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REVIEW

Regulatory T-cell vaccination independent of auto-antigen

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To date, efforts to treat autoimmune diseases have primarily focused on the disease symptoms rather than on the cause of the disease. In large part, this is attributed to not knowing the responsible auto-antigens (auto-Ags) for driving the self-reactivity coupled with the poor success of treating autoimmune diseases using oral tolerance methods. Nonetheless, if tolerogenic approaches or methods that stimulate regulatory T (T_{reg}) cells can be devised, these could subdue autoimmune diseases. To forward such efforts, our approach with colonization factor antigen I (CFA/I) fimbriae is to establish bystander immunity to ultimately drive the development of auto-Ag-specific T_{reg} cells. Using an attenuated *Salmonella* vaccine expressing CFA/I fimbriae, fimbriae-specific T_{reg} cells were induced without compromising the vaccine's capacity to protect against travelers' diarrhea or salmonellosis. By adapting the vaccine's anti-inflammatory properties, it was found that it could also dampen experimental inflammatory diseases resembling multiple sclerosis (MS) and rheumatoid arthritis. Because of this bystander effect, disease-specific T_{reg} cells are eventually induced to resolve disease. Interestingly, this same vaccine could elicit the required T_{reg} cell subset for each disease. For MS-like disease, conventional $CD25^+$ T_{reg} cells are stimulated, but for arthritis $CD39^+$ T_{reg} cells are induced instead. This review article will examine the potential of treating autoimmune diseases without having previous knowledge of the auto-Ag using an innocuous antigen to stimulate T_{reg} cells via the production of transforming growth factor-β and interleukin-10.
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COLONIZATION FACTOR ANTIGEN I (CFA/I) FIMBRIAE AND ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC)

ETEC, the causative agent of travelers' diarrhea, is the most common bacterial diarrheal disease of children in Latin America, Asia and Africa,[1] and it is contracted upon ingestion of contaminated food or water. It is estimated that this disease is responsible for 400 million cases annually resulting in 300 000 deaths of preschool children.[2,3] *E. coli* becomes enterotoxigenic upon acquisition of a plasmid or plasmids containing the heat-stable enterotoxin[4] or the cholera-like exotoxin, which is commonly termed the heat-labile enterotoxin (LT).[5,6] Both toxins are responsible for inducing fluid loss and electrolyte imbalance in the host. Facilitating infection and subsequent colonization, ETEC also acquires a plasmid encoding for the pili or fimbriae referred to as colonization factor antigens (CFAs), which mediate 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, hairlike projections extending from the bacterial cell surface to epithelial mannose-containing glycoproteins.[7] This adherence is generally host-specific for intestinal epithelium.[8] While a specific natural receptor for CFA/I fimbriae has yet to be identified in the small intestine, some studies suggest that in eukaryotes a sialylated glycoprotein is the receptor,[9,10] although others suggest that epithelial mannose-containing glycoproteins and/or glycosphingolipids may also serve as receptors.[11,12] The low incidence rates in adults from ETEC-endemic regions have correlated with the presence of anti-LT and anti-CFA antibodies (Abs), suggesting that acquired immunity to these virulence factors are protective.[13] Epidemiological studies show that children aged <3 years from these endemic regions are susceptible to multiple ETEC infections, which may provide for broad-spectrum immunity

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later in life,[14,15] while adult travelers to these endemic regions are unprotected and remain susceptible to infection.[2,3,16,17]

CFA/I fimbriae are an archetype of class 5 fimbriae characterized by a common four-gene operon.[18,19] For CFA/I, this is *cfaABCE*.[11] This operon contains four genes in the following order: periplasmic chaperone *cfaA*, major fimbrial subunit *cfaB*, outer membrane usher protein *cfaC*, and minor fimbrial subunit *cfaE*.[11,18] CfaA functions as a chaperone to facilitate proper folding of other components of the operon to the outer membrane, while CfaC 'ushers' the structural fimbrial proteins and orchestrates their assembly at the cell surface. The extracellular portion of CFA/I fimbriae comprises two proteins, CfaB and CfaE, and assembles with a single copy of CfaE followed by multiple copies of CfaB.[19,20] In fact, CfaB is the major pilin subunit, and it is present as approximately 1000 copies on the cell surface per single copy of the minor subunit, CfaE.[19]

CFA/I FIMBRIAE ARE HIGHLY IMMUNOGENIC AND CONFER PROTECTION AGAINST ETEC

Attempts to successfully vaccinate against ETEC have met with varied success. Oral vaccination of human volunteers with CFA/I or CFA/II fimbriae failed to induce significant serum immunoglobulin G (IgG) or secretory IgA (SIgA) Abs.[21] As a result of poor anti-fimbriae Ab titers,[22,23] the human volunteers were not protected against virulent ETEC.[22] Despite neutralization of their stomach acidity,[23] poor SIgA anti-CFA Ab responses were obtained in these volunteers. Subsequent work showed that gastric proteases altered the CFA fimbriae antigenicity even at a neutral pH.[24] Overcoming the deleterious effects of the gastrointestinal tract, oral vaccination of rabbits with microencapsulated CFA/I fimbriae still failed to induce serum and fecal IgA anti-CFA/I Abs.[21] Although in a separate study, microencapsulated CFA/II fimbriae when directly intubated into the rabbit duodenum revealed that CFA/II fimbriae-specific Ab-forming cell responses could be induced in the Peyer's patches and spleens.[25] Once elicited, Abs to these fimbriae do protect against ETEC infection.[26,27] An effective vaccine for ETEC still remains elusive, but promising results have been obtained from recent human trials using heat-killed ETEC plus recombinant LT-B/CT-B,[1] suggesting the fimbriae are optimally immunogenic when associated with the bacilli.

Attenuated, live *Salmonella* vectors have been extensively used as a means to vaccinate against salmonellosis[28–31] and heterologous diseases.[32] Such attenuated *Salmonella* strains have been shown to be effective in delivering heterologous antigens (Ags) because of their ability to stimulate both mucosal and systemic immune compartments[29,33] most likely via infection of Peyer's patches and followed by subsequent spread into systemic immune compartment.[31,34] Therefore, an ETEC vaccine adapted as an attenuated, balanced-lethal *ΔaroA Δasd S. Typhimurium* vaccine carrying an asd^+ plasmid encoding the CFA/I operon.[35] The expression of this fimbriae appears similar[36,37] to wild-type ETEC with long, hairlike projections extending from the bacterial cell surface.[23] Upon oral immunization, *Salmonella*-CFA/I was quite adept in stimulating elevated mucosal IgA and serum IgG Abs to the fimbriae.[35,38] Interestingly, the CFA/I fimbriae stimulated a biphasic T helper (Th) cell response with a rapid induction of Th2 cells within the first week of vaccination followed by a progressively increasing Th1 cell response to eliminate the salmonellae.[38] This was corroborated by the stimulation of elevated serum IgG1 Abs relative to IgG2a shortly after vaccination.[38] This is atypical to immune responses to *Salmonella* vaccines, which generally are Th1 cell-dependent promoting serum IgG2a Abs.[39–48] Despite this obvious difference in Th cell profiles, the expression of CFA/I fimbriae did not alter its capacity to protect against wild-type *Salmonella* challenge.[49] Given the highly proinflammatory nature of *Salmonella*,[39,42,50] subsequent analysis assessed whether the CFA/I fimbriae interfered with the normal recognition of the bacilli. Upon infection of RAW264.7 or thioglycolate-induced macrophages with low infection ratios of *Salmonella*-CFA/I, minimal-to-no interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor (TNF)-α production was observed in contrast to its isogenic *Salmonella* vector strain eliciting all of these proinflammatory cytokines with as few as one bacterium/80 macrophages.[51] To ascertain why such a profound disparity in proinflammatory cytokine production, subsequent analysis could not find any differences in *Salmonella* colonization or increased susceptibility to macrophage cell death nor were there increases in anti-inflammatory IL-10 or IL-12p40 cytokines.[51] Although the mechanism for the stealth-like qualities of *Salmonella*-CFA/I was not discerned, a possible explanation for these observations may be that the CFA/I fimbriae thwart innate immune responses by hindering detection by individual or a combination of pathogen-recognition receptors, including Toll-like receptor 4 (TLR4), TLR5, CD14, MD2 and lipopolysaccharide-binding protein,[52] to indicate *Salmonella*'s presence. Hence, we hypothesized that the *Salmonella*-CFA/I may be an anti-inflammatory vaccine.

SALMONELLA-CFA/I AS AN ANTI-INFLAMMATORY VACCINE FOR AN ANIMAL MODEL OF MULTIPLE SCLEROSIS (MS)

The surprising results from the macrophage infection studies indicated that *Salmonella*-CFA/I was not eliciting the proinflammatory arm of immunity. Moreover, the stimulation of Th2 cells and associated anti-inflammatory cytokines suggests that this vaccine may also be therapeutic in treating autoimmune diseases, such as MS or arthritis. MS is an inflammatory demyelinating disease of the central nervous system (CNS) with destruction of white matter by autoreactive T cells.[53–56] This neurodegenerative disease affects as many as 400 000 people in United States and >2 million people worldwide.[57] The frequency of MS is thought to be age and gender dependent, as it most frequently affects young and middle-aged adults and occurs twice as often in females as in males.[54,57,58] Although not considered fatal, MS can progress into considerable neurological disability, impacting the quality of life, and resulting in a shortened lifespan.[59]

Experimental autoimmune encephalomyelitis (EAE) is one of the best and most frequently studied rodent model that mimics the neuropathology and clinical disease of MS.[60–65] 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.[66] It is induced upon immunization with restricted CNS peptides, such as myelin oligodendrocyte glycoprotein,[67] myelin basic protein[67] or proteolipid protein (PLP),[68] into susceptible mice. This injection results in the activation of myelin-specific $CD4^+$ T cells in naive animals where in the CNS inflammatory cells are recruited that secrete interferon (IFN)-γ, TNF-α and IL-1, resulting in perpetuation of inflammation along with tissue damage, including axonal damage, demyelination and perivascular inflammatory lesions.[69] Due to demyelination, new epitopes become exposed, and exposure of these neoAgs acts as an immunization process, thereby causing further epitope spreading.[62,70–72] Studies have shown that the proinflammatory-promoting cytokine, IL-23, is primarily responsible for encephalitogenic T-cell development in EAE.[73–75] IL-17 is the principal mediator of the inflammation observed in EAE[76–79] and in large part induced by IL-23.[73–76] IL-17 is cross-regulated by both IL-4 and IFN-γ.[76] Neutralization of IL-17 has been shown to be protective,[76–79] and protection can also be mediated via regulatory T (T_{reg}) cells.[80–83] The proinflammatory cytokines IFN-γ[84–87] and TNF-α[73,74,82,85,88] can also impact disease primarily via Th1 cells. Protection to EAE correlates with Th2/T_{reg} cell dependence as IL-4,[80,89–91] IL-10[71,72,85,92–94] and transforming growth factor (TGF)-β[80,82,89,95–97] can reverse or prevent EAE. IL-27 has also been shown to be important for induction of protective T_{reg} cells.[98–100]

As for many autoimmune diseases, patients have lost the capacity to be tolerized to self, and consequently, the patient begins to mount an immune response to self much like recognition of foreign Ags. This T-cell dysfunction exhibited in these patients results in chronic activation of inflammatory Th1 and Th17 cells.[101,102] Often the course of treatment involves anti-inflammatory drugs and does not address the cause of the disease. Certainly, an approach that has had experimental success is the induction of tolerance. Tolerance is the inability to recognize self or to defined Ags (reviewed in Faria and Weiner,[103] Mueller[104] and Bilate and Lafaille[105]) and past efforts to induce tolerance in humans by delivering Ags have proven successful for treating allergies.[106–108] The major obstacle in human tolerance is in part attributed to the requirement for large doses of Ag or repeated administrations of therapeutics.[106] Although feeding auto-Ags is effective in treating experimental autoimmune diseases, when applied to patients, oral feeding with auto-Ags has been deemed unsuccessful.[109,110]

As noted, the *Salmonella*-CFA/I vaccine elicited Th2-type responses to the fimbriae[38] much like an effect of soluble Ag with adjuvant.[12] The observed Th2 cell bias was directed to the fimbriae but not as much as to the *Salmonella* vector, which still retained the capacity to stimulate Th1-type responses against *Salmonella* Ags.[111] With this backdrop, subsequent studies began to analyze the potential of *Salmonella*-CFA/I to augment disease, first with $PLP_{139\text{-}151}$-induced EAE.[112] As *Salmonella*-CFA/I elicits a biphasic Th cell response, groups of mice were orally vaccinated 1 week or 4 weeks before EAE challenge to coincide with a more Th2 cell- or Th1 cell-prone environment, respectively. In either case, *Salmonella*-CFA/I still conferred protection recovering completely from disease unlike those mice vaccinated with an isogenic *Salmonella* vaccine lacking CFA/I fimbriae or those mice treated with phosphate-buffered saline (PBS).[112] However, mice vaccinated with the isogenic *Salmonella* vector did show reduced disease relative to PBS-treated mice but still bore greater disease than those mice vaccinated with *Salmonella*-CFA/I. In addition to the clinical scores, increased disease pathology of the CNS was evident from the enhanced demyelination and inflammatory cell infiltration relative to *Salmonella*-CFA/I-vaccinated mice. This protection was supported by increased production of IL-4, IL-10 and IL-13 and diminished production of IFN-γ by $PLP_{139\text{-}151}$- or CFA/I fimbriae-restimulated $CD4^+$ T cells.[112] In contrast, $CD4^+$ T cells isolated from unprotected or *Salmonella* vector-immunized mice[112] displayed elevated IFN-γ and minimal-to-no Th2-type cytokines. Thus, *Salmonella*-CFA/I was successful in reducing EAE severity in a bystander fashion when administered before disease induction.

Although these prophylactic results were clearly promising, often with human autoimmune diseases, it is unknown which patients will develop disease and at what time point. Therefore, to address the therapeutic potential of oral *Salmonella*-CFA/I, additional studies were conducted to determine whether it could impact ongoing disease.[68] Adapting the same $PLP_{139\text{-}151}$ challenge model, mice were subjected to intervention with *Salmonella*-CFA/I, *Salmonella* vector or PBS 6 days post-EAE induction. As per the prophylactic studies, both the *Salmonella*-CFA/I and *Salmonella* vector were able to subdue disease to different degrees, but only *Salmonella*-CFA/I was able to inhibit CNS inflammation unlike the *Salmonella* vector-treated mice, which showed extensive neutrophil, macrophage and T-cell infiltration into the CNS.[68] Protection to EAE by *Salmonella*-CFA/I was attributed to the stimulation of anti-inflammatory cytokines, IL-4, IL-10 and IL-13 with concomitant reductions in IFN-γ and IL-17.[68]

Because of the observed anti-inflammatory cytokines, we queried whether these were generated by T_{reg} cells, possibly induced by *Salmonella*-CFA/I. T_{reg} cells were originally delineated in thymectomized neonatal mice that showed increased manifestations of autoimmune disorders.[113,114] As a result, T_{reg} cells have been shown to maintain peripheral tolerance and are responsible for protection against a variety of autoimmune diseases, including colitis,[115] arthritis[116] and EAE.[81,83,105,117] T_{reg}-cell subsets are varied in their expression of phenotypic markers, but natural T_{reg} cells are identified by the expression of IL-2 receptor α-chain.[113] These are also transcriptionally regulated by forkhead box transcription factor (Foxp3),[117–120]

and when Foxp3 function is ablated, $CD4^+CD25^+$ T_{reg} cells are abrogated producing a wasting disease and inflammatory bowel disease in mice.[118,119] Co-inhibitory molecules, activation-induced cytotoxic T lymphocyte associated protein-4 and glucocorticoid-induced TNF receptor may also be expressed and contribute in mediating T_{eff} cell suppression.[121,122] In lieu of the phenotypic markers, earlier studies relied on functional characterization of these T_{reg} cells, and these were distinguished by the cell surface expression or secretion of TGF-β[120,123,124] referred to as Th3 cells[125] or production of IL-10 referred to as $T_{regulatory}$ 1 cells.[126,127] TGF-β production by $CD25^+CD4^+$ T_{reg} cells has been shown to be responsible for recovery from EAE,[68,89,95–97,123,128] and the presence of TGF-β has been shown to be necessary for conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ $FoxP3^+$ T_{reg} cells.[120,121,124,129] More recently, IL-35, a member of the IL-12 family and produced by T_{reg} cells, was found to have inhibitory activity capable of potently suppressing arthritis,[130,131] colitis[132,133] and EAE.[134] IL-35 can mediate its effects via the stimulation of IL-10.[130,131,135]

To examine the possible role for induction of T_{reg} cells by *Salmonella*-CFA/I, a kinetic analysis was done. It was discovered that both the *Salmonella* vector and *Salmonella*-CFA/I could stimulate the induction of $CD25^+CD4^+$ T cells, but the percentage of $Foxp3^+$ T_{reg} cells was particularly augmented in mice treated with *Salmonella*-CFA/I.[68] To assess their relative contribution, *in vivo* CD25 neutralization was performed resulting in the diminution of *Salmonella*-CFA/I's protective response demonstrating the importance of these T_{reg} cells to abate autoimmune disease.[68] To assess their relative potency, adoptive transfer of T_{reg} cells from each of the treatment groups (*Salmonella*-CFA/I, *Salmonella* vector and naive) was tested.[68] T_{reg} cells derived from mice vaccinated with *Salmonella*-CFA/I showed the greatest potency in $PLP_{139\text{-}151}$-challenged mice exhibiting nearly complete protection. Conversely, although T_{reg} cells obtained from *Salmonella* vector-vaccinated mice did confer protection, some disease was still evident that was subsequently protracted. Recipients adoptively transferred with naive T_{reg} cells only showed a delayed onset of disease, and all mice developed EAE. Interestingly, partial protection could be achieved with $CD25^-CD4^+$ T cells adoptively transferred from *Salmonella*-CFA/I-vaccinated mice unlike those same cells from *Salmonella* vector-immunized mice that were unable to confer protection. The adoptively transferred T_{reg} cells from *Salmonella*-CFA/I-vaccinated mice produced both TGF-β and IL-10, but the majority of the IL-10 was derived from $CD25^-CD4^+$ T cells. Moreover, these $CD25^-CD4^+$ T cells produced IL-4 and IL-13, suggesting these are potentially Th2 cells. T_{reg} cells from *Salmonella* vector-vaccinated mice produced little TGF-β and IL-10, and their $CD25^-CD4^+$ T cells did not produce IL-4, IL-10 or IL-13, which accounts for the lesser potency in reducing EAE.[68] As TGF-$\beta1^{-/-}$ mice succumb to death *in utero* and those surviving succumb early in life,[136] additional adoptive transfer studies were performed to test the dependence on TGF-β for protection to EAE.[137] Upon adoptive transfer of T_{reg} or $CD25^-CD4^+$ T cells from *Salmonella*-CFA/I-vaccinated mice, recipients induced with EAE were neutralized of their TGF-β using a monoclonal Ab (mAb), and this abrogated much of the protective effect by *Salmonella*-CFA/I's T_{reg} cells. Complete abrogation was observed upon treating recipients with *Salmonella*-CFA/I's $CD25^-CD4^+$ T cells.[137] As with this latter finding, TGF-β neutralization resulted in the complete loss of any partial protection conferred by *Salmonella* vector T_{reg} cells, and no protection was evident in recipients given $CD25^-CD4^+$ T cells from *Salmonella* vector-immunized mice.[137] TGF-β neutralization also impacted the expression of Foxp3 by the T_{reg} cells enabling IL-17 to be augmented, which would account for the loss of function and ultimately protection. Thus, TGF-β is an essential regulatory element induced by *Salmonella*-CFA/I therapeutic.

In an attempt to disrupt the protective capacity of CFA/I fimbriae, a mutant was developed to alter the cell surface expression of the fimbriae. This mutant bears only the *cfaAB* portion of the operon and lacks the genes for the outer membrane usher protein *cfaC*, and minor fimbrial subunit *cfaE*, and is referred to as *Salmonella*-$CFA/I_{(intracellular)}$ (*Salmonella*-CFA/I_{IC}).[138] This restricted the major subunit CfaB primarily to the *Salmonella*'s periplasm. Upon oral vaccination, mice showed reduced fecal SIgA and reduced serum IgG Abs to the fimbriae but remained as effective in protecting mice against EAE as did adoptive transfer of their T_{reg} cells into EAE recipients.[138] What was particularly interesting of this finding was the difference in cytokine profiles from *Salmonella*-CFA/I-vaccinated mice. Examination of cytokine production by T_{reg} cells from *Salmonella*-CFA/I_{IC}-treated EAE mice revealed no change in TGF-β production but considerably augmented IFN-γ and IL-13 production relative to similarly treated mice with *Salmonella*-CFA/I.[138] The percentage of $Foxp3^+$ T_{reg} cells was similar. To test the relevance of the observed IFN-γ and IL-13, *in vivo* Ab neutralization studies were conducted. Adoptive transfer of *Salmonella*-CFA/I_{IC}'s T_{reg} cells into EAE recipients neutralized of their IFN-γ using a mAb resulted in no differences in susceptibility to EAE, and both groups of mice given T_{reg} cells with normal rat IgG or rat anti-mouse IFN-γ mAb showed protection against disease. In contrast, EAE recipients neutralized of their IL-13 using a polyclonal anti-IL-13 Ab and adoptively transferred with *Salmonella*-CFA/I_{IC}'s T_{reg} cells lost their protection further showing the importance of IL-13 in defense against EAE.[138] This further implicated the ability of IL-13 to directly affect T_{reg} cell function or enhance the action of T_{reg} cells.[138] Thus, these collective studies show the effectiveness of *Salmonella*-CFA/I and *Salmonella*-CFA/I_{IC} as therapeutics to defend against EAE.

SALMONELLA-CFA/I AS AN ANTI-INFLAMMATORY VACCINE FOR AN INFLAMMATORY MODEL OF ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune disorder and chronic inflammatory disease of the joints impacting ~1% of

the population in North America and the United Kingdom,[139] with women being three times more likely to be afflicted than men.[139,140] Although the etiology of this disease remains to be discerned, it is manifested as a chronic synovitis and progressive destruction of the joints, leukocyte infiltrates and cartilage destruction and bone erosion. This destruction is believed to be supported and perpetuated by proinflammatory cytokines. Past studies have indeed shown that pro-inflammatory cytokines are overexpressed in RA joints (reviewed in Feldmann *et al.*,[141] Brennan *et al.*[142] and Kannan *et al.*[143]). To understand how such cytokines are regulated, a rodent model sharing many of the same features for human disease (reviewed in Kannan *et al.*[143]) was developed. This autoimmune disease, collagen-induced arthritis (CIA), is induced by immunizing rodents, typically with heterologous (bovine or chick) type II collagen in combination with adjuvant, to elicit immune attack of the host's native collagen. Thus, components of both the innate and adaptive immune systems are involved. Emphasis on regulating proinflammatory cytokines, particularly TNF-α, is key to minimizing disease as it can be detected in joints of RA patients.[144–146] Treatment with TNF-α antagonists decreases inflammation and attenuates the destruction of cartilage and bone.[141,147–151] Such treatment is also believed to inhibit other inflammatory cytokines, including IL-1, IL-6, IL-8 and granulocyte macrophages colony-stimulating factor.[141,142] Components of both the adaptive and innate immune systems contribute as a source for TNF-α and other proinflammatory cytokines.[143,152,153] IFN-γ also contributes to disease, but it is phase dependent[154–157]. Th17 cells, which can regulate both Th1 and Th2 cells,[158,159] may be important for mediating RA disease as $ICOS^{-/-}$ DBA/1 mice showed depressed IL-17 production, but not TNF-α or IFN-γ, and still conferred complete resistance to CIA.[160] IL-17 is also expressed by the human synovium and is particularly elevated in patients with RA[161,162] and is IL-23 dependent.[163] *In vivo* neutralization of IL-17 results in significantly reduced CIA and could also lessen the progression of the established disease.[164]

Given the results from the EAE studies, we queried whether *Salmonella*-CFA/I would be effective in treating CIA, a rodent model for RA.[153] DBA/I mice are susceptible to chick or bovine collagen II (CII) challenge, and develop a progressive disease affecting multiple joints.[153,165] As such, DBA/1 mice were orally dosed with *Salmonella*-CFA/I, *Salmonella* vector or PBS 7 days before CII challenge. Mice were followed for a course of 42 days, and *Salmonella*-CFA/I protected against CIA as evidenced by minimal clinical disease and significantly reduced incidence unlike mice treated with the *Salmonella* vector or PBS.[166] The observed reduction in disease was supported by the production of the cytokines IL-4, IL-10 and TGF-β by $CD4^+$ T cells. In addition, mononuclear cells from *Salmonella*-CFA/I-treated animals had decreased levels of TNF-α, IL-1β, IL-6 and IL-27.[166] To distill which $CD4^+$ T cells were responsible for the regulatory and anti-inflammatory cytokines in DBA/1 mice void of disease, mice were orally vaccinated with *Salmonella*-CFA/I, and cytokine profiles from $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were assessed. The $CD25^+CD4^+$ T cells produced significantly more IL-4, IL-10 and TGF-β than the $CD25^-$ T cells. Interestingly, IFN-γ and IL-17 were significantly elevated relative to $CD25^-CD4^+$ T cells but less than $CD25^+CD4^+$ T cells from *Salmonella* vector-immunized mice.[166] As the $CD25^+CD4^+$ T cells from similarly vaccinated mice were highly protective against EAE,[68] adoptive transfer studies were performed to measure the potency of these T_{reg} cells in conferring protection against CIA. Surprisingly, neither the individual $CD25^+CD4^+$ nor $CD25^-CD4^+$ T-cell subset was adequate in treating CIA relative to whole $CD4^+$ T-cell isolates with respect to disease onset and mice with reduced clinical disease.[166] Thus, it appeared that both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were required for protection against CIA. Subsequently, adoptive transfer studies using total $CD4^+$ T cells in combination with anti-IL-4 mAb or anti-TGF-β mAb were performed and revealed that inhibition of either cytokine resulted in disease and loss of the protective response.[166] Collectively, these data showed that *Salmonella*-CFA/I could treat CIA via the induction of diverse populations of T_{reg} cells.

As neither $CD25^+$ nor $CD25^-CD4^+$ T cells could completely protect following adoptive transfer into DBA/1 mice, our data suggested that perhaps a different T_{reg}-cell subset was being induced. To investigate such a possibility, the *Salmonella*-CFA/I-induced $CD4^+$ T cells were screened for expression of alternative T_{reg}-cell markers other than CD25. One such alternative is CD39. These $CD39^+$ T_{reg} cells are also able to suppress Th17 cells, and their absence has been linked to MS.[167] Specifically, CD39 is an ectonuleoside triphosphate diphosphohydrolase, and it is expressed on the cell surface of $Foxp3^+$ T_{reg} cells, dampening proinflammatory cells by ultimately converting proinflammatory extracellular ATP to anti-inflammatory adenosine.[166,168,169] Subsequent evaluation of $CD39^+$ expression was conducted in C57BL/6 male mice, which also are susceptible to arthritis.[170] Thus, CIA mice were orally dosed with *Salmonella*-CFA/I, and it was revealed that only half of the $CD39^+CD4^+$ T cells was $Foxp3^+$ $CD25^+CD4^+$, although CD39 was also expressed on the $CD25^-CD4^+$ T cells.[170] To determine whether these $CD39^+$ T cells were protective against disease, C57BL/6 mice were adoptively transferred with $CD39^+CD4^+$ or $CD39^-CD4^+$ T cells from *Salmonella*-CFA/I- or *Salmonella* vector-dosed mice into recipients challenged 14 days earlier with CIA. Only mice receiving $CD39^+CD4^+$ T cells from *Salmonella*-CFA/I-dosed mice were protected against CIA, indicating that again the CFA/I fimbriae are essential in stimulating this therapeutic subset of T_{reg} cells. As these $CD39^+CD4^+$ T cells were composed of both $Foxp3^+$ and $Foxp3^-$ cells, further analysis was performed to determine whether $Foxp3^+$ $CD39^+CD4^+$ T cells were protective against CIA. Notably, CIA recipients given either $Foxp3^+CD39^+CD4^+$ or $Foxp3^-CD39^+CD4^+$ T cells protected equally to disease but not as effectively as total $CD39^+CD4^+$ T cells.[170] These subsets were further teased to discern how they differ. Cytokine analysis revealed that IL-10 and TGF-β segregated

with Foxp3$^+$CD39$^+$CD4$^+$ and Foxp3$^-$ CD39$^+$CD4$^+$ T cells, respectively.[170] Neutralization of TGF-β reduced the percentage of CD39 expression, implicating the importance of TGF-β for induction of CD39.[170]

CONCLUSIONS

Outside of our studies, there have only been a few reports where bacterial infections have been used to subdue EAE,[171,172] and even less for CIA. However, it is important to emphasize that the induction of T_{reg} cells is mediated not so much by the attenuated *Salmonella* vaccine strain as these cells are induced by the fimbriae or the combination of bacteria with fimbriae. Current studies are addressing these possibilities. Nonetheless, our studies demonstrate the feasibility of a simple oral treatment with *Salmonella*-CFA/I to render protection against EAE and CIA without having previous knowledge of the auto-Ag. Consequently, in a bystander fashion, both fimbriae- and $PLP_{139-151}$-specific, TGF-β-producing, FoxP3$^+$CD25$^+$CD4$^+$ T cells were induced for EAE and CII-specific, IL-10-producing FoxP3$^+$CD39$^+$ and TGF-β-producing FoxP3$^-$CD39$^+$CD4$^+$ T cells. For the protective T_{reg} cells in CIA, co-expression of CD25 did not specifically segregate with either subset.[170] The advantage of using *Salmonella*-CFA/I is that intervention of autoimmunity can be achieved upon vaccination with an innocuous Ag, and in this case the side-effect would be the additional protection against the diarrheal diseases, ETEC and salmonellosis. Moreover, this therapeutic can be administered orally enabling patient compliance. Additionally, this approach does not polyclonally activate T_{reg} cells, which have been shown to have a cataclysmic outcome.[173]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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