune responses in advance of Th1-type development. These Th2-type responses were evidenced by elevated serum IgG1 and mucosal IgA. Furthermore, *Salmonella*-CFA/I failed to induce proinflammatory cytokines on infection of macrophages despite identical infectivity with the isogenic *Salmonella* vaccine.

EAE is a pro-inflammatory CNS disease mediated by myelin-specific Th1 cells, induced by either immunization with myelin antigen or passive transfer of myelin-specific Th1 cells. Studies have shown the suppression of EAE with provision of Th2-type cytokines by adoptive transfer of myelin-specific Th2 cells or direct delivery into the CNS. Experimental induction of Th2 cells for the treatment of EAE was achieved in either an antigen-specific or –nonspecific manner represented by oral tolerance and bystander suppression. Based on the studies outlined above, we hypothesized that oral immunization with *Salmonella*-CFA/I or *Salmonella*-CFA/I bearing encephalitogenic T cell epitope (*Salmonella*-CFA/I-PLP) could protect against the development of EAE via either the bystander suppression or induction of epitope-specific Th2 cells, respectively.
In the following section, we examined the EAE-prophylactic efficacy of Salmonella-CFA/I and Salmonella-CFA/I-PLP. To carry out this analysis, we compared clinical development, histology and immunopathology, and cytokine profile among different immunization groups (PBS, isogenic Salmonella strain [H647], Salmonella-CFA/I [H696], or Salmonella-CFA/I-PLP [JYL002]).

Protection from EAE via Bystander Suppression with Immunization of Salmonella-CFA/I and Salmonella-CFA/I-PLP

Results

Cloning of PLP\textsubscript{139-151} into CFA/I Structural Gene cfa/B and Its Expression. To obtain encephalitogenic epitope-specific anti-inflammatory Th2 cell-inducing vaccine, we cloned encephalitogenic PLP\textsubscript{139-151} into CFA/I fimbrial structural gene cfaB for expression by Salmonella. Mutagenesis of cfaB gene with PLP\textsubscript{139-151}-specific oligonucleotides does not affect the formation of CFA/I fimbriae, as confirmed by the CFA/I-specific Western blotting. Figure 3.1 describes the cloning procedure and fimbrial expression.
Figure 3.1: Construction of pCFA/PLP and its expression. Encephalitogenic PLP$_{139-151}$ oligonucleotides were cloned into $cfaB$ gene by PCR mutagenesis method and subsequently inserted into 315 DNA position of $cfaB$ gene of pJGX-$asd$ plasmid, and designated as pCFA/PLP ($A$, $B$). The expression of pCFA/PLP was confirmed by probing with polyclonal rabbit anti-CFA/I serum ($C$). The cloning procedure is described in detail in materials and methods fimbriae chapter. H696 ($Salmonella$ strain expressing pJGX15C-$asd$). JYL002 ($Salmonella$ strain expressing pCFA/PLP).

To determine whether mice were effectively immunized by $Salmonella$-CFA/I-PLP, CFA/I-specific copro-IgA and serum IgG endpoint titers at week 1 and 2 were measured by ELISA without PLP$_{139-151}$ challenge. CFA/I-specific Ab titers (Fig 3.2$A$, $B$) were similar to endpoint titers obtained in BALB/c mice as was the dominant IgG1 subclass responses when compared to IgG2a.
The *Salmonella*-CFA/I-PLP dosed group showed similar clinical EAE course and resolution to the *Salmonella*-CFA/I vaccinated group (Fig 3.4A, B). However, in an antigen recall assay with various tissues from *Salmonella*-CFA/I-PLP immunized mice, PLP<sub>139-151</sub>-specific cytokines were not detected, suggesting the failure of PLP<sub>139-151</sub> antigen presentation (Fig 3.3). Thus, further studies with *Salmonella*-CFA/I-PLP were halted.

![Chart A](image1.png)

**Figure 3.2:** Immunization with *Salmonella*-CFA/I (H696) and *Salmonella*-CFA/I-PLP (JYL002) shows similar responses to *Salmonella*-CFA/I immunized BALB/c mice. Copro-IgA, serum IgG titers (A) and IgG subtype (B) were examined either after 2 weeks of *Salmonella*-CFA/I immunization in 1 wk and 4 weeks post immunization challenge group, or 1 wk and 2 wks after *Salmonella*-CFA/I-PLP immunized group without PLP<sub>139-151</sub> challenge.
Reduced EAE Clinical Development and Resolution by the Oral Immunization of \textit{Salmonella-CFA/I} (H696)

To test whether the bystander suppression hypothesis with oral vaccination of Th2 cell-promoting \textit{Salmonella-CFA/I} vaccine could alter the course of EAE development, three groups of SJL/J mice were orally vaccinated with PBS, the isogenic empty \textit{Salmonella} vaccine vector (H647), or \textit{Salmonella-CFA/I} (H696). One or four weeks after oral immunization, each group of mice was challenged with PLP\textsubscript{139-151} and PT. To determine whether mice were effectively immunized by \textit{Salmonella-CFA/I}, CFA/I-specific copro-IgA and serum IgG antibody endpoint titers at week 2 were measured by ELISA. CFA/I-specific Ab titers (Fig. 3.2A) were similar to endpoint titers obtained in BALB/c mice (162). In addition, IgG subtype titer was also determined, and it showed strong Th2 bias, as indicated by elevated levels of CFA/I-specific IgG1 Ab (Fig. 3.2B). The
unvaccinated control group showed the expected disease course with a mean day onset of disease of 8.8 ± 1.2 days (Table 3.1) with disease peaking between days 12 – 20 after PLP_{139-151} challenge (Fig. 3.4A). Mice orally vaccinated with the *Salmonella* vector showed similar disease kinetics (Fig. 3.4A) with a mean day onset of 7.83 ± 1.37 days (Table 3.1). However, while the *Salmonella*-CFA/I vaccinated mice did not show a significant delay in disease onset (Fig. 3.4A and Table 3.1), the overall clinical outcome was significantly milder in these mice (*P* < 0.001). These data suggest that at the peak of the Th2 cell dominance, previously observed for vaccination with strain H696 (162), the disease course was clearly altered, with these mice recovering by 21 days after EAE induction. Unvaccinated and *Salmonella* control groups showed disease persistence throughout the observation period.

To determine whether *Salmonella*-CFA/I was capable of providing protection during the IFN-γ dominated phase, a second set of experiments was performed in which oral immunization occurred 4 weeks prior to EAE induction (Fig. 3.4B). We have previously shown that at this time point, the Th2 cell response was reduced, while the Th1 cell responses became elevated (162). To test this, SJL/J mice were orally vaccinated with PBS, H647 or H696 strains and challenged (immunized) with PLP_{139-151} on day 28 and evaluated for clinical disease for 33 days (Fig. 3.4B). As with the 1-wk vaccination study, the unvaccinated control group developed a similar, but more severe course (Table 3.1). Both the PBS- and H647-vaccinated groups showed similar mean day onset of clinical disease (Table 3.1 and Fig. 3.4B). In contrast, the *Salmonella*-CFA/I-vaccinated groups showed a significantly delayed mean day onset of 10.5 ± 1.5 days when compared to PBS- or H647-dosed mice (*P* < 0.001). Although the *Salmonella*-CFA/I-vaccinated mice showed higher clinical scores than mice vaccinated one wk prior to challenge, all the vaccinated mice resolved
their EAE by 24 days after challenge. The H647-vaccinated mice, while showing reduced disease compared to the PBS-dosed group, had elevated clinical scores when compared to the H647-dosed mice in the one week post-immunization challenge. The PBS-dosed group also showed elevated clinical scores when compared to PBS-dosed mice in the one week post-immunization challenge. The elevated clinical scores in the four weeks post-immunization group may reflect the influence of the age of the mice at the time of challenge on the severity of clinical EAE expression (224, 225). Thus, the protective effect of *Salmonella*-CFA/I strain persists throughout the Th2 and Th1 cell phases of vaccine-specific immune responses.

Histopathological Analysis of CNS: Myelin Status and Nucleated Cell Infiltration to CNS

Typically, EAE is characterized by CNS inflammatory cell infiltration and loss of myelin. To determine the extent of demyelination and inflammatory cell infiltration among the one week vaccination groups, sections of spinal cord were stained with LFB and H&E. A striking difference in the extent of demyelination was observed among the different treatment groups when compared to normal SJL/J mouse spinal cord (Fig. 3.5). H696-immunized mice showed no demyelination or only mild demyelination (Fig. 3.5). Spinal cord sections from PBS- and H647-immunized mice showed demyelination with the H696-vaccinated mice, revealing the most mild myelin loss compared with PBS- and H647-vaccinated mice (Table 3.2; \( P < 0.001 \)). Since demyelination is caused the inflammatory cells, the degree of inflammatory cell infiltration in the CNS (Fig. 3.6) was evaluated in spinal cord sections stained with H & E (Fig. 3.6). The H696-vaccinated group showed little or no inflammation similar to that observed in normal mice. By contrast, PBS- and H647-vaccinated mice had clear inflammatory infiltrates in the meninges and CNS parenchyma regions.
The degree of cellular infiltration in the spinal cords is summarized in Table 3.2. The H696-vaccinated group scored 0.4, which represented either no infiltration or minimal meningeal infiltration. PBS- and H647-vaccinated groups had scores in the 2.5 - 2.7 range, indicating between one to four small perivascular infiltrations and more than four small perivascular/or large parenchymal invasions. Thus, protection from clinical disease in H696-vaccinated mice was clearly associated with reduction in inflammatory CNS infiltrates.

**Salmonella-CFA/I Vaccine Halts CD4+ T Cell and Mac-1+ Cell Influx into the CNS.** To determine the general composition of the inflammatory infiltrates, immunohistochemistry was performed to identify cell types typically present in EAE tissue (Fig. 3.7). Infiltrates in spinal cord sections from PBS-vaccinated mice consisted of CD4+ T cells, Mac-1+ cells, and neutrophiles (SK208+). By contrast, spinal cords from H647-vaccinated mice showed staining for a small number of Mac-1+ cells and only a few neutrophils. Finally, CD4+ T cells and SK208+ neutrophiles were absent from infiltrates in Salmonella-CFA/I-vaccinated mice; only a few Mac-1+ cells were detected. These data suggested that H696 vaccination suppressed development of myelin-specific, pathogenic Th1 cells and/or prevented migration of inflammatory cells to the CNS.
Figure 3.4: Oral vaccination with *Salmonella*-CFA/I (H696) or *Salmonella*-CFA/I-PLP (JYL002; designated as Epitope in legend) was protective against PLP139-151 challenge in SJL/J mice. Mice were challenged with PLP139-151 one (A) or four wks (B) after oral vaccination with *Salmonella* vaccines or PBS and given pertussis toxin on day 0 and 2 of challenge. *Salmonella*-CFA/I- or *Salmonella*-CFA/I-PLP-immunized mice showed significantly reduced EAE in both 1 and 4 wks post-vaccinated groups and delayed onset in the 4-wk group (Table 3.1). All H696- or JYL002-vaccinated mice recovered. The vector control, H647-immunized group also showed reduced disease when compared to PBS-immunized mice; however, clinical disease persisted, and mice did not recover. Depicted are the results from two combined experiments each *P* < 0.001 for PBS vs. H647, PBS vs. H696, and PBS vs. JYL002.
Table 3.1: Vaccination with *Salmonella*-CFA/I (H696) one week prior to PLP139-151 challenge protects SJL/J mice from EAE

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<tr>
<td>7. PBS</td>
<td>8. 10/10</td>
<td>9. 8.8 ± 1.2</td>
<td>10. 5</td>
<td>11. 61.22</td>
</tr>
<tr>
<td>12. H647</td>
<td>13. 9/9</td>
<td>14. 7.83 ± 1.37</td>
<td>15. 4</td>
<td>16. 33.54*</td>
</tr>
<tr>
<td>17. H696</td>
<td>18. 9/9</td>
<td>19. 10.13 ± 3.8</td>
<td>20. 2</td>
<td>21. 6.32**</td>
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Vaccination with *Salmonella*-CFA/I (H696) four weeks prior to PLP139-151 challenge protects SJL/J mice from EAE

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<tr>
<td>27. PBS</td>
<td>28. 10/10</td>
<td>29. 8.0 ± 0.57</td>
<td>30. 5</td>
<td>31. 91.09</td>
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<tr>
<td>32. H647</td>
<td>33. 8/8</td>
<td>34. 8.0 ± 0.8</td>
<td>35. 5</td>
<td>36. 53.07*</td>
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<tr>
<td>37. H696</td>
<td>38. 9/10</td>
<td>39. 10.5 ± 1.5*</td>
<td>40. 2</td>
<td>41. 11.47*</td>
</tr>
</tbody>
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SJL/J mice were challenged s.c. with 200 µg PLP139-151 in complete Freund’s adjuvant 1 wk post-vaccination with PBS, H647, or H696. Mice received i.p. 200 ng PT on days 0 and 2.

b Number of mice with clinical EAE over total in the group.

c Mean day of clinical disease onset.

d Maximum daily clinical score.

e Cumulative clinical scores were calculated as the sum of all clinical scores from disease onset to the day of sacrifice (25 - 27 days), divided by the number of mice in each group. *P < 0.001 for PBS vs. H647, PBS vs. H696, and H647 vs H696.

f EAE was induced by immunization against PLP139-151 at 4 wks post-vaccination with PBS, H647, and H696. MDO: *P < 0.001 for PBS vs. H696 and H647 vs H696.
Figure 3.5: Spinal cord sections from different immunization regimens were stained with LFB to detect demyelination. Spinal cord sections from PBS- and H647-immunized mice showed significant demyelination (indicated by arrows); however, the H696-dosed group showed minimal to no pathology.
Figure 3.6: To visualize cell infiltration into the CNS, spinal cord sections from the different immunization groups were stained with H&E. Inflammation of the meningeal and parenchymal regions of CNS were found in PBS- and H647-immunized mice (see arrows). Spinal cord sections from H696-immunized mice showed absence of inflammation and resembled normal spinal cord. Samples were collected between day 11 and 12 after PLP139-151 challenge.
Table 3.2: Histopathological changes in vaccinated mice with PLP$_{139-151}$ induced EAE$^a$

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>N</th>
<th>Inflammation</th>
<th>Demyelination</th>
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</thead>
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<tr>
<td>PBS</td>
<td>6</td>
<td>2.5 ± 0.7</td>
<td>2.67 ± 0.6</td>
</tr>
<tr>
<td>H647</td>
<td>6</td>
<td>2.67 ± 0.27</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>H696</td>
<td>6</td>
<td>0.33 ± 0.27*</td>
<td>0.17 ± 0.16*</td>
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$^a$ Spinal cord sections were stained with H&E to evaluate cellular infiltrates, and LFB to assess demyelination. The sections were scored separately for mononuclear cell infiltrates, and for the presence of demyelinating lesions. Inflammatory cell infiltrates: 0, normal; 1, meningeal mononuclear infiltrates; 2, between one and four small perivascular infiltrates/section; 3, more than four small perivascular infiltrates and/or one or more large infiltrates invading parenchyma; 4, extensive mononuclear infiltrates involving 20% or more of the white matter/section. Myelin damage: 0, normal; 1, one small area of demyelination/section; 2, two to three small area of demyelination; 3, one to two large demyelinating areas; 4, extensive demyelination involving 20% or more of the white matter/section.

$^b$ Mice were challenged s.c. with 200 µg PLP$_{139-151}$ in complete Freund’s adjuvant 1 wk post-vaccination with PBS, H647, or H696. On days 0 and 2 post-challenge, they received i.p. 200 ng PT.

$^c$ Number of mice examined per group. *P < 0.001 for PBS vs. H696
Figure 3.7: To identify the types of inflammatory cells infiltrating CNS, IHC was performed. Spinal cords from PBS-dosed mice showed mostly CD4 cells and macrophages, evidenced of minimal neutrophil infiltration (indicated by Mac-1$^+$ and SK208 mAb staining). Spinal cords from H647-vaccinated mice showed mostly Mac-1 and only few SK208 cells. Only a few Mac-1$^+$ cells were detected in spinal cords from H696-vaccinated mice. Mice were sacrificed between days 11 and 12 after PLP$_{139-151}$ challenge. Depicted are representative examples of 6 mice / group.
PLP_{139-151} -Specific Immune Deviation from Th1 to Th2 Cell by Vaccination with *Salmonella*-CFA/I.

The participation of Th1/Th2-type cytokines in the development of organ-specific autoimmune disease has been well-documented (246, 247). To determine whether the protective effects of *Salmonella*-CFA/I vaccination is Ag-specific, cytokine responses were measured ex vivo in lymphocytes isolated from spleens, PP, CLN, or spinal cords from vaccinated and unvaccinated mice, and pulsed in vitro with OVA, CFA/I fimbrae, or PLP_{139-151} (Fig. 3.8). Unstimulated lymphocytes were included as controls. As we previously observed (162), cytokine responses were clearly Th2 cell-dominant in H696-vaccinated mice, exceeding IFN-γ production (Fig. 3.8). Unstimulated lymphocytes were included as controls. Not having previously measured IL-13, we observed considerable enhancement in H696-vaccinated mice, but not in the PBS-dosed mice. Some splenic and CLN IL-13 was induced in mice orally vaccinated with the *Salmonella* vector, but not to the levels observed with H696-vaccinated mice. Upon restimulation with PLP_{139-151} peptides, the IFN-γ levels in H696-vaccinated mice were significantly reduced in all tissues examined when compared to unvaccinated controls or H647-vaccinated mice. The major production of PLP_{139-151}-specific IFN-γ was primarily observed with CLN from PBS- and H647-vaccinated mice, suggesting that this was the site of T cell activation. Conversely, the PBS- and H647-vaccinated mice showed minimal-to-no Th2-type cytokine production in any of the tissues further examined, suggesting that the *Salmonella*-CFA/I vaccine imparted a Th2-type bias and, in an Ag-independent fashion, biased the development of PLP_{139-151} CD4^+ T cells to becoming Th2-type. Thus, oral immunization with *Salmonella*-CFA/I vaccine induced PLP_{139-151}-specific immune deviation for ultimate protection against EAE.
Figure 3.8 (A) and (B)
Figure 3.8: Lymphocytes from Salmonella-CFA/I-vaccinated mice showed increased production of Th2-type cytokines and reduced IFN-γ secretion following in vitro restimulation with PLP\textsubscript{139-151} peptide. Lymphocytes were collected from mice vaccinated for one wk and harvested between 11 and 12 days post-EAE induction. Lymphocytes from (A) spleen, (B) CLN, and (C) PP of H696-vaccinated mice showed significant increases in the production of Th2-type cytokines (IL-4, IL-10, and IL-13) in response to PLP\textsubscript{139-151} restimulation compared to similar cultures from PBS- and H647-vaccinated mice. In contrast, PBS- and H647-vaccinated mice showed greater production of IFN-γ including CLN and spinal cord (SC) than lymphocytes obtained from the H696-vaccinated mice. Unstimulated cultures (M) or cultures restimulated with nonspecific Ag (OVA) showed minimal IFN-γ secretion. Data depict the mean of three experiments. *\(P < 0.001\), ***\(P \leq 0.018\) depict differences between PLP\textsubscript{139-151} restimulated cultures from PBS-dosed mice versus cultures from H647- or H696-vaccinated mice.
Discussions and Conclusions

Previous works show that *Salmonella*-CFA/I vaccine fails to elicit proinflammatory cytokines from infected macrophages unlike its isogenic strain, which, in a dose-depend fashion, induces the expected elevation in TNF-α, IL-1α, IL-1β, and IL-6 (163). It is apparent from this study that the presence of the CFA/I fimbriae impedes the expected proinflammatory response. This lack of an inflammatory response is not attributed to increased production of IL-10 or IL-12p40, since these did not significantly differ from those levels induced by the *Salmonella* vector strain (163). Further evidence that the CFA/I fimbriae alters the *Salmonella*’s behavior is supported by in vivo studies in which CFA/I fimbria-specific Th2 cells were dominated by IL-4, IL-5, IL-6, and IL-10 and accompanied by elevated serum IgG1 and mucosal IgA Ab titers (162). We hypothesized that this property of the *Salmonella*-CFA/I vaccine could be exploited to develop an anti-inflammatory vaccine.

To test this hypothesis, the EAE-susceptible SJL/J mice received the anti-inflammatory vaccine, which was engineered to express encephalitogenic PLP$_{139-151}$, its isogenic construct (lacking the CFA/I operon), or PBS one or fours wks prior to EAE induction by active immunization against PLP$_{139-151}$. We first questioned whether SJL mice would make an appropriate immune response to the vaccine, as previously shown for BALB/c (162) and B6 mice (218). The data clearly indicate that SJL/J mice are vaccine responders generating a robust anti-CFA/I Ab response that is similar in magnitude and kinetics to those exhibited by similarly vaccinated BALB/c and B6 mice. However, antigen recall experiments with various tissues from the mice immunized with *Salmonella*-CFA/I-PLP, which was originally designed to induce PLP$_{139-151}$-
specific EAE suppression, failed to stimulate PLP\textsubscript{139-151}-cytokine production. The next series of studies showed that \textit{Salmonella}-CFA/I-vaccination significantly reduced clinical and histopathological EAE in PLP\textsubscript{139-151}-immunized mice. The duration of acute disease was shorter; peak clinical scores were lower; and, more importantly, all of the vaccinated mice recovered from the acute phase of disease. By contrast, disease in control mice receiving the \textit{Salmonella} vector strain alone or PBS was sustained throughout the 33 day observation period, with minimal recovery after the acute phase. Although the severity of EAE observed in mice receiving \textit{Salmonella}-CFA/I vaccine 4 weeks prior to PLP\textsubscript{139-151} immunization was worse than observed in mice with EAE induced 1 week post-vaccination, all vaccinated mice in both vaccination regimens recovered completely. It was not surprising that the \textit{Salmonella}-CFA/I-vaccinated mice in the one week regimen showed less severe clinical disease, since the Th2 cell response is dominant during the early stages of \textit{Salmonella} inflection, and since CFA/I fimbria-specific Th1 cells generally do not peak until three or four wks post-infection (162). We also noted a difference in the magnitude of EAE in PBS-dosed mice, which may be related to the differences in age of the challenged mice. Such differences may be attributed to age, as others have previously suggested that EAE is age-dependent (224, 225).

Surprisingly, mice vaccinated with the \textit{Salmonella} vector strain H647 also showed reduced EAE in both dosing regimens, which was a consistent finding in 4/4 experiments conducted independently. In addition, this occurred in the presence of an intact IFN-\(\gamma\) response and in the absence of a clear Th2 cell deviation. An explanation for this finding may be that pro-inflammatory responses induced by \textit{Salmonella} infection alone may be competing with PLP\textsubscript{139-151}-induced
inflammation. In all four experiments, this was consistently observed. Similar observations have been made for EAE protection (206) and treatment of relapsing-remitting MS in humans (207-209) following vaccination with BCG. BCG, a Th1-type promoting bacterium, caused the formation of granulomas in spleen and liver of infected mice, and the investigators demonstrated that MOG-specific Th1 cells were recruited into the liver granulomas rather than to the CNS (206). This suggests that vaccination with Th1 cell-inducing microbial agents may redirect a Th1 cell response away from an Ag-targeted site of inflammation. Additional studies are warranted to directly address this possibility in the Salmonella-CFA/I vaccination model.

In addition to reduction in clinical disease, mice receiving oral Salmonella vaccine also exhibited clear evidence of reduced tissue pathology. Salmonella-CFA/I-vaccinated mice showed minimal demyelination and inflammatory cell infiltration during peak EAE, and the composition of inflammatory cell infiltration differed significantly among the three experimental groups. CD4+ T cells and Mac-1+ cells and some neutrophils dominated the spinal cord infiltrates in PBS-vaccinated mice, while infiltrates in Salmonella vaccinated mice consisted almost exclusively of Mac-1+ cells (not neutrophils). Thus, a reduction in T cells may account for the observed reduced pathology in Salmonella-vector vaccinated mice. The lack of T cells in spinal cord infiltrates limited the ability to study T cell cytokines in the CNS of Salmonella-CFA/I-vaccinated mice. This evidence supports the notion that oral vaccination with Salmonella-CFA/I vaccine can reduce peripheral or distal inflammation in a tissue other than the mucosal compartment. This ability to reduce inflammation in the CNS occurred in an Ag-independent fashion because there was no primary structural homology between PLP139-151 and CFA/I fimbria.
Since no primary structural homology could be identified between PLP\textsubscript{139-151} and CFA/I fimbrial subunit, we hypothesized that the mechanism for the observed efficacy against EAE involved immune deviation. As previously indicated, the CFA/I fimbriae alter how the host recognizes the *Salmonella* vaccine, and it is evident that potent Th2 cells were induced despite the proinflammatory challenge produced by the EAE regimen. In this context, the development of inflammatory PLP\textsubscript{139-151}-specific Th cells was diminished, as indicated by the reduced PLP\textsubscript{139-151}-specific IFN-\(\gamma\)-producing T cells in *Salmonella*-CFA/I-vaccinated mice. Instead, PLP\textsubscript{139-151}-specific Th2 cells were induced and sustained for at least four weeks. In contrast, the PBS- and *Salmonella* vector-immunized mice showed elevated levels of IFN-\(\gamma\) and minimal to no production of Th2-type cytokines. Thus, it appears that the *Salmonella*-CFA/I vaccine altered the microenvironment, possibly in the draining LN of the head and neck, to prevent the development of encephalitogenic Th1 cells.

We were also surprised to find the quite robust IL-13 responses by Ag-restimulated lymphocytes since we had not previously evaluated this Th2-type cytokine. IL-13 is a signature Th2-type cytokine that exhibits functions similar to those observed for IL-4. It was evident in this study that while IL-13 was induced against the CFA/I fimbriae, the majority was induced by PLP\textsubscript{139-151}, especially in the CLN. It has been reported that IL-13 can suppress EAE, using transfection methods (194) or adoptive transfer of Th2 cells (184), and it has been suggested IL-13 suppresses EAE by inhibiting the production of proinflammatory cytokines, such as IL-1\(\beta\) and TNF-\(\alpha\), from macrophage/monocyte (196, 197) or nitric oxide by glial cells (198). Although IL-13 and IL-4 share a receptor, the ability of IL-13 to suppress EAE appears distinct from that of IL-4 (195). In our studies, the concomitant stimulation of IL-4 and IL-13 by oral vaccination with a live
vaccine suggested a clear advantage for our *Salmonella*-CFA/I vaccination strategy to prevent inflammatory autoimmune diseases.

In addition to the stimulation of IL-4 and IL-13, the *Salmonella*-CFA/I vaccine also stimulated production of IL-10 (162), which has a well-documented beneficial impact on EAE (184, 190-192). IL-10 delays disease onset and reduces clinical disease when directly administered at the time of disease induction (190) and in transgenic mice over-expressing IL-10 (183, 191). Moreover, targeted disruption of IL-10 results in loss of EAE suppression (183, 191). IL-10 acts during the induction, rather than in the effector stage of disease, since it fails to suppress EAE induced by adoptive transfer of myelin-specific cells (193). Administration of IL-10 may actually be detrimental in the adoptive transfer model (193). In our study, IL-10 was already present at the time of disease induction and may have contributed to deviation of PLP139-151-specific CD4+ T cells toward a Th2 cell phenotype, either independently or in conjunction with IL-4 and IL-13.

Data from other infection models suggest that preconditioning the host with a Th2-inducing organism may bias and alter the development of encephalitogenic T cells. Infection with *S. mansoni* initially produces a Th1 cell response, which then shifts to Th2-type response during egg production, implying that the eggs are responsible for inducing Th2-type responses (211). However, the mechanism for protection by *S. mansoni* differs between a live infection or an exposure to eggs alone. *S. mansoni* infection results in the down-regulation of IFN-γ and IL-12p40, but leaves IL-10 and IL-4 unchanged, suggesting mechanisms other than Th2 cell activation in EAE protection, and possibly involving macrophages (212). By contrast, immunization with *S. mansoni* eggs results in EAE protection that is accompanied by the down-regulation of IFN-γ and increases production of IL-4, IL-10 (213, 214), and TGF-β (213). Consequently, adapting the host to a Th2 cell-dominated
environment by helminth infection is currently being considered as a strategy for the treatment of MS (210, 248).

In conclusion, this study showed that oral administration with the anti-inflammatory vaccine, *Salmonella*-CFA/I, in an Ag-independent fashion, can prevent the development of the inflammatory murine disease, EAE. Expression of the ETEC fimbriae was necessary since mice receiving the “naked” *Salmonella* vector showed persistent clinical disease throughout the observation period. Although mice exposed to naked vector showed less disease severity than PBS treated controls, the *Salmonella*-CFA/I-vaccinated mice completely resolved EAE as early as 20 days after disease induction. Protection from EAE in *Salmonella*-CFA/I, but not naked vector-vaccinated mice, was accompanied by elevated production of PLP_{139-151}-induced Th2-type cytokines and diminished IFN-γ production. Protected animals showed reduced infiltration of inflammatory cells, especially CD4⁺ T cells and macrophages, in the CNS. Thus, encephalitogenic T cells were converted from a pathogenic Th1 to a protective Th2 phenotype that was clearly capable of preventing CNS immune infiltration and clinical disease. Future studies will address the ability of this vaccination strategy to induce long-term changes in cytokine environment and whether it can treat ongoing disease in a treatment paradigm.
CHAPTER FOUR

MUCOSAL VACCINE DEVELOPMENT AGAINST ENTEROTOXIGEIC (ETEC) - AND ENTEROHEMORRHAGIC (EHEC) *E.Coli*

Intranasal ETEC Vaccine Targeting

Introduction

In the early 1970s, several diarrhea causing *E. coli* were isolated in humans with various serotypes, producing an enterotoxin similar to cholera toxin (252-254). Later, two species of cholera toxin-like enterotoxins were determined and designated as heat stable enterotoxin (ST) and heat labile enterotoxin (LT). The ST is stable in boiling for 30 minutes, while the LT is destroyed by such treatment (221-223, and 255). The *E. coli* strains producing either one or both toxins were classified as ETEC.

LT is 85kD protein consists of a single 240 residue A subunit and five identical B subunits (222, 223, and 256). The specific binding of B subunits to GM1 glycolipid ganglioside on the brush border of small intestine form a pentameric structure with a central pore in which the C-terminal tail of the A subunit extends to hold the A/B5 holotoxin together (256-258). The A subunit functions as a permanant adenylate cyclase activator and results in the increase of cyclic AMP (cAMP) levels in the enterocyte which causes loss of electrolytes, as well as a water, into intestinal lumen (259). The ST is 18 amino acids, 18 kD small protein (221, 260). The binding of ST to the brush border of the host initiates the activation of guanylate cyclase with concomitant increase of intracellular cyclic GMP (cGMP) levels (221, 260). This chain reaction causes the lowering of the sodium and chloride levels in cytosol, and resulting in fluid loss (221, 261).
Along with toxin production, ETEC virulence depends on the presence fimbrial adhesins or colonization factors, which confer binding and colonization of bacteria. Fimbriae provides tissue tropism and host specificity. For example: K88 is porcine-specific, K99 is for bovine- and porcine-specific, and CFAs are human-specific (250, 262). The receptors for the fimbriae tend to be very specific for carbohydrate or glycolipid moieties (150, 263).

ETEC is an important cause of diarrhea in humans as well as in animals. In developing countries, ETEC contributes to 380,000 infant deaths annually (264) and causes diarrhea for travelers to endemic areas (220, 264). For animals, it is one of the leading causes of diarrhea scours for calves and piglets and is a considerable factor in mortality of piglets and newborn calves (250). Previous studies show that immunization with cholera toxin subunit B or ST-LT conjugate confers protection against ETEC in human and animal studies (265-267). Fimbrial adhesins were also investigated as a vaccine target. Oral immunization of calves with purified K99 failed to induce significant immune responses (151), and volunteers immunized with purified CFAs failed to protect against ETEC challenge in human trial (152). In addition, oral vaccination with microsphere-encapsulated purified CFA/I, which is designed to protect antigen from the gastric environment, induced strong and sustained antigen-specific serum IgG, but rarely induced protective S-IgA (153). Thus, novel delivery systems are needed to protect antigens from gastric environment, as well as for proper targeting to induce protective immunity. Previous studies in our lab show that oral *Salmonella* vaccine vector with CFA/I (162) or K99 (250) elicits strong antigen-specific serum IgG and S-IgA, and confers protection against ETEC challenge (250). Another mucosal targeting means was also investigated in our lab with reovirus protein σ1. Reovirus is an enteric pathogen that infects host through the M cell binding (301), and the binding is mediated by reovirus adhesin, protein σ1.
Previous studies showed that the recombinant protein σ1 binds to its receptor on rodent and human cell lines (251) and nasal-associated lymphoid tissue (NALT) M cells (302). Intranasal (i.n) immunization with a protein σ1 polylsine (PL) model DNA vaccine complexes elicited enhanced S-IgA and cytotoxic T lymphocyte responses when compared to naked DNA immunization (302, 303).

Intranasal (i.n) immunization has several attractive features over conventional vaccination. It has proven to be effective for inducing both upper and lower respiratory immunity, as well as distal mucosal immunity with less vaccine requirement and higher S-IgA induction when compared to conventional immunization (304-307). Thus, we hypothesize that i.n vaccination with K99 major structural subunit, FanC, as a protein σ1 recombinant conjugate can elicit protective S-IgA responses against ETEC challenge.

Results

Plasmid Construction for FanC/protein σ1 Fusion Vaccine and Its Expression in E.coli.

To obtain the protein σ1/FanC fusion protein, we cloned single- (p1FS) or 3x fanC (p3FS) under N-terminus of protein σ1 to exploit C-terminus M cell targeting property. Sequencing (data not shown) and restriction analysis confirms the adequate construction of p1FS or p3FS. Fig 4.1 describes the proposed p1FS and p3FS restriction map and representative p1FS restriction analysis. However, we experienced difficulties in purification of FanC/protein σ1 fusion protein from the E. coli expression system due to the formation of inclusion bodies.
FanC/protein σ1 Plasmid Construction for Yeast Expression. To overcome the problem of inclusion body formation, we adapted yeast (*Pichia pastoris*) expression system to exploit its slow and regulated protein expression. To obtain the yeast clone that expresses FanC/protein σ1 fusion vaccine, we cloned *fanC/protein σ1* into yeast expression vector, pPICZ A, multi-cloning sites (EcoRI and Kpn I, Fig 4.2A), and named it as pPI1FS. Positive yeast clones were selected on zeocin/YPD plates. The successful expression of FanC/protein σ1 fusion vaccine was determined by SDS-PAGE and Western blotting probed with a monoclonal anti-K99 antibody. Fig 4.2 describes the restriction map of yeast expression vector, pPICZ A, and *fanC/protein σ1* yeast expression construct, pPI1FS, and its expression. However, despite expression in yeast, the suspected malformed protein conformation disallowed any anti-K99 antibodies to be induced. Thus, we terminated this project.
Figure 4.1: Construction of protein FanC/protein $\sigma_1$ expression plasmid. (A) represents the proposed plasmid map for single- or 3x FanC/protein $\sigma_1$ fusion construct. (B) shows the restriction digestion of p1FS and confirms appropriate DNA fragments.
Fig 4.2: Construction of yeast expression vector and its expression. (A) Yeast expression construct pPI1FS constructed by cloning \textit{fanC/protein \sigma 1} gene into pPICZ A multi cloning site (MCS) (EcoRI/Kpn I). (B) FanC/protein \sigma 1 fusion protein was purified by using Ni\textsuperscript{++} -NTA column chromatography and confirmed with SDS-PAGE and Western blotting with K99-specific monoclonal antibody. Lane 1; blank, lane 2; purified FanC/protein \sigma 1.
Live Vaccine for EHEC

Introduction

In late 1977, “Vero” cell active cytotoxin, which was distinct from ST and LT, was identified from several *E. coli* strains (268). Later, the Vero cell cytotoxin (VT) producing *E. coli* and its association with hemolytic uremic syndrome (HUS) and hemorrhagic colitis were determined, and it was identified as O157:H7 serotype (269-271). The EHEC VT was found to be similar to Shiga toxin from HUS-causing *Shigella dysentriae* type 1, and thus was called “shiga-like” toxin (SLT) (272, 273). Two different SLT were identified: SLT I and SLT II (273, 274).

SLTs are phage encoded and consist of 6 subunits, 1x A and 5x B (274). SLTs act on protein synthesis. The binding of pentameric B subunits to globotriaosylceramide (Gb3)-lipid complex on cell surface causes endocytosis of SLT (275-277). Once in cytosol, the A subunit is released, and targets to ribosome, N-glycosidase activity of A subunit cleaves N-glycosidic bond of the adenine (position: 2324) of 28S ribosomal RNA (rRNA), and causes subsequent inactivation of 60S ribosome (278). Previous research showed that the primary target for SLTs is the vascular endothelium (279-282). In fact, the first signal of EHEC infection is bloody diarrhea due to the failure of renewing endothelium in gut resulting from protein synthesis inhibition by SLTs, which eventually lead to a breakdown of lining and hemorrhage (283). In severe cases, SLTs target to glomerular endothelial cells of the kidney and cause HUS (281, 282).

Another virulence factor of EHEC is intimin, encoded by the *eae* gene in the chromosome (284), producing an attaching and effacing (A/E) region in the small intestine brush border (285). Intimin is also found in *Citrobacter rodentium* (286), which causes transmissible murine colonic
hyperplasia in laboratory mice (287). Mutation of eae causes the failure of colonization and disease induction by EHEC or C. rodentium (284, 288, and 289).

Cattle have been identified as a major reservoir of EHEC (290-292). Deer (293), sheep (294), and water (295-297), which is contaminated by EHEC from the fecal waste of cattle, are minor ones. Ground beef has been responsible for cases of EHEC-related disease outbreaks (298). The infectious dose in a food-born outbreak of EHEC is very low (2-2000 CFU) due to its acid tolerance (299). Thus, preventing the colonization of EHEC in cattle intestine has been considered as the most effective means of preventing human disease. However, past experiments have shown that cattle preexposed to EHEC were not protected from reinfection, even with prompt and sustained Ab production to EHEC’s LPS and SLT I (300). This lack of protection is ascribed to poor mucosal colonization of EHEC in adult cattle as evidenced by the lack of attaching/effacing region (291, 292), and it is conceivable that the specific mucosal immune responses were not efficiently stimulated by natural infection. Thus, we hypothesized that by adapting the targeting capabilities of a live vector system with an EHEC-specific antigenic epitope, we will be able to effectively stimulate mucosal tissues for optimal induction of S-IgA for protection against EHEC colonization.

To express EHEC antigenic epitope on the Salmonella vaccine, we exploited K99 fimbriae, which was described previously (250), as a molecular scaffold. The K99 operon is encoded by a virulence plasmid consists of 8 different genes, referred to as fan ABCDEFGH. These genes have unique functions in the biogenesis of K99 fimbriae, such as positive regulator (A and B), major fimbrial protein (C), translocator (D), molecular chaperon (E), and minor fmbrial subunits (F, G, and H). Previous studies by other groups have shown that mutation in fanG or fanH had no influence on the
formation of K99 fimbriae (308). Therefore, we decided to clone the EHEC epitope to a selected region of \textit{fanH}.

\textbf{Results}

\textbf{Expression of EHEC LPS Peptide Mimetic on Salmonella Vaccine Vector.} The consensus peptide sequence that mimics EHEC LPS structure was identified by LigoCyte Pharmaceuticals (Bozeman, MT) by using a phage display system. Fig 4.3 shows the EHEC peptide mimetic sequence.

\begin{verbatim}
11: GRGQFEGLAA
14: DVTGQFLGLAA
15: NQSGQFVGLAR
22: ADNGQFFELGR
CON: SGQFXGLARV
\end{verbatim}

Fig 4.3: EHEC peptide mimetic sequence. Following three rounds of affinity selection and amplification, immunoreactive sequence was selected that bind specifically with EHEC LPS-specific monoclonal EC114. Four representative clones are shown with a numerical designation. From these, a consensus (con) sequence was identified (LigoCyte Pharmaceuticals, Bozeman MT).

To clone and express this mimetic, we first analyzed prokaryotic codon usage by using the \textit{E. coli} Codon Usage Analysis 2.0 program (Morris Maduro) and identified optimal sequences for prokaryotic expression (TCT GGC CAG TTC GAA GGC CTG GCG CGT GTT GAA TCT TGC). The oligonucleotides were synthesized and annealed \textit{in vitro} and cloned into the K99 fimbriae minor structural protein gene \textit{fanH}. Fig 4.4 shows the EHEC LPS peptide mimetic expression construct (pFH:LPS). The cloning was confirmed with sequencing (data not shown), restriction analysis, and Western blotting with polyclonal anti-K99 Ab. Fig 4.5 shows the restriction-
and Western blot analysis. Restriction digestion with Nsi I reveals the corresponding size increase of LPS mimetic sequence insertion. Western blot with polyclonal anti-K99 Ab tells us that the mutagenesis in the \textit{fanH} gene with LPS mimetic sequence did not influence the formation of K99 fimbriae and suggested that this region can be exploited as a further cloning site for other antigenic epitopes. The \textit{Salmonella} vaccine with pFH:LPS is designated as JY002.

\textbf{Antibody Responses by \textit{Salmonella} Vaccine with Chimeric K99/LPS Peptide Mimetic.} To of the EHEC peptide mimetic, we immunized mice with $5 \times 10^9$ (Fig 4.6 \textit{A}) or $1 \times 10^{10}$ (Fig 4.6 \textit{B}) CFU of \textit{Salmonella} vaccine (JY002). Both low and high dose regimens elicited significant serum IgG titers toward K99 fimbriae, as well as the LPS mimetic, but failed to induce S-IgA against the LPS mimetic. Even boost with $5 \times 10^9$ CFU at week 5 in low dose immunization did not influence S-IgA induction (Fig 4.6 \textit{A}: indicated by the arrow). The importance of S-IgA for the protection of the colonization of EHEC was previously described (300). Thus, without eliciting LPS mimetic-specific S-IgA, we terminated further studies.
Figure 4.4: Cloning of EHEC LPS peptide mimetic into K99 minor structural gene fanH. Briefly, 
fanH gene was amplified with fanH Sac I-forward (TAG AGC TCA AAA CCT TTT GAG CGG) 
and Hind III-reverse (TAA AGC TTT GAA TTT CTT ATT CCC CTC) primers using pMAK99-
asd as a template. The amplified fanH fragment was cloned into pUC18 vector, and designated it as 
pFanH. Further, the pFanH was transferred into MH6083 E.coli strain (dam) to obtain 
unmethylated Bcl I EHEC LPS mimetic cloning site because Bcl I restriction enzyme is 
methylation-sensitive. The corresponding peptide mimetic nucleotides were annealed in vitro 
(contain Bcl I site and Bcl I-treated) and inserted into Bcl I-treated pFanH: pFanH:LPS. Then, the 
LPS mimetic containing fragment of pFanH:LPS was amplified (fanH Nsi I-forward: TAT ATG 
CAT ATC AAA ACC TTT TGA GCG, fanH Nsi I-reverse: TAT ATG CAT TGA ATT TCT TAT 
TCC) and cloned into TA vector: pTA/fanH:LPS. The Nsi I-treated LPS mimetic-containing 
fragment from pTA/fanH:LPS was replaced with corresponding Nsi I fragment from pMAK99-asd, 
and resulted in pFH:LPS.
Figure 4.5: Restriction analysis of pFH:LPS and expression of chimeric K99 fimbriae in Salmonella vaccine, JY002. There are two Nsi I restriction sites in pMAK99-asd+ plasmid, located in 5899 and 6466 of K99 operon from the starting codon. Restriction digestion of pMAK99-asd+ and pFH:LPS shows size difference corresponding to the cloned LPS peptide mimetic nucleotides (A). Lane 1; pMAK99-asd+, lane 2, 4; pFH:LPS, lane 3; self-ligated Nsi I treated pMAK99-asd+. The chimeric K99 expression was confirmed by probing and developing with polyclonal rabbit anti-K99 serum and goat anti-rabbit IgG HRP-conjugated antibody (B). Lane 1, 2; K99, lane 3; chimeric K99 with LPS mimetic.

Figure 4.6: Immunogenecity of LPS peptide mimetic. (A): $5 \times 10^9$ JY002 CFU dose and arrow indicates the boost at wk 6 with same amount of CFU. (B): $1 \times 10^{10}$ CFU vaccination. ELISA plates were coated with EC-OVA (ovalbumin-LPS mimetic peptide conjugates) and purified K99 fimbriae.
Conclusions

In an effort to develop a smart subunit vaccine for ETEC with exploitation of reovirus adhesin protein σ1, initially, we made recombinant ETEC K99 fanC/protein σ1 expression plasmid in *E. coli*. When this fusion protein expressed in *E. coli*, it formed an inclusion body, thus making it difficult to purify for i.n immunizations. To overcome this obstacle, we turned to a yeast expression system and obtained FanC/protein σ1 fusion protein without forming inclusion bodies. However, i.n. vaccination with this protein failed to elicit any immune responses supposedly due to the malformation either in FanC or protein σ1. The FanC/protein σ1 binding assay to nasal epithelial cells would tell whether protein σ1 retains its mucosal targeting capacity or not when it presents in chimeric form. Further, above binding assay also could show protein σ1’s influence on antigenecity of target proteins. We temporarily halted this work at this point.

Using another approach to induce S-IgA to inhibit EHEC colonization, we cloned and expressed the peptide mimicking EHEC LPS structure on *Salmonella* vaccine using ETEC K99 fimbriae as a molecular scaffold. Cloning of peptide mimetic into K99 minor structural gene, *fanH*, did not influence for the formation of K99 fimbriae. However, immunization with *Salmonella* having chimeric K99 fimbriae (K99 with LPS mimetic) failed to elicit S-IgA against the peptide mimetic, but induced serum IgG. At this point, we stopped further investigation, because without peptide-specific S-IgA induction, inhibition of EHEC colonization can not be expected.
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