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Tolerance in the Absence of Autoantigen

D.W. Pascual[*],

J. Ochoa-Repáraz,

A. Rynda,

X. Yang

Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717, USA

Abstract

Regulatory T (T_{reg}) cells show promise for treating autoimmune diseases, but their induction to elevated potency has been problematic when the most optimally derived cells are from diseased animals. To circumvent reliance on auto-antigen reactive T_{reg} cells, stimulation to vaccine antigens (Ags) may offer a viable alternative while maintaining potency to protect against proinflammatory diseases. Our *Salmonella* vaccine expressing colonization factor Ag I (CFA/I) possesses anti-inflammatory properties, evident by elevated Th2 cell responses, reduced inflammatory cell infiltrates in the Peyer's patches, and an absence of proinflammatory cytokine production by infected macrophages. Given these findings, we hypothesized whether this vaccine would be protective against experimental autoimmune encephalomyelitis (EAE). As such, *Salmonella*-CFA/I protected in both prophylactic and therapeutic paradigms against proteolipid protein ($PLP_{139-151}$)-mediated EAE in SJL mice. The protected mice showed significantly reduced clinical disease and subsequent resolution when compared to PBS-treated controls. Histopathological studies showed reduced demyelination and no inflammation of spinal cords when compared to PBS- or *Salmonella* vector-treated mice. To ascertain whether the observed immune deviation was in part supported by T_{reg} cells, analysis revealed involvement of $FoxP3^+$ $CD25^+$ $CD4^+$ T cells. Adoptive transfer of induced TGF-β^+ T_{reg} cells from vaccinated mice showed complete protection against $PLP_{139-151}$ challenge, but not by naive T_{reg} cells. Partial protection to EAE was also achieved by the adoptive transfer of $CD25^-$ $CD4^+$ T cells, suggesting that Th2 cells also contributed. Thus, these data show that T_{reg} cells are induced by oral vaccination with *Salmonella*-CFA/I contributing to the efficacious treatment of autoimmune disease.

Keywords

Vaccine; regulatory T cells; Th1\Th2\Th17 cells; EAE; bacteria

[*]Address correspondence to this author at the P. O. Box 173610, Bozeman, Montana 59717-3610, USA; Tel: (406) 994-6244; Fax: (406) 994-4303; dpascual@montana.edu.

INTRODUCTION

Salmonella, a gram-negative, rod-shaped bacterium, is responsible for enteric disease in humans and livestock. *S. enterica* serovar Typhi, is the principle serovar responsible for typhoid fever in humans, and remains a major health threat with >16 million cases and 600,000 deaths/year [1]. Although *S.* Typhi is not the natural host in experimental animals, much of the work to understand *Salmonella* infections relies upon studies using the related *S. enterica* serovar Typhimurium which is infectious in rodents and cattle. *S.* Typhimurium is commonly used in murine studies [rev. in 2, 3] producing mostly a systemic infection in rodents. Despite the debate over the adequacy of this model, both Typhi and Typhimurium infect subsequent oral ingestion. Attenuated strains of both *Salmonella* serovars have been developed as vaccines [4, 5], as well as carriers of heterologous vaccines [6].

S. Typhimurium is an intracellular pathogen that infects antigen-presenting cells, e.g., macrophages [2, 7] and dendritic cells [8, 9], and ultimately disseminates throughout the host upon entering the thoriac duct and spreading *via* the efferent lymph into circulation causing a bacteremia. In the phagocytic cells, as well as other cell types, that *S.* Typhimurium persists and replicates. Consequently, the innate immune system is important to initially limit the *Salmonella* infection, as evident by the phagocytosis of *Salmonella* both by macrophages and PMNs [10, 11]. Natural killer cells also become stimulated to produce the initial IFN-γ response needed to activate macrophages' [12, 13] bactericidal mechanisms by reactive oxygen and nitrogen intermediates [11, 14]. While clearly the innate arm of immunity is responsible for the clearance of *Salmonella*, ultimately, long-term immunity is CD4 T cell-dependent, which is evident by the inability of TCRαβ-deficient mice [15] or nude mice [16] to control *Salmonella* infections. Control of *Salmonella* infection is IFN-γ-dependent since IFN-γ-deficient mice succumb even to attenuated *Salmonella* vaccine strains [17,18].

Multiple sclerosis (MS) is a human autoimmune degenerative disease associated with inflammation and destruction of the central nervous system (CNS) white matter contributed by autoreactive T cells [19–21]. The frequency of MS is thought to be gender-, and age-dependent. The first manifestation of clinical symptoms is often seen during young adulthood, affecting females twice as often as males [22,23]. Although the etiology of MS remains unknown, genome-wide studies reveal that susceptibility to MS is linked to genes in the major histocompatibility complex (MHC) on chromosome 6 [24–26]. Susceptibility to MS is not entirely a genetic predisposition; however, the likelihood of inheriting the disease is greater than 1:50, and only about 20-30% of identical twins will both develop MS [27,28]. Environmental factors may also contribute to MS, possibly, *via* molecular mimicry in which inappropriate cross-reactivity occurs between foreign and self-antigen [29,30]. This cross-reactivity is usually associated with the conserved structural motifs shared by both pathogen and host [23,29,31,32]. From the clinical perspective, MS can be categorized as either relapsing-remitting MS (RRMS observed in vast majority of patients) or primary progressive MS (PPMS) [21, 23]. In 30% of the cases, RRMS progresses into the secondary chronic progressive state. The chronic phase responsible for the majority of MS pathology [33] is due to degeneration of the both the myelin sheath synthesized by oligodendroglial cells and the underlying axons [23].

Rodent models have been developed that mimic MS, which is referred to as experimental autoimmune encephalomyelitis (EAE) [rev. in 34–40], and generally require immunization with myelin peptides or proteins. EAE is a T cell-dependent disease, and these encephalitogenic T cells secrete Th1-type cytokines, including IFN-(and IL-2, which are responsible for local activation of macrophage and microglial cells. Chemokine production by these mononuclear cells results in further recruitment of inflammatory cells into the CNS (23). Inflammatory cells infiltrate from peripheral lymphoid tissues into the CNS resulting in demyelination (21, 41–43). More recently, however, IL-17 has been implicated as the major inducer of EAE [44–47].

Herein this review, we will describe how it is possible to vaccinate against inflammatory diseases, such as EAE, and confer protection, as recently described [48]. We found that the expression of colonization factor antigen I (CFA/I) fimbriae from enterotoxigenic *E. coli* (ETEC) by an attenuated *Salmonella*-CFA/I [49, 50] exhibits anti-inflammatory properties [50, 51]. Based upon these findings, we hypothesized that oral immunization with *Salmonella*-CFA/I could behave as an anti-inflammatory vaccine and may prevent onset and treat inflammatory disease such as EAE. As such, it was found that oral immunization with *Salmonella*-CFA/I could prevent EAE *via* immune deviation by the production of IL-4, IL-10, and IL-13 [48]. The potential of this approach is that treatment of inflammatory diseases may be feasible in the absence of auto-antigen (auto-Ag), eliminating the need for defining the auto-Ag, especially if the auto-Ag is not known, or in some cases, it may eliminate the need to custom design vaccines for patients with auto-active MHC.

A. LIVE ORAL *SALMONELLA* VACCINES

Live *Salmonella* Vector Vaccines Stimulate a Predominance of Th1 Cells

Reproducibly, live oral *Salmonella* vaccines confer protection through Th1-driven cell-mediated responses, such as IFN-γ-secreting $CD4^+$ T cells and delayed-type hypersensitivity [52–57]. As anticipated, *Salmonella* vaccine vectors elicit similar cellular responses against passenger (vaccine) antigens. Early studies have demonstrated the efficacy of adapting *Salmonella* vaccine vectors for protection against intracellular pathogens [rev. in 2, 3]. The advantage of *Salmonella* vectors is that these are Th1 cell-biased, as evident by the production of IFN-γ following oral immunization [55,56,58,59]. Strikingly, no detectable levels of Th2-type cytokines are noted, and the induced Th1-driven cellular responses conferred protection against challenge, showing the shared requirement for Th1 cell-dependent immunity. This is exemplified by data showing that an orally delivered *Salmonella* vector expressing *Leishmania major* gp63 engendered protection against *L. major* in highly susceptible BALB/c mice [58, 60] and against other intracellular pathogens [61–64].

Mucosal $CD4^+$ T cell responses to passenger antigens were also predominated by T cells displaying a Th1 cell phenotype, as shown in our recent studies with tetanus toxoid [56]. Mice orally immunized with an attenuated *Salmonella* vaccine carrying the *toxC* gene (fragment C of tetanus toxin) induced a mucosal Th1 cell response to tetanus toxoid, in contrast to Th2 cell responses obtained for mice orally immunized with tetanus toxoid and the mucosal adjuvant, CT. Isolated Peyer's patches (PP) $CD4^+$ T cells produced IFN-γ and

IL-2, but no IL-4, in response to *in vitro* restimulation with tetanus toxoid [56]. Thus, the mode of vaccine delivery will elicit distinct subsets of mucosal $CD4^+$ T cells compared to those that developed after oral immunization with soluble antigens [56,65,66]. Moreover, *Salmonella*-elicited S-IgA responses are IL-4-independent and can develop in IL-4-deficient mice [67].

Immunogenicity of Passenger Antigens Varies With Antigen Placement in *Salmonella* Vector

Aside from the amount of *Salmonella* vaccine vectors given, foreign antigen dose is controlled by two additional factors: *in vivo* stability and expression. With respect to the former, there are well-described systems available that genetically stabilize foreign expression cassettes in the absence of antibiotic selection markers, including chromosomal integration systems [68,69] and balanced-lethal stabilization systems [49,70,71]. Foreign gene expression levels in *Salmonella* have also been successfully manipulated by altering promoters [59,72–75] and foreign gene copy number [59]; the combination of these approaches increased foreign antigen expression to levels as high as 5 - 10% of the total vector protein [59] and demonstrated a directed relationship between the foreign antigen expression level and immunogenicity [59]. More recently, to circumvent the use of antibiotic selection, an operator-repressor titration system was used to achieve stable and high level expression of *Yersinia pestis* F1-Ag in *Salmonella* [71].

Another factor that can contribute to poor immunogenicity by *Salmonella* vectors is how the passenger antigens are displayed, which is usually in the cytoplasmic compartment of the vectors [76]. Some foreign antigens are highly protease resistant and probably display a long half-life in the bacterial cytoplasm, such as ToxC, and are immunogenic in this compartment [56]. However, high-level expression of eukaryotic and viral proteins in the bacterial cytoplasm commonly produces inclusion bodies and a shorter product half-life [77], a problem, which, in many cases, is alleviated by secretion [61].

B. ENTEROTOXIGENIC *E. COLI* (ETEC) AND THEIR COLONIZATION FACTOR ANTIGENS (CFA)

Virulence by enterotoxigenic *E. coli* is in part contributed by the acquisition of the plasmid for the pili or fimbriae colonization factor antigens (CFA), which enhances the colonization of *E. coli* in the gastrointestinal tract. The CFA pili are a heterogenous group of fimbrial adhesins responsible for adherence to small intestinal epithelial cells *via* their fimbriae or long, hair-like projections extending from the bacterial cell surface [78]. A number of potential ligands for CFA/I fimbriae have been identified, including epithelial mannose-containing glycoproteins [79], GM2 gangliosides [80], and more recently described glycosphingolipids [81]. This adherence appears to be host-specific for human intestinal epithelium [79], but possibly could interact with other animal glycoproteins [79,80] and glycolipids [81].

Oral delivery of CFA/I or CFA/II fimbriae fails to induce significant serum IgG antibodies (Abs) or S-IgA Abs [82]. As a result of poor anti-fimbriae Ab titers [78,83], the human

volunteers were not protected when challenged with pathogenic ETEC [83]. Despite neutralization of stomach acidity in these subjects [84], poor S-IgA anti-CFA Ab responses were obtained; later, it was shown that gastric proteases altered the CFA fimbriae antigenicity even at a neutral pH [84]. These studies indicated that in order to obtain protective mucosal immune responses to CFA fimbriae, the antigenic properties of the pili must remain intact. Microencapsulation of CFA/I was shown to overcome potential deleterious effects of the stomach when they were administered intragastrically to rabbits [82]; however, minimal serum and fecal IgA anti-CFA/I Abs could be detected. In a separate study, direct administration of microencapsulated CFA/II into the rabbit duodenum was able to elicit CFA/II-specific antibody-forming cell responses in the PP and spleens [85]. An effective vaccine for ETEC still remains elusive despite human trials with heat-killed ETEC given with cholera toxin B subunit [86].

The use of attenuated, live vectors has proven to be an effective means to deliver antigens to mucosal inductive sites of the small intestine [87–90], presumably through the PP and subsequent dissemination into systemic lymphoid tissues [3,87,88,91,92], where mucosal and systemic immune protection can be induced. Along these lines, to develop an effective vaccine against ETEC, a prototypic *Salmonella* vaccine was generated capable of eliciting both neutralizing mucosal and systemic Abs against CFA/I-expressing ETEC [49]. An attenuated, balanced-lethal *ΔaroA Δasd S.* Typhimurium vaccine carrying an *asd*$^+$ plasmid encoding the CFA/I operon was produced [49] that mimicks native ETEC fimbriae expression on the *Salmonella*'s cell surface [93]. Studies showed that the CFA/I fimbriae structural integrity [93] was sufficiently retained to prevent wild-type ETEC infection of human intestinal Caco-2 cells. Our studies have shown that the *Salmonella*-CFA/I vaccine is capable of inducing rapid, elevated Ab titers to CFA/I fimbriae [49,50]. Interestingly, the observed immune response proceeded *via* a Th2 cell-dependent phase [50], unlike that observed in previous studies where Th cells' responses to *Salmonella* expressed antigens were predominantly IFN-γ-dominated [52–60,63]. Such findings suggested that the expression of CFA/I fimbriae, possibly because of cell surface expression, influenced how the host recognizes this vaccine. Subsequent *in vitro* studies revealed that infection of macrophages with *Salmonella*-CFA/I failed to elicit proinflammatory cytokine, i.e., IL-1α, IL-Iβ, IL-6, and TNF-α, production unlike its isogenic *Salmonella* vector strain which elicited these proinflammatory cytokines with as few as one bacterium/80 macrophages [51]. This absence of proinflammatory cytokine production was not attributed to differences in *Salmonella* colonization nor to increased macrophage cell death [51]. Thus, it appears that the *Salmonella*-CFA/I exhibits anti-inflammatory properties.

C. EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

One model of T cell-dependent inflammatory disease is EAE, a highly reproducible disorder in rodents that can be induced by immunizing susceptible strains with TCR-reactive peptides [rev. in 34–40]. SJL mice show susceptibility to proteolipid protein (PLP) peptide$_{139\text{-}151}$, HSLGKWLG HPDKF, [39,94,95] and myelin oligodendrocyte glycoprotein (MOG) peptide$_{92\text{-}106}$, DEGGYTCFFRDHSYQ, [96], whereas C57BL/6 mice show susceptibility to MOG$_{35\text{-}55}$, MEVGWYRSPFSRVVHLYRNGK, [36,39,97,98]. T cell recognition of these peptides eventually causes a progessive demylination [34,39] that is

further amplified by inflammatory macrophage [36,40,99–101] and neutrophil infiltration [102–107]. In these mouse strains, EAE manifests as an ascending disease in the spinal cord where initial symptoms begin as a limp or paralyzed tail, followed by rear leg paralysis that can eventually progress into forearm paralysis [108]. In SJL mice, PLP-induced EAE can eventually develop into a chronic relapsing disease. It is also believed that because of this demyelination, new epitopes become exposed, and exposure of these neoantigens acts as an immunization process, thereby causing further epitope spreading of this disease [37,109–111]. Protection to EAE appears to be Th2 cell-dependent since IL-4 [112–115], IL-10 [110,111,116–120], and TGF-β [112,114,121–124] can reverse or prevent EAE. While IL-12 can promote Th1 cell development, studies have shown that the proinflammatory promoting cytokine, IL-23, is primarily responsible for encephalitogenic T cell development in EAE [101,125,126]. The proinflammatory cytokines, IFN-γ [106,119,127,128] and TNF-α [101,119, 124,125,127–131] can effect disease primarily *via* Th1 cells. Interestingly, IFN-γ can be protective in EAE if introduced directly into the CNS [132], or it can make resistant mice susceptible to EAE when IFN-γ is genetically deleted [106, 133], suggesting that IFN-γ is protective by limiting neutrophil infiltration and activation [106]. IL-17 is believed to be the principle mediator of the inflammation observed in EAE [44–47]. Since IL-23 is the major inducer of inflammation in EAE, more so than IL-12 [101,125,126], IL-23 was shown to stimulate IL-17-producing T cells, which are regulated by both IL-4 and IFN-γ [44]. Neutralization of IL-17 was shown to be protective [44–47], and protection to EAE can also be mediated *via* T_{reg} cells [111,114,120,124,134].

D. REGULATORY T CELLS

Tolerance is the inability or diminished capacity of the immune system to react to defined Ags [rev. in 135–137]. Tolerance is important to eliminate self-reactivity, and when this resistance is broken, autoimmune disorders can occur. How tolerance is induced dictates the mechanism or type of tolerance produced. In reference to past studies, two types of tolerance have been described based upon Ag dose administered. The low-dose tolerance favors the induction of regulatory T cells that leads to active suppression either by Ag-specific or Ag-nonspecific mechanisms [137]. In contrast, high dose tolerance is believed to result in T cell anergy. PP are thought to be required for induction of tolerance [135, 138,139] based upon the findings that treatment of female mice with soluble lymphotoxin-β receptor-Ig during gestation results in the disruption of the peripheral LN and PP in their offspring, leaving the mesenteric, sacral, and cervical LN intact [140]. Offspring of such PP-null mice subjected to high-dose OVA oral tolerance regimen do not respond to peripheral OVA challenge [138], yet, the lymphotoxin-β-deficient mice, in which mesenteric LN are intact but lack PP, are able to show tolerance [141] unless the mice are also deficient in mesenteric LN [142]. Whether this dependence is related to the actual lymphoid structures, or to the dendritic cells [143] required for tolerance induction, remains to be determined.

Considering recent observations for stimulating tolerance, specific mechanisms have been identified to define how tolerance can be maintained [rev. in 135], including Th3 cells producing TGF-β, $T_{regulatory}$ (T_R) 1 cells that produce IL-10, and T_{reg} ($CD25^+$ $CD4^+$ cells preventing autoreactivity, $T_{suppressor}$ cells, and γδ T cells [144]. Weiner [137] refers to a specific subset of T_R cells,Th3 cells, which produces TGF-β to actively suppress Ag-specific

T cell responses and has been shown to prevent EAE in SJL mice [145]. Whether this suppression is due to TGF-β or IL-10 has led to another defined T_R cell subset based upon its production of IL-10 observed in murine colitis models [146]. Since IL-10 inhibits the proinflammatory pathway that supports hapten-induced colitis, it is believed that the observed colitis in the absence of IL-10 may be due to inappropriate regulation of TGF-β [147]. More recently, a considerable focus has been on $CD25^+$ $CD4^+$ T cells originally described for the development of immune diseases in thymectomized 3 day-old mice [148]. While not all $CD25^+$ $CD4^+$ T cells are T_{reg} cells, a subset of these does exhibit suppressor activity [rev. in 149]. A $CD8^+$ equivalent to T_{reg} cells has also been recently described and defined as being $CD8^+$ $CD122^+$ (IL-2/IL-15 receptor β chain) and does not require antigen-presenting cell intervention [150,151].

D. HOW DOES ORAL IMMUNIZATION WITH A *SALMONELLA* VACCINE PROTECT AGAINST A PROINFLAMMATORY DISEASE?

As previously described, *Salmonella* vaccine vectors are adept to delivering vaccines to the mucosa [2,3,6,91,92]. Typically, these vaccines are given orally and enable immunization of the mucosal immune system. Because of the large surface area in the gut, it is able to stimulate mucosal B and T cell responses. In addition, the peripheral immune compartment also becomes responsive to the expressed vaccines with the end result of immunity in both mucosal and systemic tissues becoming immunized. Due to their versatility, attenuated *Salmonella* vectors have been selected to express ETEC fimbriae [49,93] to circumvent the fimbriae-sensitive environment of the gut [82–85], which denatures the fimbriae preventing subsequent immunization.

In our experimental systems, the *Salmonella*-CFA/I construct is highly immunogenic, stimulating long-lived IgA antibody-producing cells subsequent to its oral immunization with this live vaccine [48–50]. Interestingly, these induced mucosal IgA responses are supported by both Th1 and Th2 cells stimulated in a biphasic fashion [50]. A robust serum IgG1 and mucosal IgA responses are evident by as early one week post-infection [6,48,50], suggesting that the fimbriae remains sufficiently immunogenic when ferried by the *Salmonella* vector. At this same time point, minimal to no IFN-γ responses are detected in both the mucosal and systemic compartments, yet elevations in Th2 cell cytokines, IL-4, IL-5, and IL-6 are observed [50]. This Th2 cell dominance remains for at least two weeks, but subverts to IFN-γ responses by four weeks post-immunization, and at this time, it appears that Th1 cells dominate presumably to clear the intracellular pathogen. Nonetheless, sufficient memory Th2 cells remain and can be reactivated. It is this Th2 cell bias that is believed to enhance mucosal IgA Ab responses when compared to conventional *Salmonella* vaccines [56].

While it is unclear how such a potent Th2 cell response is induced, evidence suggests that the fimbriae mimics soluble immunization with adjuvant, whether it be for their expression on the *Salmonella*'s cell surface, and/or they are secreted. When a similar approach was adapted to produce a bovine vaccine for K99 ETEC, a scouring disease that afflicts newborn calves and piglets [152], similar elevations in mucosal IgA and serum IgG1 Ab responses

and Th2 cell dominance, were observed in mice orally immunized with *Salmonella*-K99 [153]. Likewise, oral immunization of heifers also showed enhanced IgG1 (equivalent to murine IgG1) anti-K99 Ab titers [154]. To discern the impact of cell surface expression, deletions of the K99 fimbriae gene clusters revealed, that as gene reductions (primarily chaperone genes) in the operon were done to limit cell surface expression, the K99 fimbrial subunit was more likely retained within the vector (not exported to the outer membrane) and resulted in progressive diminution in IgG1 Ab titers with concurrent increases in IgG2a and IgG2b anti-K99 Ab titers [155]. Such mode of (intracellular) vaccine expression is typical of conventional *Salmonella* vaccines [56,58,73,76,77]. Others have shown that Ag secretion [156,157] or cell surface expression [158] can result in the stimulation of IgG1 Ab responses. Thus, the expression of the CFA/I fimbriae does alter the convention by which *Salmonella* vaccine vectors stimulate host immunity producing a mixed Th cell phenotype.

Subsequent efforts have also focused on determining how the expression of CFA/I fimbriae alters host immune responses to this vaccine. The observed Th2 cell bias is not indicative of the *Salmonella* vector since it stimulates Th1-type responses against *Salmonella* antigens [159]. Rather, the expressed CFA/I fimbriae alter host recognition of this *Salmonella* vaccine. Infection studies with murine macrophages revealed that *Salmonella*-CFA/I failed to stimulate proinflammatory cytokine production, unlike its isogenic *Salmonella* vaccine vector lacking the CFA/I operon, which was able to stimulate IL-1α, IL-1β, IL-6, and TNF-α with as few as one bacterium per 80 macrophages [51]. The failure to produce these cytokines was not attributed to preferential increases in IL-10 nor IL-12p40. Infection studies revealed that both *Salmonella* vaccines colonized the macrophages to similar degree. One plausible explanation for these results may be the CFA/I fimbriae interferes with innate immune responses, possibly, by blocking anyone or combination of the normal pathogen recognition receptors, including TLR4, TLR5, CD14, MD2, and LPS-binding protein [160], that would signal the presence of *Salmonella.*

Given this lack of proinflammatory cytokine production [51], we questioned whether *Salmonella*-CFA/I could behave as an anti-inflammatory vaccine and prevent experimental autoimmune diseases. In this regard, SJL mice were tested for their ability to resist development of $PLP_{139-151}$-induced EAE following oral immunization with *Salmonella*-CFA/I [48]. To test its effectiveness, SJL mice were orally vaccinated for one or four weeks to coincide with the peak Th2 and Th1 cell responses, respectively, and then subjected to $PLP_{139-151}$ challenge. In both instances, mice showed only limited disease, and all completely recovered, whereas unprotected mice either succumbed to EAE or exhibited the relapsing disease [48]. Interestingly, mice immunized in a similar fashion with the isogenic *Salmonella* vector also exhibited reduced EAE, but still exhibited greater disease than the *Salmonella*-CFA/I-vaccinated mice. This was evident by the increased demyelination and inflammatory cell infiltration into the CNS when compared to *Salmonella*-CFA/I-vaccinated mice. Protection may have been contributed in part by the immune deviation, as evidenced by the reduced levels of IFN-γ produced with concomitant increases in Th2 cell cytokines, IL-4, IL-10, and IL-13 following restimulation with $PLP_{139-151}$, which contrasted with the responses by similarly treated $CD4^+$ T cells from unprotected or *Salmonella* vector-immunized mice [48] that showed increased IFN-γ and minimal to no Th2-type cytokine production.

The therapeutic potential of *Salmonella*-CFA/I was also recently examined to assess whether it could diminish ongoing EAE [161]. SJL mice were challenged with $PLP_{139-151}$, as previously described, and six days later, they were orally gavaged with *Salmonella*-CFA/I, *Salmonella* vector, or PBS. Mice treated with the *Salmonella* vaccines were able to resolve EAE, but significant inflammation of the CNS still occurred in *Salmonella* vector-, but not *Salmonella*-CFA/I-treated mice, as evidenced in the latter by the lack of neutrophils, macrophages, or T cells [161]. As before, similar increases in the Th2 cell cytokines, IL-4, IL-10, and IL-13 were obtained with concomitant reductions in IFN-γ and IL-17 [161].

In addition to immune deviation, we questioned whether possibly T_{reg} cells may be induced as a consequence of immunization with the *Salmonella* vaccines. Only a few studies have evaluated whether bacterial infections [162,163] or bacterial products, e.g., toxins [164,165] can elicit T_{reg} cells. Indeed T_{reg} cells could be induced, but unlike many viral infections which are able to induce T_{reg} cells at the expense of the host to promote viral infection and persistence [166–168], bacterially induced T_{reg} cells from these limited reports were protective. Thus, we questioned whether the observed protection obtained with *Salmonella*-CFA/I vaccination was T_{reg} cell-dependent. A kinetic study was performed and found that both the *Salmonella* vector and *Salmonella*-CFA/I could elicit $CD25^+$ $CD4^+$ T cells, but the percentage of $FoxP3^+$ T_{reg} cells was strikingly enhanced in mice dosed with *Salmonella*-CFA/I [161]. Upon *in vivo* neutralization of CD25, a loss of protection conferred by *Salmonella*-CFA/I vaccine was observed, suggesting that these T_{reg} cells were important for dampening autoimmunity [161]. Adoptive transfer studies were also conducted with T_{reg} cells, and interestingly, a rank-order of potency was observed: *Salmonella*-CFA/I T_{reg} cells > *Salmonella* vector T_{reg} cells > naive T_{reg} cells [161]. Partial protection was also obtained using the $CD25^-$ $CD4^+$ T cells from *Salmonella*-CFA/I-dosed mice in contrast to the same T cells from *Salmonella* vector-dosed mice that conferred no protection. The *Salmonella*-CFA/I-induced T_{reg} cells produced TGF-β, but less TGF-β was evident with the *Salmonella* vector-induced T_{reg} cells, and instead these produced IL-4 and IL-17 [161]. This finding appears paradoxical since IL-17 is important for EAE development [44–47], but its induction is TGF-β-dependent [169,170]. Nonetheless, our studies showed that oral immunization with *Salmonella*-CFA/I can confer protection *via* the stimulation of TGF-β-producing, $FoxP3^+$ $CD25^+$ $CD4^+$ T cells and independent of auto-Ag.

CONCLUSIONS

T_{reg} cells are sought to aid in the treatment of autoimmune diseases generally by eliciting Ag-specific T_{reg} cells [rev. in 171,172]. Alternative measures of polyclonally stimulating T_{reg} cells have failed clinically with adverse consequences [173]. Thus, there is a need to stimulate T_{reg} cells, preferably Ag-specific; however, such approach is hampered by the variability of human MHC and not knowing the specific peptide reactivity by individuals with autoimmune disease. These limitations may be circumvented by vaccinating with known epitopes to produce an environment suitable for disease-specific T_{reg} cell development. We have observed that oral immunization with *Salmonella*-CFA/I can stimulate T_{reg} cell development independent of auto-Ag, but additional Ag-specific T_{reg} cells are also induced during EAE [161]. The *Salmonella*-CFA/I vaccine showed optimal T_{reg} cell stimulation when compared to its isogenic *Salmonella* vector because

of the differences in potency of the induced T_{reg} cells. It appears the improved potency by *Salmonella*-CFA/I was attributed to its ability to co-stimulate elevated Th2 cells, which by immune deviation, enhanced T_{reg} cell development. Co-stimulation of Th2 cells was not evident upon immunization with the *Salmonella* vector. Thus, the stimulation of both Th2 cells and T_{reg} cells makes the *Salmonella*-CFA/I vaccine able to act as a therapeutic for EAE. Future studies will evaluate the universality of these findings with other autoimmune diseases and will address which early events are important for directing the co-stimulation of Th2 cells and T_{reg} cells by *Salmonella*-CFA/I vaccine.

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ABBREVIATIONS

Abs	Antibodies
Auto-Ag	Auto-antigen
CFA	Colonization factor antigen
CNS	Central nervous system
CT	Cholera toxin
EAE	Experimental autoimmune encephalomyelitis
ETEC	Enterotoxigenic *E. coli*
FoxP3	Forkhead box P3
IFN-(	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
OVA	Ovalbumin
PLP	Proteolipid protein

PMNs	Polymorphonuclear cells
PP	Peyer's patches
PPMS	Primary progressive MS
RRMS	Relapsing-remitting MS
S-IgA	Secretory IgA
TCR	T-cell receptor
TGF-∃	Transforming growth factor-beta
Th	T helper
TLR	Toll-like receptor
TNF-∀	Tumor necrosis factor-alpha
T_{reg}	Regulatory T

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