IDENTIFICATION OF IMMUNODOMINANT T CELL EPITOPES FROM ENTEROTOXIGENIC *E. coli* COLONIZATION FACTOR ANTIGEN I (CFA/I) RESPONSIBLE FOR T HELPER CELL CYTOKINES

by

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TABLE OF CONTENTS

1. INTRODUCTION 1

Antigen Processing and Presentation by MHC Class II 1
Colonization Factor Antigen I (CFA/I) Fimbriae 14
an Anti-Inflammatory Vaccine 18
Salmonella-CFA/I Is Therapeutic in EAE 20
Salmonella-CFA/I Is Therapeutic in CIA 23
IL-35 29

2. MATERIALS AND METHODS 33

Cloning of pPICBCFA/Ie and pETCFA/Ib 33
Growth and Purification of Histidine-Tagged Proteins 35
SDS-PAGE and Western Blot Analysis 36
CFA/I Fimbriae Purification 37
Mice 37
Oral Immunizations with H696 38
Peptide Library 38
$CD4^+$ T cell Isolation and ELISPOT 39
Cytokine ELISA 41
Experimental Autoimmune 42
Encephalomyelitis (EAE) Induction 42
Administration of Proteins in EAE-Induced Mice 43
Proliferation Assay 43
Fluorescence Activated Cell Sorter (FACS) 43
Analysis and Intracellular Staining 43

3. RESULTS 45

4. DISCUSSION 75

Immunodominant Epitopes from CFA/I Fimbriae 75
Critical Amino Acid Arginine 181 76
Immune Evasion by ETEC 77
Induction of a Biphasic Response 82
Salmonella-CFA/I as an Anti-Inflammatory 83
Vaccine and T_{reg} Cell Inducer 83
$CD4^+$ Regulatory T cell Subsets 84
IL-10, TGF-β, IL-35, and Their 85
Production by CFA/I Fimbriae Peptides 85
Number of Responding $CD4^+$ T 88
Cells and Infectious Tolerance 88

TABLE OF CONTENTS CONTINUED

CREB Regulation/Contribution 90
Additional Future Experiments 91

REFERENCES 94

LIST OF TABLES

Table Page

1 Clinical Scores for EAE 42

2 cfaB Peptide Library Pools and Response 52

3 C-terminal cfaE Peptide Library Pools and Response 53

4 N-terminal cfaE Peptide Library Pools and Response 53

5 Immunodominant Peptides and Associated Response 73

LIST OF FIGURES

Figure Page

1: MHC II molecule. 3

2: Cell surface CFA/I fimbriae major and minor fimbrial subunits........ 16

3: Schematic of individual cfaB and cfaE fimbrial subunits. 17

4: Plasmid map and protein expression of cfaE in pPICBZ vector. 46

5: Plasmid map and protein expression of cfaB in the pET-32a........ 47

6: C57BL/6 mice selected for future studies based on robust cytokine response........ 48

7: Key Th1 and Th2 cell cytokines evaluated following culture with CFA/I fimbriae or independently expressed cfaB or cfaE. 49

8: EAE challenge in C57BL/6 mice receiving CFA/I fimbriae, cfaB, cfaE, or PBS i.m. 1 week prior to challenge. 50

9: EAE challenge in mice receiving cfaE and cfaB i.n. and/or i.m. on Days +1 and +6 of EAE challenge. 51

10: IFN-γ production by $CD4^+$ T cells from H696-treated C57BL/6 mice cultured with cfaB peptide pools........ 55

11: Generation of other cytokines following culture with cfaB peptide pools. 57

12 : Subtraction assay determining if a single peptide within pool #7 generates cytokines. 58

13 : Immunodominant region from major fimbrial subunit, cfaB. 59

14: Proliferation of $CD4^+$ T cells from H696-treated C57BL/6 mice cultured with peptides from cfaB. 61

15: Cytokine production following culture with C-terminal cfaE peptide pools. 63

16: Determination of cytokine-producing peptide within C-terminal cfaE pool........ 65

17: Immunodominant region from CFA/I fimbriae minor subunit, cfaE 66

18: Cytokine production generated from C-terminal cfaE peptides
cultured with cfaB peptide 25. 68

19: IL-10 production following culture with N-terminal cfaE pool 12 with
or without cfaB peptide 25. 69

20: p35 and EBI3 production following culture with C-terminal cfaE
peptide 1 and cfaB peptide 25. 72

21: Crystal structure of cfaE and cfaB. 74

22: Immunodominant peptides from cfaE. 76

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrheal disease contracted by consuming contaminated food or water. ETEC is able to adhere to the small intestine by utilizing pili or fimbriae, one of which is the fimbriae Colonization Factor Antigen/I (CFA/I). The extracellular portion of CFA/I fimbriae is comprised of two fimbrial subunits, cfaB and cfaE. Expression of CFA/I fimbriae on the surface of an attenuated *Salmonella* vaccine vector, *Salmonella*-CFA/I, results in a biphasic T cell response in immunized mice. This response is characterized by the initial production of Th2-type cytokines, including IL-4 and IL-5, followed by a shift after 4 weeks toward an IFN-γ-associated, Th1 response. Restimulation of $CD4^+$ T cells from *Salmonella*-CFA/I-immunized mice with CFA/I fimbriae also generates the anti-inflammatory cytokine, IL-10. *Salmonella*-CFA/I is able to generate antigen-independent $Foxp3^+$ regulatory T cells, which are able to reduce symptoms of Experimental Autoimmune Encephalomyelitis in immunized SJL mice and Collagen Induced Arthritis in DBA/I and C57BL/6 mice, via production of IL-10 and TGF-β, by phenotypically distinct regulatory T cell subsets. The following research describes the contribution of cfaB and cfaE to the observed therapeutic and immunological responses. This was measured by independently expressing recombinant cfaB and cfaE proteins and evaluating the associated cytokine responses from the co-culture of these proteins with $CD4^+$ T cells from immunized mice. Major Histocompatibility Complex II-restricted immunodominant regions were also mapped for both cfab and cfae proteins using cytokine ELISAs, ELISPOTs, Proliferation Assays, and flow cytometry. We mapped an IFN-γ-producing peptide from cfaB and an IL-4-producing peptide from cfaE. We further determined that co-culture with peptides from both fimbrial proteins is able to generate regulatory T cell-associated cytokines including IL-10 and TGF-β, as well as the newly described suppressive cytokine, IL-35. These results show that the immune responses to cfaB and cfaE are mediated by multiple immunodominant regions within each protein.

INTRODUCTION

Antigen Processing and Presentation by MHC Class II

The adaptive immune system relies upon successful presentation of antigen by MHC II or Major Histocompatibility Complex II to generate a $CD4^+$ T cell-mediated responses to foreign stimuli. The process by which specific antigen peptides are selected for presentation is vital to understanding the immune system and could play a key role in vaccine development by utilizing neutralizing epitopes, a select group of peptide sequences within complex protein sequences to which $CD4^+$ and $CD8^+$ T cells respond.[1-3] The cellular components that determine which peptide sequences within a protein are selected for presentation by MHC II are complex. First, a selected peptide must be loaded onto the MHC II molecule within an endocytotic compartment and then must be transported to the cell surface.[1] Once at the cell surface, the association between the selected peptide and MHC II must be stable while the antigen- presenting cell (APC) translocates to lymph nodes in order to interact with T cells.[1] Lastly, the MHC II : peptide complex also must be maintained during persistent T cell receptor (TCR) signaling after the initiation of interaction between TCR and MHC II. The complete process of translocation, T cell recruitment, and subsequent TCR interactions, lasts 10-20 hours in total for efficient immune stimulation.[1] The following will present an overview of the current understanding of complex mechanisms by which a peptide is selected for presentation on MHC II by an APC during an immune response.

Foremost to understanding what governs binding of a specific peptide region to MHC II is the MHC II molecule itself. MHC II is a heterodimer of proteins, alpha and beta, expressed on the surface of APCs, such as dendritic cells, B cells, and macrophages (Figure 1).[4,5] MHC II is also expressed in non-APCs, including thymic epithelial cells, mesenchymal stromal cells, endothelial cells, fibroblasts, and $CD4^+$ T cells in many species[6-9], as well as enteric glial cells, keratinocytes, and eosinophils during Crohn's disease, dermatoses, and oesophagitis or esophagitis, respectively.[5] In mice, the MHC genes are located on chromosome 17, while in humans, they are located on chromosome 6.[10] In mice, the Major Histocompatibility Complex (H-2) is comprised of three regions containing the following alleles: K, IA, IE, S, and D. MHC II is associated with regions IA and IE that encode the products IA alpha/beta and IE alpha/beta, respectively. There are multiple mouse haplotypes or combinations of alleles. The research herein was primarily conducted in C57BL/6 mice, haplotype "b," and to a lesser extent in BALB/c mice, haplotype "d."

Alpha and beta, the proteins forming the MHC II heterodimer, each form half of the binding platform,[10] a structure consisting of two parallel alpha helicies positioned on top of a beta sheet (Figure 1). The space between the two alpha helicies comprises the peptide-binding groove, which binds to the peptide via an extended conformation.[10] The ends of the binding groove are open, unlike the binding groove of MHC I, allowing the binding of longer peptides, approximately 20 amino acids (a.a.), as compared to the average 7-10 a.a. length bound by MHC I.[10] Two modes of non-covalent interaction regulate the binding of peptide to the MHC II domain. The first is the sequence-

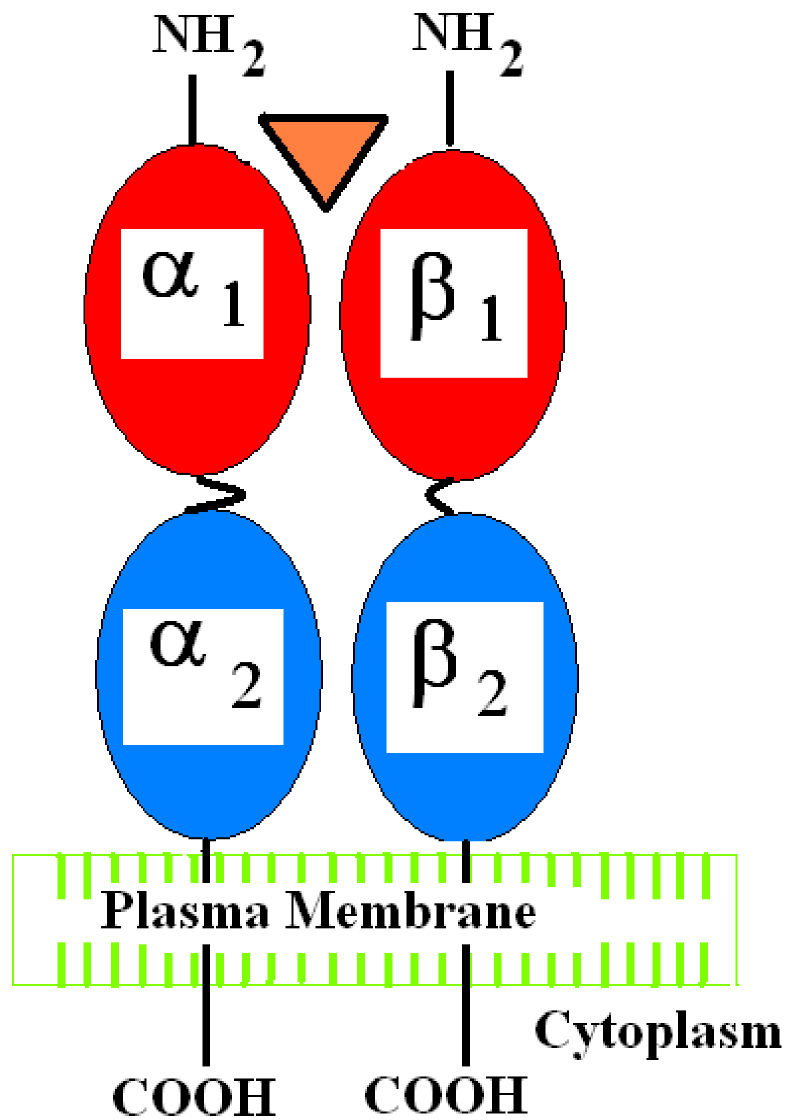


Figure 1: MHC II molecule.
The MHC II molecule is a heterodimer comprised of two polypeptides, α and β, each of which are further divided into regions 1 and 2. The α2 and β2 regions are highly conserved and interact with CD4. The α1 and β1 regions are highly polymorphic and form the peptide binding groove. The peptide binding region is denoted by the orange triangle.

dependent occupation of one of nine binding pockets where an amino acid side-chain fills or interacts with a pocket within the MHC II binding groove. The second is sequence-independent and is mediated by hydrogen bonding between the loaded peptide and the binding groove.

Binding of the peptide to the MHC II binding groove both alters and stabilizes the structure of MHC II.[4,10] Unlike MHC I, where only the ends of the peptide are secured via hydrogen bonding, in MHC II binding, hydrogen bonds are present along the length of the peptide.[10] Initially, occupation of multiple binding pockets within the MHC II binding groove was believed to regulate which peptides are selected and thus, regulate which regions are immunodominant. However, the resolution of crystal structures of known immunodominant epitopes bound to the appropriate MHC II molecule[11,12] revealed that often minimal and varying binding pockets are occupied by known immunodominant epitopes. Furthermore, studies in haplotype "k" SJL mice, examining necessity of binding certain pockets within MHC II by amino acid substitution, indicated that the existence of a "dominant pocket interaction" was the exception rather than the rule.[10]

Furthermore, the maltose binding protein expression system was used as a shuttle vector to test the contribution of specific amino acids to immunodominance *in vivo*. Leclerc *et al.* determined that there are 7 sites within this expression vector where peptides of up to 70 a.a. can be inserted without altering intrinsic folding or proteolysis effects.[13] Sant *et al.*[3] utilized this expression system to perform point mutations of amino acids at "critical" pocket residues and then evaluated changes in immunodominance. In

most proteins tested, immunodominance was either minimally or unaffected by these substitutions, indicating that immunodominance is not easily predicted by ideal pairing of optimal residues with particular pockets in the MHC II binding groove in most instances. Rather, these studies suggest that hydrogen bonding, or sequence-independent interactions, plays a larger role in determining immunodominant epitopes than sequence-dependent interactions.

The process by which MHC II is loaded with peptide and is presented on an APC surface is tightly controlled. A basic pathway for this to occur involves uptake of material by endosomes, TLR and scavenger receptor signaling to create the phagosome, removal of CLIP from the MHC II binding groove, replacement of CLIP with the peptide to be displayed, and, finally, expression of MHC II on the cell surface. The following will discuss each step in detail.

Extracellular material is first taken up via endocytosis. Endocytosis can be divided into the two following large categories based upon method of uptake: phagocytosis, the encodytosis of large particles, and pinocytosis, the endocytosis of fluid and small soluble molecules.[14] Phagocytosis begins with the direct binding of surface receptors to ligand on the target particle. This ligand can be either soluble, host-derived proteins such as antibody or complement, as well as conserved pathogen epitopes, expressed directly on the particle, such as terminal mannan or beta glucan.[15] Recognition of these ligands is mediated by many surface receptors on the APC including integrins, complement receptors, lectin receptors such as MR or Dectin-1, and Fc Receptors (FcR).[15] FcR or CR binding of antibody or complement proteins, respectively, as well as

direct binding by the other phagocytic receptors, facilitates the uptake of material accomplished by actin rearrangement and invagination of the bound material with the associated receptor into intracellular vesicles. This includes the particle, receptor(s), and an endocytosed portion of the plasma membrane and is now termed an endosome.[14] Alternatively, pinocytosis engulfs a much smaller volume of extracellular material and is categorized as receptor-mediated, adsorptive, or fluid phase.[14] Pinocytosis is mediated by clathrin-dependent and clathrin-independent mechanisms.[14] Regardless of method of uptake, once inside the cell, the newly-formed endosome is directed to other endosomal compartments, which contain machinery for further antigen processing.

The APC, particularly macrophages, must phagocytize and digest necrotic “self” cells as well as pathogen and induce immune responses to only the pathogen-containing endosomes. Toll-like Receptor (TLR) signaling dictates whether endosomes contain pathogen and are critical to elicit an immune response. In fact, in the case of apoptotic cells, the absence of simultaneous TLR signaling fails to elicit an immune response.[16] TLR signaling also aids in recruitment of crucial proteins to create the phagosome, including proteases and tetraspanins or scaffolding proteins. This phagosome fuses with endosomes containing MHC II and is now termed the phagolysosome.[4]

A key component in MHC II assembly and loading is the invariant chain (Ii). Ii (CD74) associates with newly-translated MHC II in the endoplasmic reticulum[17] via weak binding interactions with the MHC II binding groove. This interaction prevents dissociation of the heterodimer and also prevents loading of self-peptides in the early endosome compartment. The cytoplasmic tail of Ii also contains an endosomal retention

signal, which aids in guiding the MHC II to late endosomal compartments where relevant peptides are loaded. These late endosomal compartments are titled MHC II compartments (MIIC) due to the concentration of MHC II present. Here, Ii is cleaved by proteases, including the critical cathepsin S, leaving only a short peptide sequence bound to the MHC II binding groove. This MHC class II-associated Ii peptide is termed CLIP. CLIP remains bound until a suitable peptide sequence derived from processed exogenous protein is found.[4,5]

In addition to proteases and tetraspanins present in the phagolysosome, there is also a nonpolymorphic heterodimer protein termed H2-DM in mice, DM in humans. DM functions as both a chaperone and a catalyst and is required for the removal of CLIP[5] from the MHC II binding groove, allowing binding by processed peptides. [1,4] Speculation is that DM might also affect final peptide selection[2] by functioning as a "peptide editor," facilitating the exchange of multiple peptides in the MHC II binding groove by antagonizing those which bind with low stability to MHC II and thereby selecting for high stability peptide complexes for APC presentation and eventual immunodominance. Once peptides are successfully loaded onto MHC II, the MHC II: peptide complex is then transported to the cell membrane via microtubules where $CD4^+$ T cell activation occurs.[17] It is not completely understood if proteases cleave the protein before or after a region has bound to MHC II. The "determinant capture model" proposes that immunodominant peptides are first to unfold in a given protein and thus, are most likely to bind MHC II after which cryptic regions are degraded via proteases.[3] Others propose proteins are cleaved into smaller peptides before being loaded onto MHC II.[18]

T cell hybridomas generated to both known immunodominant peptides, as well as to cryptic peptides, were used to examine how APCs that either contained DM or were void of DM function in terms of antigen presentation. DM presence was found to enhance antigen presentation with regards to immunodominant peptides.[3] Conversely, presentation of cryptic peptides was negatively affected by DM presence.[3] This suggests that DM does function to select specific peptides, which will form stable peptide: MHC II complexes, while negatively impacting the binding of less stable peptide complexes. Furthermore, the observation that DM-deficient APCs are capable of displaying cryptic peptides indicates that these peptides are not inefficiently processed in lysosomes nor are they degraded quickly by proteases, a hypothesis termed “differential proteolysis.”[3] Since all immunodominant peptides are affected by DM in a positive manner despite variations in sequence and source, the idea of DM’s contribution to immunodominance by an intrinsic property of protein: MHC II complexes has evolved.[3] In conclusion, although the mechanism by which DM is able to select a particular peptide is not yet understood, its critical role in MHC II loading generally and selection of immunodominant epitopes specifically is clear.

Interestingly, the requirement for TLR signaling in MHC II presentation is independent of DM. This is shown by loading of the same peptides by DM regardless of TLR stimulation.[16] Blander *et al.* suggest that since DM functions to remove CLIP and screen potential antigens after cleaving of Ii, TLR signaling must impact MHC II loading prior its localization to the late endosome.[16] Therefore, they hypothesize that TLR activity modulates MHC II presentation at the level of Ii cleaving rather than via DM.

TLR signaling has also been implicated in regulation of MHC II expression although the mechanism through which this occurs is still under investigation. CpG-DNA and LPS, which signal through TLR9 and TLR4, respectively, up-regulate MHC II expression and do so more effectively than stimulation with IFN-γ, which acts through the transcription factor MHC class II transactivator (CIITA).[19,20] Since TLR9 and TLR4 signaling results in activation of the MyD88/IL-1R-associated kinase (IRAK) pathway, and since IRAK activates the transcription factor NF-κB, it is hypothesized that TLR signaling regulates MHC II expression via NF-κB.[21] However, experiments directly linking TLR-driven NF-κB expression to MHC II expression have not yet been shown. However, the transcription factor, CIITA, is not the sole regulator of MHC II expression since stimulation of TLR9 and TLR4 in an MHC II reporter line shows MHC II expression even following neutralization of CIITA via small interfering RNA (siRNA).[21] This fact, coupled with recent experiments showing NF-κB activation is necessary for up-regulation of some MHC II molecules *in vitro,*[21] is suggestive that this transcription factor might be the means of CIITA-independent MHC II up-regulation via TLR signaling.

Once loaded with peptide, MHC II is shuttled to the plasma membrane via either microtubule-based motors, dynein or the kinesin family, or the actin-based myosin motor family.[5] Tetraspanins also play a role in assembly of loaded MHC II at the cell surface. Although their function is not fully understood, they appear to form large protein networks by interacting with other transmembrane proteins and may help to form lipid rafts of loaded MHC II found clustered near the cell surface of DC.[17] Activated APCs

then migrate to the lymphoid system where they present to $CD4^+$ T cells and mediate the adaptive immune response. These responses can vary greatly from Th1 to Th2, but also can meditate other Th cells, including iT_{reg} cells, Th17 cells, Th9 cells, etc. Previous reports show that the induction of this response is dependent upon the type of APC involved, as well as the type of responding T cell.

The hypothesis that immunodominant $CD4^+$ T cell epitopes may not be defined by their ability to outcompete cryptic peptides for MHC binding[3] has led to significant experimentation to determine the mechanism for immunodominant peptide selection. Work by Hartman *et al.* indicates immunodominance is greatly affected by stability of peptide: MHC II interactions, but that immunodominance is primarily governed by the rate at which a peptide *dissociates* from MHC II rather than by the rate at which it *associates*.[2] Specifically, cryptic peptides appear to assemble with MHC II at a rate similar to the assembly with immunodominant peptides but appear to dissociate much more quickly from MHC II.[10] Using FITC-labeled immunodominant and cryptic peptides, Lazarski *et al.* demonstrate that each binds MHC II at a similar rate, although cryptic peptides dissociate at a rate much faster than immunodominant epitopes.[1] Furthermore, many known immunodominant epitopes such as $Myo_{102-118}$, Lack $_{156-173}$, OVA $_{273-288}$, and HA $_{126-138}$ have half-lives of greater than 150 hours when complexed with MHC II, while cryptic eptitopes, such as CLIP $_{85-99}$, HEL $_{11-25}$, and HEL $_{20-35}$, have half-lives of less than 10 hours.[1]

Additionally, experimentally determined cryptic epitopes expressed in the MalE vector were subjected to amino acid substitutions and were then empirically tested for

increases in peptide: MHC II stability. Substitutions increasing stability from 26 hours to 80 hours were sufficient to increase T cell recognition of the peptide from less than 3% of CD4$^+$ T cells to more than 20% of T cells, indicating that immunodominance is affected positively or negatively by increasing or decreasing the stability of the peptide: MHC II complex.[3] Furthermore, decreases in the stability of these complexes is even able to overcome the effect of T cell precursors observed in the peptide $LACK_{156-173}$ from *Leishmania major*.[3] In summary, stability of the peptide: MHC II complex is crucial to immunodominance and the subsequent CD4$^+$ T cell response. In addition, this stability is reflected in the rate at which the complex dissociates rather than the rate at which it associates with MHC II.

Clearly, expression of MHC II on cells will affect the associated immune response.[5,21] While commonly expressed on APCs, other cells can be induced to increase its expression. Furthermore, modulation of MHC II can impact the efficacy of APC presentation. The expression of MHC II proteins is regulated by the transcription factor CIITA.[5] CIITA must undergo monoubiquitination, as well as phosphorylation, before being activated as an MHC II transcription factor. In immature DCs, CIITA's promoter is bound by PU.1, nuclear factor-kB (NF-κB), interferon-regulatory factor 8 (IRF8), and SP1 yielding high levels of CIITA expression stimulating high levels of MHC II expression.[5] Upstream regulators of CIITA have also been identified and, in short, can be classified as signaling by external factors or chromatin regulation, such as epigenetics, and include many factors such as mitogen-activated protein kinase 1 (MAPK1), cell division cycle-associated protein 3 (CDCA3), pleckstrin homology domain-containing

family A member 4 (PLEKHA4), RMND5B, and CCR4-NOT transcription complex subunit 1 (CNOT1).[5] Interestingly, cytokines are also implicated in regulating CIITA activation. For example, recent systems analysis shows that one key upstream external signal activating CIITA is the transforming growth factor-β (TGF-β).[5]

In summary, the MHC II: peptide complex must be stable enough to translocate to the lymph nodes and the spleen where interaction with T cells occurs for maximal immune stimulation. Next, the TCR must be able to make a prolonged interaction with the MHC II: peptide for optimal immune responses. Immunodominance is the ability of a single peptide or possibly a limited number of peptides from a complex protein to preferentially bind with MHC II and generate an adaptive immune response on behalf of the entire protein. Initial observations of immunodominance resulted in two proposed explanations. First, "determinant selection" hypothesized that MHC could only bind a limited number of antigens from a given complex protein. The second hypothesis, "holes in the repertoire," suggested that the limiting factor is actually the TCR, which could only recognize a limited number of peptides. Experimentally, both theories were disproven.[3] More recently, the "determinant capture model" proposed that immunodominant peptides are first to unfold in a given protein and thus, most likely to bind MHC II after which cryptic regions are degraded via proteases.[3] Recent work shows that immunodominance is likely more complex than this explanation and likely relies upon the biochemical stability of the peptide: MHC II interaction itself, an interaction overseen by the protein DM in order to moderate which peptides are able to be presented in the context of MHC II on the cell surface in an effort to streamline the adaptive immune response.

While the above material describes methods by which exogenous protein is endocytosed by APC, cleaved into peptides, and eventually expressed on the cell surface in the context of MHC II, the method by which exogenously provided peptides are loaded onto MHC II is somewhat different. The method by which such peptide is presented in the context of MHC II is important due to the abundance of epitope mapping experiments currently underway as well as experiments directed at disciphering mechanisms of antigen presentation themselves. MHC I-expressing APC have been shown to load extracellular peptides on cell-surface MHC I.[22] Others suggest that this loading of extracellular peptides by bystander APC expressing MHC I is not unique to MHC I and in fact is also a property of MHC II.[22-27] However, other research disputes this theory indicating that antigen must be endocytosed prior to effective MHC II presentation.[22,23,25,28] In truth, both observations can be correct depending on the nature of the peptide in question. Specifically, like MHC I, MHC II peptides require cysteinylation, the reduction of peptide-included cysteine residues, prior to MHC II loading.[22] The requirement for cysteinylation clarifies observations that endocytosis is required prior to MHC II loading. Peptides which contain cysteine must be reduced, a process which would naturally occur within late endosomal compartments, prior to MHC II loading while peptides void of cysteine are able to load bystander APC-expressed MHC II directly and stimulate T cells. *In vitro* experiments utilizing cysteine-containing peptides can overcome the need for endocytosis-driven reduction by utilizing a reducing agent for peptide suspension.

Colonization Factor Antigen I (CFA/I) Fimbriae

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrheal disease and are contracted by ingesting contaminated food or water. *E. coli* becomes enterotoxigenic after acquiring plasmid(s) that allow for the production of heat-stable enterotoxin and/or the heat-labile enterotoxin.[29] Additionally, ETEC also acquires a plasmid that produces pili or fimbriae, which bind surface receptors in small intestine.[30,31] Colonization factor antigen I (CFA/I) is one such surface fimbriae of human ETEC.[32] ETEC utilizes these fimbriae to adhere to the host intestine during the early phases of infection.[31,32] After adherence to the small intestine, ETEC secretes the heat-labile or heat-stable enterotoxins resulting in diarrhea.[33] A specific natural receptor for CFA/I fimbriae has yet to be identified in the small intestine, but many studies suggest that in eurkaryotes, a sialylated glycoprotein is the receptor,[34,35] although other studies suggest that epithelial mannose-containing glycoproteins and/or glycosphingolids may also be receptors.[36] CFA/I fimbriae have been shown to bind both human and bovine erythrocytes, as well as to human small intestine mucosa.[34]

CFA/I fimbriae are an archetype of class 5 fimbriae characterized by a common 4-gene operon.[31,32] For CFA/I, this is *cfa*ABCE.[37] 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.*[31,37] cfaA functions to transport other components of the operon to the outer membrane, while cfaC assembles the fimbriae and “ushers” them to the cell surface. The extracellular portion of CFA/I fimbriae is comprised of two proteins, cfaB and cfaE, and assembles with a single copy

of cfaE followed by multiple copies of cfaB.[32,33] In fact, cfaB , the major pilin subunit, is present in a magnitude of 1,000 copies on the cell surface per single copy of the minor subunit, cfaE (Figure 2).[32]

Class 5 fimbriae, which contains 8 members of similar genetics,[31] are also categorized by the utilization of the alternate chaperone pathway for assembly.[32] The alternate chaperone pathway utilizes the immunoglobulin-like fold shared by all fimbrial subunit structures, save class IV, and leaves a hydrophobic region exposed, which is then filled by a β-strand from the next subunit in a method termed "donor strand exchange." Li *et al.* determined via electron microscopy (EM) and X-ray crystallography that the donated strand is conserved during the assembly of the CFA/I minor fimbrial subunit, cfaE, to the major fimbrial subunit, cfaB, structures, as well as between individual major subunit copies.[32] Furthermore, this donor sequence, VEKNITVTASVD, is conserved between all class 5 fimbriae examined to date and is categorized by alternating patterns of hydrophobicity in the side chains of amino acids.[32] These side chains fill hydrophobic pockets within the binding groove of the exposed immunoglobulin fold in the preceding subunit.[32]

Assembled extracellular CFA/I fimbriae assume two structures; the first is a thin, extended form termed fibrillar, and the second is a more compact helical structure.[32] Li *et al.* suggest that the conserved proline residue immediately adjacent to the 3' end of the conserved donor sequence may play a key role in determining the extracellular conformation of CFA/I fimbriae.[32] Specifically, if this proline is present in *cis*, then CFA/I fimbriae assembles in a helical structure, while if this proline is in *trans*, then the

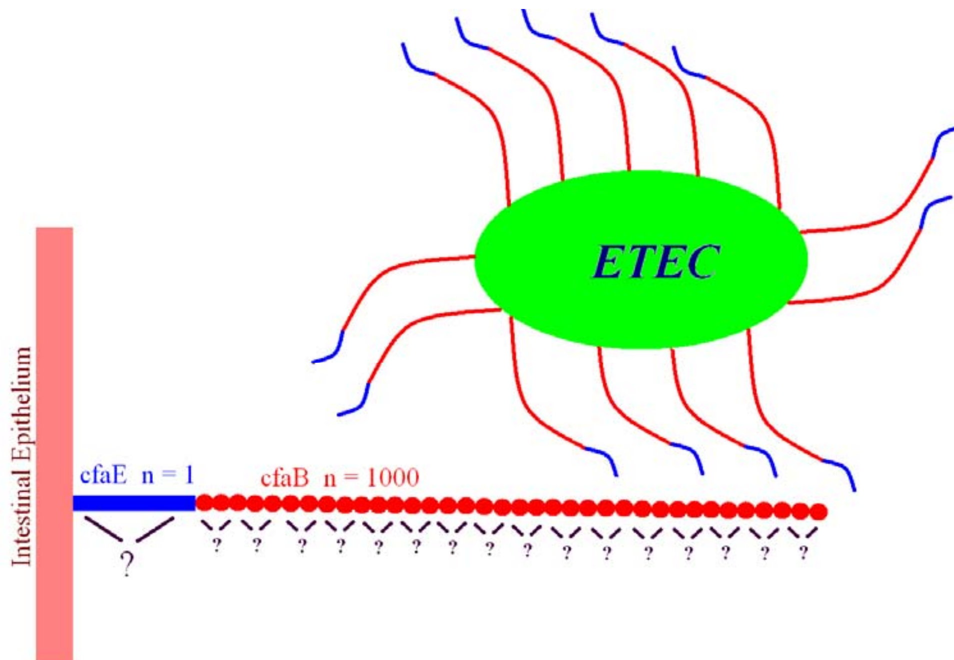


Figure 2: Cell surface CFA/I fimbriae major and minor fimbrial subunits. Schematic of location of cfaE and cfaB on the cell surface of ETEC. cfaE is shown binding its unknown receptor in the intestinal epithelium. The relative frequency of cfaE to cfaB is 1 : 1000. The as yet undefined immunodominant epitopes from cfaE and cfaB are represented by "?".

extended fibrillar structure forms. Additionally, Li *et al.* suggest that cfaC, the outer membrane usher protein, may function to facilitate *trans*-to-*cis* isomerization in Pro,[32] although this function has yet to be definitively proven.[32] CFA/I fimbriae can assume either the fibrillar or helical structure, but the latter is the dominant structure found extracellularly.[32] Amino acid sequences of cfaB and cfaE are shown in Figure 2 and Figure 3. The immunodominant epitopes from CFA/I fimbriae, which could be found within cfaB or cfaE, remain to be determined (Figure 2). Highlighted regions found in Figure 3 are crucial to fimbriae assembly as well as to the immunodominant regions to be discussed within this thesis.

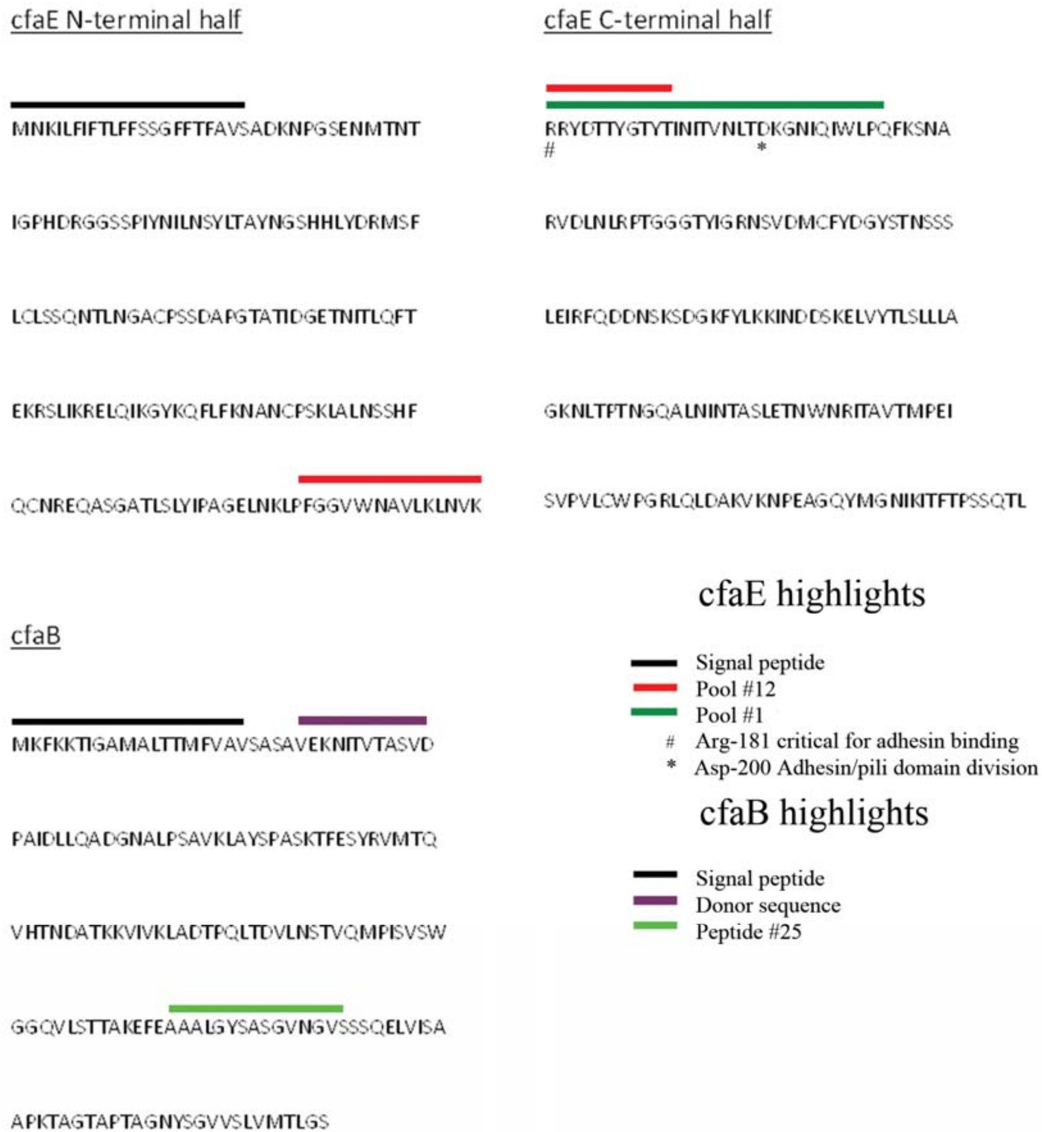


Figure 3: Schematic of individual cfaB and cfaE fimbrial subunits.
Amino acid sequence of cfaE and cfaB. Prominent peptides, pools, and protein regions are labeled including Asp-200. Structure upstream of this aspartic acid is part of the cfaE Adhesin Domain while downstream is part of the Pili Domain. Pool #12, which spans the N-terminal and C-terminal halves of cfaE, is labeled in red. C-terminal cfaE pool #1 is labeled in dark green. The subsequently identified immunodominant region of cfaE is comprised of portions of pool #12 and pool #1.

Salmonella-CFA/I Behaves as
an Anti-Inflammatory Vaccine

In an effort to develop an effective vaccine against ETEC, CFA/I was expressed on the surface of attenuated *Salmonella enterica* serovar Typhimurium[1,4] utilizing *asd*-stabilization rather than antibiotic selection.[38] Hypothesizing that since *Salmonella* attaches to M cells in the small intestine at the Peyer's patches, passenger antigen, in this case CFA/I fimbriae, would be more readily taken up by or exposed to immune cells such as T cells, B cells, macrophages, and dendritic cells found within Peyer's patches.[38] In accordance, this CFA/I-expressing *Salmonella,* strain H696, is able to generate elevated serum and mucosal antibodies against CFA/I fimbriae, while *E. coli* expressing CFA/I cannot.[38]

Classically, immunization with *Salmonella* strains results in Th1 cell responses characterized by IFN-γ, serum IgG2a, and some production of mucosal secretory IgA (SIgA) directed both at the *Salmonella* vectors, as well as toward any genetically engineered passenger antigen.[39] Rather than observing classic Th1-type responses, a biphasic T cell response is observed in mice orally vaccinated with *Salmonella*-CFA/I. This response is characterized by production of Th2-type cytokines initially and then shifts by 4 weeks to the classic Th1 response.[39] Comparison of antibody responses from mice immunized with *Salmonella* -CFA/I (H696) or the empty *Salmonella* vector (H647) revealed that SIgA anti-CFA/I fimbriae are produced following a single oral immunization with H696, but not in mice orally immunized with H647. Immunization with H696 also produced significantly elevated serum IgG levels. Interestingly, this IgG

response was dominated by Th2 cell-associated IgG1 one week after immunization. By 4 weeks post-immunization, Th1 cell-associated IgG2a increased to levels similar to the noted IgG1 levels. These findings prompted the evaluation of Th2-type cytokines, which would support the levels of SIgA and IgG1 observed. It follows that fresh splenic lymphocytes isolated one week following H696 immunization produce significantly more IL-4 and IL-5 than levels of IFN-γ. A similar trend was observed in lymphocytes from the Peyer's Patches where IFN-γ was not even detected.

To evaluate the role of the surface expression of CFA/I fimbriae in this observed biphasic response, lymphocytes from *Salmonella*-CFA/I treated animals were cultured with purified CFA/I fimbriae. $CD4^+$ T cell ELISPOT revealed that lymphocytes from H696-treated mice restimulated with purified CFA/I fimbriae produced significantly more IL-4 and IL-5 than with restimulation of medium alone demonstrating CFA/I fimbriae expression redirects cytokine responses.[39] The effect of CFA/I fimbriae presence was also emphasized that following $CD4^+$ T cell restimulation with CFA/I fimbriae, significant levels of IL-10 were detected in the Peyer's Patches compared to cells cultured with media alone.[39] Subsequent studies revealed a lack of pro-inflammatory cytokines, including TNF-α, IL-1α, IL-1β and IL-6, produced by H696-infected macrophages, which supported the Th2-type response observed in early H696 immunization. Of note, immunization with the isogenic vector alone does resulted in diminished production of the aforementioned cytokines from infected macrophages, highlighting the requirement for CFA/I fimbriae expression to generate the biphasic response.[40]

Salmonella-CFA/I Is Therapeutic in EAE

Previous studies show that Th2 cells, as defined by cytokine secretion, are therapeutic in experimental autoimmune encephalitis (EAE).[29] EAE is an induced model for multiple sclerosis (MS) characterized by inflammation of the central nervous system (CNS) coupled with demylelination.[41,42] It is induced upon immunization with restricted CNS peptides, such as myelin oligodendrocyte glycoprotein (MOG),[42] myelin basic protein (MBP),[42] or proteolipid protein (PLP),[43] into susceptible mice. This injection results in activation of myelin-specific $CD4^+$ T cells in naïve animals where, in the CNS, inflammatory cells are recruited that secrete IFN-γ, TNF-α, and IL-1, resulting in perpetuation of inflammation along with tissue damage, including axonal damage, demyelination, and perivascualr inflammatory lesions.[41] Disease is mediated by $CD4^+$ T cells evidenced by the observation that passive transfer of $CD4^+$ cells from diseased animals causes disease in naïve animals.[41] Since EAE shares many characteristics with MS, therapies that are functional in EAE have potential for treating MS.

Jun *et al.* examined if H696 could be protective in EAE since it generates Th2 cells. H696-treated mice displayed significantly decreased clinical scores if administered either 1 or 4 weeks prior to PLP-dependent EAE induction, although treatment 1 week prior to induction yielded the best protection. Additionally, these mice recover completely from the disease during the observation period.[29] This protection was supported by increased production of IL-4, IL-10, and IL-13 and decreased production of IFN-γ by lymphocytes upon restimulation with CFA/I fimbriae or $PLP_{139\text{-}151,}$ emphasizing the protective role of H696 in EAE was supported by Th2-type cytokines.[29] Lastly,

H696-immunized mice also displayed decreased demyelination in the spinal cords when compared to H647- or PBS-treated animals.[29] Thus, H696 is successful at reducing severity of EAE disease in a bystander, Th2-type manner when administered prior to disease induction.

H696 was similarly tested for its ability to have a therapeutic role in the PLP-dependent EAE model.[43] H696 is therapeutic in this EAE model if given 6 days after PLP-challenge and is signified by lessened clinical scores when compared to mice treated with either the isogenic H647 strain or PBS. These diminished clinical scores were supported by increased production of IL-4, IL-13, and IL-10 coupled with decreases in IFN-γ and IL-17 production following restimulation with purified CFA/I fimbriae or $PLP_{139\text{-}151}$.[43] Interestingly, mice receiving the empty vector, H647, also experienced lower clinical scores, although not as low as observed with H696-treated animals. However, examination of the spinal cords from H647-treated animals revealed that although they showed reduced clinical scores, the histological scores from these animals were similar to those from PBS-treated animals suggesting that the isogenic vector was insufficient in preventing inflammation within the CNS.[43] Such prevention was only observed in H696-treated mice. This work demonstrates that, in addition to a prophylaxis for EAE, H696 also had a therapeutic effect.

Regulatory T (T_{reg}) cells share many properties with Th2 cells. Furthermore, adoptive transfer of anti-encephalitogenic T_{reg} cells from diseased animals to naïve animals prior to EAE induction is therapeutic in EAE.[43] Ochoa-Repáraz *et al.*[43] have evaluated whether H696-stimulated T_{reg} cell formation could account for the H696-

mediated protection against EAE. First, confirmation of T_{reg} cell development was determined by evaluating Foxp3$^+$ expression following immunization with H696. Lymphocytes from cervical lymph nodes (CLNs) of PLP-challenged SJL mice were examined for co-expression of CD25$^+$ and the transcription factor Foxp3$^+$ following oral treatment with H696, H647, or PBS. A significant increase of Foxp3 expression was found in CD25$^+$ CD4$^+$ T cells from H696-immunized animals where more than 90% of CD25$^+$CD4$^+$ cells were Foxp3$^+$. Thus, in this scenario, CD25$^+$ expression correlated with Foxp3$^+$ expression, and these T_{reg} cells were capable of inhibiting effector T (T_{eff}) T cell proliferation.[43]

In order to determine if nonencephalitogenic T_{reg} cells generated by H696 are protective in PLP-dependent EAE, Ochoa-Repáraz *et al.* adoptively transferred T_{reg} cells (CD25$^+$CD4$^+$) and non-T_{reg} cells (CD25$^-$CD4$^+$) from H696- and H647-treated mice into naïve mice one day prior to EAE challenge.[43] To evaluate the contribution of innate T_{reg} cells, CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells from naïve mice were also tested by adoptive transfer. Mice receiving CD25$^+$CD4$^+$ cells from H696 mice experienced a delay in disease onset by one week or more coupled with reduced overall clinical scores.[43] In fact, mice receiving T_{reg} cells from H696 mice displayed lower clinical scores and later disease onset than mice orally immunized with H696,[43] indicating that the generation of T_{reg} cells following H696 immunization is critical for intervention in EAE.

Mice receiving CD25$^-$CD4$^+$ cells from H696-treated mice also showed lessened clinical scores than PBS-treated mice, but higher scores than mice receiving T_{reg} cells. Evaluation of the cytokine production by these CD25$^-$CD4$^+$ cells showed they produced

Th2-type cytokines, such as IL-4 and IL-13. Although they also produced IL-10, unlike their T_{reg} cell counterpart, they did not produce TGF-β. The protection rendered from adoptive transfer of these $CD25^{-}CD4^{+}$ cells shows that Th2 cells, independent of T_{reg} cells, can also dampen disease effects in EAE.[43] Collectively, these data show that T_{reg} cells generated following H696 immunization are responsible for the observed delay in disease, reduced clinical scores, and low histological scores in EAE challenged SJL mice, although Th2 cells also contribute to dissolution of disease.

Adoptive transfer of T_{reg} cells from H647-immunized mice is partially protective against EAE; however, these mice do get sick and do not experience a delay in disease onset, whereas T_{reg} cells from H696-immunized animals nearly abolishes disease symptoms. On the contrary, $CD25^{-}CD4^{+}$ T cells from this group do not confer protection against EAE. Of note, $CD25^{-/+}CD4^{+}$cells from naïve mice do not confer protection against EAE, indicating that innate or natural T_{reg} cells are insufficient for protection against EAE.

Salmonella-CFA/I Is Therapeutic in CIA

Since H696 is effective at treating the autoimmune disorder EAE, it was also tested to determine efficacy in its ability to treat another mouse model of a common human autoimmune disorder, rheumatoid arthritis (RA). The mouse model selected was collagen-induced arthritis (CIA) and is on a different MHC II background either H-2q or H-2b, for DBA/I mice or C57BL/6 mice, respectively, versus H-2k utilized by SJL mice. In CIA, $CD4^{+}$ T cells, just as in EAE, are misdirected against self, thus driving the CIA-

induced inflammatory responses. In addition to self-reactive T cells, the proinflammatory cytokines IL-1β, TNF-α, and IL-6 are also central to CIA, as are B cells that generate antibodies against Collagen II (CII).[44] H696 administered 7 days prior to disease induction protected against CIA and resulted in minimal clinical scores and significantly reduced incidence of disease. This disease reduction was supported by the production of the cytokines IL-4, IL-10, and TGF-β by $CD4^+$ T cells. In addition, mononuclear cells from H696-treated animals had decreased levels of TNF-α, IL-1β, IL-6, and IL-27.[44] In contrast, treatment with H647 neither reduced clinical scores nor decreased disease incidence. Thus, unlike EAE in which the isogenic strain played a diluted role in ameliorating disease, in CIA , treatment with H647 yielded no improvement in CIA animals.[44] This implies that H696 alone functions in a prophylactic manner in CIA in DBA/I mice.

In an effort to determine which subset of $CD4^+$ T cells were generating regulatory and anti-inflammatory cytokines in DBA/I mice void of disease, animals were immunized with H696, and cytokine profiles from $CD25^+CD4^+$ and $CD25^-CD4^+$ cells were compared. The $CD25^+$ cell subset from H696 mice produced significantly more IL-4, IL-10, and TGF-β than the $CD25^-$ T cell subset. Significant decreases in the production of IFN-γ and IL-17 were seen from H696-treated mice in the $CD25^-$ and $CD25^+$ $CD4^+$ T cell subsets, respectively.[44] However, adoptive transfer of neither $CD25^+CD4^+$ nor $CD25^-CD4^+$ T cell subset was adequate in treating CIA when compared with the whole $CD4^+$ T cell subset where disease onset was delayed by over 10 days, and mice had lessened clinical scores after 40 days.[44] This indicates that optimal disease

protection via H696-immunization utilized effects from both $CD25^{+/-}CD4^{+}$ subsets. Evaluation of cytokines from DBA/I recipients receiving total $CD4^{+}$ T cells adoptively transferred yielded significant increases in IL-4, IL-10, IL-13, and TGF-β and significantly less IL-17 than animals receiving H647 $CD4^{+}$ T cells or PBS-treated animals.[44] This suggests that *Salmonella*-CFA/I is able to effectively treat CIA in an antigen-independent manner through $CD4^{+}$ T cells producing Th2 cytokines, as well as regulatory cells producing IL-10 and/or TGF-β.

To determine the contribution of Th2 cells versus T_{reg} cells, DBA/I mice received $CD4^{+}$ T cells from H696-treated mice 15 days post-CIA induction, while also receiving anti-TGF-β or anti-IL-4 mAbs.[44] Upon TGF-β neutralization recipient mice lost all protection to CIA as clinical scores and incidence of disease were restored to PBS control levels. Neutralizing IL-4 resulted in a partial loss of protection based on increases in clinical scores. Although said scores did not attain levels of either PBS-treated animals or animals receiving TGF-β neutralizing antibodies, neutralization of IL-4 did result in a significant increase in incidence of disease. As expected, based on observed clinical scores, mice which received TGF-β neutralizing antibodies produced TNF-α, IL-6, IL-17, and IL-27 at levels similar to PBS control (diseased) mice. Mice receiving IL-4 neutralizing antibodies also experienced increases in all pro-inflammatory cytokines, but still experienced less IL-17 and TNF-α production than mice subjected to TGF-β neutralization or PBS-treated animals. Collectively, these data demonstrate that both TGF-β and IL-4 are important for H696-mediated CIA protection.[44]

Since neither subset of $CD4^+$ T cells, $CD25^+$ or $CD25^-$, completely protected following adoptive transfer in DBA/I mice, and since Foxp3 expression correlated with both $CD25^+$ and $CD25^-$ $CD4^+$ T cells, the induction of a different T_{reg} cell subset was hypothesized. Thus, the generated $CD4^+$ T cells were screened for expression of T_{reg} cell markers other than CD25. One alternate marker for T_{reg} cells is CD39, which also can correlate with $Foxp3^+$ expression. These $CD39^+$ T_{reg} cells are also able to suppress Th17 cells, and their absence has been linked to MS disease.[45] Evaluation of $CD39^+$ expression in H696-immunized CIA mice revealed that half of all $CD4^+$ T cells that were $Foxp3^+CD25^+CD4^+$ were also $CD39^+$ although CD39 expression also associated with $CD25^-CD4^+$ T cells. However, CD39 was preferentially expressed with $Foxp3^+$ cells and suggested further examination of CD39 expression following H696 immunization is important.[44]

This novel subset of $CD39^+$ T_{reg} cells regulates via control of extracellular ATP, which is released during tissue damage and/or apoptosis, by utilizing its extracellular ectonucleoside triphosphate diphosphohydrolase-1 enzyme activity.[46] Extracellular ATP induces proinflammatory responses, including nitric oxide (NO) production and TNF-α release from macrophages, but CD39 is able to convert ATP to a single adenosine[47] abolishing the pro-inflammatory effects of ATP. In addition, adenosine exhibits anti-inflammatory properties.[48] Specifically, one adenosine receptor, A2A, is present on $CD4^+$ T cells and functionally terminates proliferation of T_{eff} cells when added to $CD25^-$ $CD4^+$ cells.[48] Thus, $CD39^+$ T_{reg} cells confer immunomodulation, in part, via conversion of inflammatory ATP to the anti-inflammatory adenosine.

CD39 expression was up-regulated on $CD4^+$ cells following H696-immunization compared to mice immunized with its isogenic strain.[44] These H696-induced $CD39^+$ $CD4^+$ T cells include $CD25^+$, $CD25^-$, $Foxp3^+$, and $Foxp3^-$ cell subsets.[44] Since protection against CIA following H696-immunization was not segregated into either $CD25^+CD4^+$ or $CD25^-CD4^+$ cells, possibly $CD39^+$ cells in conjunction with Foxp3 expression might delineate the protective subset of $CD4^+$ T cells, and thus warrant closer examination.

H696 was also examined for its ability to abrogate CIA symptoms in C57BL/6 mice as seen in DBA/I mice.[44] In addition to testing H696's efficacy in treating CIA on a different MHC II background, H696 was also tested for its ability to therapeutically treat CIA since it has been used prophylactically in DBA/I mice.[44] C57BL/6 mice receiving H696 14 days following induction of CIA were protected with an incidence of disease of only 10% and significantly lower histological scores and cartilage loss; however, extending administration of H696 another week, to 21 days, resulted in a 60% incidence of disease and higher overall average clinical scores. Cytokine profiles of collagen II-specific $CD4^+$ T cells from the 14 day group revealed diminished levels of IFN-γ and IL-17 in mice receiving H696 coupled with increased levels of IL-4, IL-10, and TGF-β. While mice treated at 21 days still displayed significantly more IL-4 and regulatory cytokines, they did not experience decreases in pro-inflammatory IFN-γ and IL-17 which likely accounted for the observed clinical scores. Interestingly, treatment with H647 yielded clinical scores greater than PBS-treated animals.[48] Thus, while the isogenic strain was partially protective in EAE[43], it seemed to exacerbate disease when tested in a therapeutic capacity in C57BL/6 mice.[48]

Initial studies showed immunization of DBA/I mice with H696 prior to CIA challenge resulted in the generation of $CD39^+CD4^+$ T cells,[44] and the generation of this population was confirmed in C57BL/6 mice induced with CIA.[48] The expressed CD39 was functional based on an observed increase in ATP hydrolysis in mice receiving H696 versus H647 or PBS, confirming that H696 generates a population of $CD39^+CD4^+$ T_{reg} cells that are functional based on the release of phosphate upon ATP conversion to adenosine. In order to confirm that the observed CIA protection from H696 immunization was indeed attributable to this population of T_{reg} cells, C57BL/6 mice were adoptively transferred with $CD39^+CD4^+$ or $CD39^-CD4^+$ T cells from H696- or H647-dosed mice into recipients previously challenged 14 days earlier with CIA. Only mice receiving $CD39^+CD4^+$ T cells from H696-immunized mice were protected against CIA, indicating that CFA/I fimbriae are critical to the generation of this therapeutic population of T_{reg} cells.[48]

Further analysis revealed that the majority of these $CD39^+$ T_{reg} cells were also $Foxp3^+$. Interestingly, adoptive transfer of either $Foxp3^{+/-}$ subset of $CD39^+CD4^+$ T_{reg} cells resulted in a delay of disease onset, as well as reduced clinical scores although mice did get sick. Evaluation of Foxp3 status 40 days after adoptive transfer indicated a high frequency of mice receiving $Foxp3^-CD4^+$ T cells had converted to $Foxp3^+CD4^+$ cells, the majority of which were $CD39^+$.[48] These data imply a role for both Foxp3 subsets exists in CIA. Cytokine analysis of $CD39^+CD4^+$ T_{reg} cells with respect to Foxp3 expression revealed TGF-β was primarily expressed by $Foxp3^-CD39^+CD4^+$ cells, which were

$CD25^-$, while IL-10 was produced by $Foxp3^+CD39^+CD4^+$ T_{reg} cells.[48] In conclusion, CIA in C57BL/6 mice yields a population of functional regulatory T cells that are $CD39^+CD4^+$ and either $Foxp3^{+/-}$ with IL-10 production polarizing toward $Foxp^+$ cells and TGF-β to $Foxp3^-$ cells. Similarly, we determined that treatment of C57BL/6 mice with recombinant IL-35 (rIL-35) could also generate a population of $CD39^+CD4^+$ T cells.[47] These cells had an increased expression of Foxp3 within the $CD39^+CD4^+$ subset, but IL-35 treatment did not appear to increase $CD25^+$ expression,[47] which supports the previous findings in DBA/I mice that $CD39^+$ expression does not necessarily correlate with $CD25^+$ expression.[44]

IL-35

IL-35 is a heterodimer of Epstein-Barr virus-induced gene 3 (EBI3) and p35,[49] each of which also encodes for a subunit of IL-27 and IL-12, respectively.[50] IL-35 is secreted by T_{reg} cells, but not T_{eff} cells,[50] and suppresses proliferation of T_{eff} cells, inhibits *in vivo* differentiation of Th17 cells, and expands $Foxp3^+$ $CD25^+CD4^+$ T cells.[49] Restriction of expression of IL-35 T_{reg} cells is due, at least in part, to EBI3 regulation by the transcription factor Foxp3.[50] It follows that EBI3 mRNA can be detected in T_{reg} cells, but not in T_{eff} cells.[50]

Studies exploring potential therapies with recombinant IL-35 (rIL-35) show that it can also suppress proliferation of T_{eff} cells *in vitro*.[50] *In vivo*, rIL-35 blocks experimental colitis and partially suppresses CIA in DBA/I mice by promoting IL-10 production and suppressing Th1 and Th17 cells via reduced proliferation, as well as by reduction in pro-

inflammatory cytokines.[49] In C57BL/6 mice, rIL-35 protects against CIA, and treated mice display significantly less cartilage loss, resulting in a decreased histological score supported by decreases in IFN-γ and IL-17 and increases in IL-10.[47] These rIL-35-treated mice also display increases in $CD39^{+}$ $CD4^{+}$ T cell populations, with increased $Foxp3^{+}$ expression, which are protective against CIA when adoptively transferred into naïve mice. Of note, adoptive transfer of $CD39^{-}CD4^{+}$ cells from IL-35-treated mice did not protect against CIA, demonstrating the necessity of the $CD39^{+}$ phenotype.[47] Adoptive transfer of $CD39^{+}CD4^{+}$ T cells from rIL-35-treated $IL\text{-}10^{-/-}$ mice did not protect against CIA, highlighting the important role that IL-10 plays in IL-35 mediated suppression.[47]

$CD39^{+}CD4^{+}$ T_{reg} cells are further categorized by evaluating the role that CD25 expression plays within this population with regard to potency. C57BL/6 mice were induced with CIA followed by adoptive transfer of either $CD25^{+}CD39^{+}CD4^{+}$ or $CD25^{-}CD39^{+}CD4^{+}$ cells from rIL-35-treated donors. The ensuing disease course revealed mice receiving the $CD25^{-}$ subset were completely protected against CIA. While fewer mice receiving the $CD25^{+}$ subset developed disease when compared with PBS controls, these mice did get sick indicating that the $CD25^{-}CD39^{+}CD4^{+}$ subset of T_{reg} cells is most potent in CIA.[47]

These findings suggest that rIL-35 generates a unique population of T_{reg} cells that expand during CIA in C57BL/6 mice in an IL-10-dependent manner and are associated with CD39 expression. Since a similar $Foxp3^{+}$ $CD39^{+}CD4^{+}$ T cell population is generated in H696-treated DBA/1 mice, [44] possibly IL-35 is produced in this scenario, as

well. It is tempting to speculate that IL-35 may itself be driving the generation of $CD39^+$ T_{reg} cells.

The following research further describes the function of the two extracellular components of CFA/I fimbriae, the major fimbrial subunit, cfaB, and the minor fimbrial subunit, cfaE. First, the ability of each component to independently affect EAE disease in C57BL/6 mice was evaluated followed by co-treatment with each independently expressed protein. Next, both cfaB and cfaE were subdivided into peptide libraries and utilized to map immunodominant region (s) for each subunit (Figure 3) based upon their ability to restimulate $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice. This response was measured by the generation of relevant cytokines described above; cytokines that provided the ability for H696, *Salmonella*-CFA/I, to confer protection to autoimmune diseases in an antigen-independent manner. Mapping of immunodominant regions from cfaB yielded a single peptide capable of inducing robust amounts of IFN-γ. Conversely, mapping of cfaE revealed an immunodominant region responsible for the generation of the anti-inflammatory cytokine, IL-4 by $CD4^+$ T cells. In order to generate T_{reg} cell-associated cytokines, TGF-β and IL-10, from *Salmonella*-CFA/I restimulated $CD4^+$ T, we found that immunodominant regions from both cfaB and cfaE were required. Furthermore, this restimulation of $CD4^+$ T cells with immunodominant peptides from both fimbrial subunits also resulted in the production of the newly-described suppressive cytokine IL-35. This observed synergism between epitopes from both proteins, which comprise the CFA/I fimbriae, sheds light on how ETEC may be able to cause immune evasion as well as why a biphasic shift is observed following *Salmonella*-CFA/I

immunization. It is possible that this information can be utilized to manipulate the immune response to *Salmonella*-CFA/I to promote either pro- or anti-inflammatory responses, depending on the use of the vaccine.

MATERIALS AND METHODS

Cloning of pPICBCFA/Ie and pETCFA/Ib

cfaE was amplified via PCR, and primers were used to add the restriction sites, EcoRI and Kpn1, to the N-terminal and C-terminal ends of the gene, respectively, as well as an N-terminal start codon and Kozak sequence (Figure 4A). The resulting product was then cloned into the TOPO TA vector (Invitrogen Life Technologies, Grand Island, NY), and positive clones were Mini-Prepped (Qiagen, Valencia, CA) and sequenced to ensure the entire DNA sequence was correct. The resulting *cfaE* product was then cut from the TA vector and ligated to pPICZB vector, which was digested with EcoR1 and Kpn1. The pPICZB vector contains a C-terminal myc epitope followed by a 6-histidine tag. The resulting product was then chemically transformed into Top 10 *E. coli* cells, and positive colonies were selected based on Zeocin resistance. Positive clones were again sequenced to ensure sequence integrity. The resulting pPICBCFA/Ie was linearized using PmeI and transformed into X-33 *Pichia pastoris* electrocompetent cells and plated onto yeast extract peptone dextrose agar plates with sorbitol (YPDS) containing zeocin for selection. *Pichia pastoris* is a methyltrophic yeast that is able, but not required, to utilize methanol as its sole carbon source by expressing alcohol oxidase, which is under the control of two alcohol oxidase promoters, AOX1 and AOX2[51-53]. The multiple cloning site is downstream of the AOX1 promoter so expression of *cfaE* is inducible with methanol. Positive clones were then tested for their ability to express the cloned protein by growing first in minimal medium containing glycerol (YNB-G) to increase cell density followed

by switching to minimal media containing methanol (YNB-M) that utilizes the AOX1 promoter, which controls the cloned protein's expression (Invitrogen, Pichia Manual, Version A).

cfaB was amplified via PCR, and primers were used to add restriction sites, HindIII and XhoI, to the N-terminal and C-terminal ends of the gene (Figure 4B). An N-terminal start codon and Kozak sequence was also added. The resulting PCR product was cloned into the TOPO TA vector (Invitrogen Life Technologies, Grand Island, NY), as described above, after which it was ligated to pET-32a vector (Novagen, Darmstadt, Germany) and digested with HindIII and XhoI. This vector has a T7lac promoter, as well as an ampicillin-resistance gene. Although the pET-32a vector has both an internal and C-terminal histidine tag, as well as an internal S-tag and N-terminal Trx-tag, our protein was intentionally cloned out of frame with all N-terminal tags and was modified to possess its own start codon so the resulting product was our gene of interest cloned to only one tag, the C-terminal 6x histidine tag. This manipulation was performed to reduce the expression of unnecessary tags, which would have increased final protein size. Also, placement of the histidine tag at the C-terminal end of the gene rather than at the N-terminal end ensured that complete protein translation had occurred. The resulting plasmid was transformed into chemically competent chloramphenicol-resistant BL21(DE3)pLysS *E. coli* cells. Resulting clones were tested for expression via induction using isopropyl-D-thiogalactopyranoside (IPTG).

Growth and Purification of Histidine-Tagged Proteins

pPICBCFA/Ie was grown on a large-scale by first growing to an optimal density of OD_{600} 0.8 in YNB-G O.N. at 30˚C followed by a switch to a YNB-M media, thus, driving protein expression via the AOX1 promoter. The yeast grew in the methanol-based media for 40-48 hrs. Since the expressed protein was not secreted, the resulting biomass was subjected to disruption with glass beads in the presence of 0.1% Triton X-100 detergent (Sigma-Aldrich, St. Louis, MO). Cell debris was separated from the protein-containing lysate via centrifugation.

pETCFA/Ib was grown in Luria-Bertani (LB) broth supplemented with 1% glucose to an OD_{600} of 0.6-0.8. Then cultures were induced for protein production with 1 mM IPTG O.N. at 25˚C. Since the expressed protein was not secreted, resulting bio-mass was subjected to three freeze/thaw cycles to aid in cell disruption followed by sonication until cell walls were broken. Cell debris was separated from the protein-containing lysate via centrifugation.

The resulting proteins were both purified over a BD TALON resin (BD Biosciences, Sparks, MD) that utilizes immobilized metal affinity chromatography (IMAC), specifically a Co^{2+} ion, to bind the expressed 6x histidine tag. Bound proteins were eluted from the column with 150 mM Imidazole (Sigma-Aldrich).

Since pETCFA/Ib was expressed in *E. coli*, it underwent a further purification step in order to remove it from LPS. This was accomplished via ion exchange chromatography using a Q2 Column (Bio-Rad, Hercules,CA). LPS removal was

confirmed via the Limulus amebocyte lysate test (Associates of Cape Cod, E. Falmouth, MA).

SDS-PAGE and Western Blot Analysis

After purification, each protein was then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE, and evaluated for protein quality. The purified protein was also subjected to a Western blot by transfer to nitrocellulose membrane via wet transfer using the Mini Trans-Blott Electrophoretic Transfer Cell (Bio-Rad) overnight (O.N.) at 4mAmps. The nitrocellulose membrane was then blocked for 10 minutes at room temperature (R.T.) with 5% dry milk. pPICBCFA/Ie was detected using an anti-histidine monoclonal primary antibody (Amersham, Pittsburgh, PA) applied O.N. at 4˚C at a dilution of 1:3000. pETCFA/Ib was detected using an anti-CFA/I polyclonal primary antibody (made in-house) applied O.N. at 4˚C at a dilution of 1:100. After washing, a horseradish peroxidase (HRP) linked anti-mouse IgG (Southern Biotech, Birmingham, AL) for anti-histidine primary Ab or a HRP-linked anti-rabbit IgG (Southern Biotech) for anti-CFA/I fimbriae primary antibody was applied at 1:1000 for 2 h at R.T. The membrane was then washed and developed by exposure to a 4-chloronapthanol tablet (Sigma-Aldrich) dissolved in MeOH diluted with Tris-buffered saline (TBS) and hydrogen peroxide.

CFA/I Fimbriae Purification

CFA/I fimbriae were produced by an *asd*+ balanced-lethal plasmid in *E. coli* referred to as H695.[38,39] First, starter cultures were grown O.N. at 37˚C at 125 rpm. These starter cultures were used to streak 6 13"x9" pans filled with 1L of Minca agar[54] and were grown upside down for 65 h at 37˚C. The resulting biomass was harvested, and the cells were disrupted via homogenized mixing. The protein-containing lysate was removed from cell debris via centrifugation. Next, the protein was precipitated using 20% ammonium sulfate O.N. at 4˚C. After centrifugation, the protein was resolubilized by dialysis into sterile reverse osmosis (RO) water. Insoluble proteins that co-precipitated were then removed via ultracentrifugation at 18,000 rpm for 1 hour. Finally, the CFA/I fimbrial protein was precipitated again with 20% ammonium sulfate followed by centrifugation and resuspension and finally dialysis to phosphate buffered saline (PBS). CFA/I fimbriae preparation was subjected to ion exchange chromatography, as described above, to remove LPS. CFA/I fimbriae quality and identity were confirmed via SDS-PAGE and Western blot using rabbit anti-CFA/I polyclonal antibodies at 1:100, as described above.

Mice

Female C57BL/6, BALB/c, or SJL mice were used for these studies and were maintained in the Montana State University (MSU) Animal Resources Center, Bozeman, Montana. They were fed sterile food, provided with sterile water *ad libitum*, and

maintained under HEPA-filtered barrier conditions in individually ventilated cages. Mice were free of bacterial and viral infections.

Oral Immunizations with H696

Mice were orally immunized with attenuated *Salmonella* typhimurium expressing CFA/I fimbriae, abbreviated H696. The *cfa*I operon was maintained as a balanced-lethal *asd*$^{+}$ mutation described above. Mice received 5 x 10^{9} CFUs of H696 via oral gavage after neutralization of stomach acid by oral gavage with 50% saturated sodium bicarbonate solution. CFA/I fimbrial expression on the surface of the attenuated *S.* typhimurium was confirmed via agglutination test with anti-CFA/I antibodies prior to immunization. The administered oral dose was confirmed via dilution plating.

Peptide Library

Peptide libraries were synthesized by Proimmune Inc./Sigma-Aldrich (Springfield, VA) and spanned the length of the described protein. All peptides were 15mer sequences. The cfaB peptides overlapped by 10 amino acids while both the C-terminal and N-terminal cfaE peptides overlapped by 12 amino acids. Peptide purity was >70%, and each peptide was quantified by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry. Peptides arrived lyophilized, and upon arrival were solubilized by vortexing in the reducing agent, DMSO, at a concentration of 80% DMSO/20% sterile RO water at 10 mg/mL. Once dissolved, this solution was further diluted in sterile RO water to a concentration of 1 mg/mL and was

aliquoted into 20 μL aliquots. Peptides were stored at -80˚C until use. Peptides were titrated prior to use, and culture concentration ranged from 1 μg/mL to 20 μg/mL of peptide. Peptides were determined to be LPS-free via the Limulus amebocyte lysate test (Associates of Cape Cod).

$CD4^+$ T cell Isolation and ELISPOT

Mice were sacrificed two weeks post-immunization with *Salmonella*-CFA/I. Spleens and mesenteric lymph nodes (MLN) from immunized mice were pooled and homogenized using glass douncers. Red blood cells (RBCs) were lysed by resuspending in ACK lysis buffer for 5 minutes at R.T. Lysate was removed from cell debris via centrifugation and passage through sterile Nitex. $CD4^+$ T cells were then sorted using Dynal CD4 negative isolation kit (Invitrogen). Syngenic feeder cells were depleted of $CD4^+$ and $CD8^+$ cells using a 1:3 dilution of 30-H12, GK1.5, and 2.43 hybridoma supernatant fluid made in-house followed by incubation with complement prior to irradiation, which was 100 rads/minute for 25 minutes (2500 rads). ELISPOT plates (MultiScreen HTS with Cellulose Ester Membrane, Millipore , Billerica, MA) were prepared by coating O.N. at 4˚C with the following antibodies at 5 μg/mL: anti-IFN-γ (eBioscience, Inc., San Diego, CA), anti-IL-4 (BD Pharmingen, Sparks, MD), anti-IL-13 (R and D Systems, Minneapolis, MN), anti-IL-10 (BD Pharmingen), and anti-TGF-β (R and D Systems). Plates were then blocked for 1-2 h in complete medium (cRPMI) supplemented with 0.1% 50mM β-mercaptoethanol at 37˚C and 5% CO_2. Cells were resuspended in cRPMI and cultured at either 2 $x10^6$ cells/mL or 2.5 x 10^6 cells/mL with

10 units/mL of human IL-2 in the presence of irradiated feeder cells at a concentration of 1:1 and peptides or proteins at indicated concentrations for two days at 37˚C and 5% CO_2. Where indicated, "naïve" cultures, were $CD4^+$ cells from naïve mice cultured with the immunodominant pool as described and indicated within each figure. "Unstimulated" cultures, where indicated, were $CD4^+$ cells from *Salmonella*-CFA/I mice not restimulated with protein or peptide. "DO11.10" cultures, where indicated, were $CD4^+$ cells from *Salmonella*-CFA/I mice restimulated with $OVA_{323\text{-}339}$.

Twelve-well or 24-well BD Falcon plates were utilized for tissue culture. Cells were cultured in duplicate for 48 h. Then duplicate wells were pooled and cells were counted and plated onto coated ELISPOT plates in triplicate at 1:1, 1:2, and 1:4 and were cultured an additional 24 h at 37˚C and 5% CO_2. Next, ELISPOT plates were washed 3x with PBSTween followed by 2 washes with PBS and biotinylated secondary antibodies (BD Pharmingen for IFN-γ, IL-4, and IL-10; or R and D Systems for TGF-β and IL-13) were applied at 1:1000 O.N. in a humidifying chamber at 4˚C. Then the plates were washed again, as previously described, and an anti-biotin-HRP (Vector Laboratories) labeled antibody was applied at 1:1000 O.N. in a humidifying chamber at 4˚C. The plates were again washed and developed in a solution of 2% 50X peroxidase substrate (Moss, Inc., Pasadena, MD) and 10% AEC 20X buffer (Moss, Inc.) dissolved in sterile RO water. The developing reaction was allowed to proceed for 1 hour and was stopped with tap water. Plates were allowed to dry overnight, and wells were then evaluated for spot formation. ELISPOT data was normalized to report as cytokine-forming cells (CFCs) /10^6 $CD4^+$ T cells.

Cytokine ELISA

CD4$^+$ T cells and feeder cells were sorted from spleens and MLNs two weeks post-immunization with *Salmonella*-CFA/I and prepared, as described above, for T cell ELISPOT assays. Cells were cultured with peptides or proteins at indicated concentrations for three days at 37˚C and 5% CO_2. Unstimulated, naïve, and/or DO11.10 $OVA_{323-339}$ were utilized as described above. Twelve-well or 24-well plates by BD Falcon were used for culture. After three days, cultures were collected and spun via centrifugation; supernatants were collected and frozen at -20˚C until evaluated for cytokine production.

Capture ELISAs were performed by first coating 96-well flat-bottom plates from Greiner Bio-One (Monroe, NC) with unlabeled antibodies at 10 μg/mL (anti-IFN-γ), 4 μg/mL (anti-IL-13), or 2 μg/mL (anti-IL-4, anti-IL-10, anti-TGF-β) O.N. at 4˚C. Plates were blocked for a minimum of 2 h at 37˚C in 1% bovine serum albumin (BSA) in PBS. Plates were then washed 3x in PBS with Tween followed by 2 washes in PBS. Supernatants were applied in triplicate in a 50 μL volume to each well. Two to three rows on each plate were also devoted to a standard curve comprised of dilutions of the purified cytokine of interest. Supernatants and standard curve proteins were incubated in triplicate O.N. at 4˚C. After washing, a biotinylated antibody (BD Pharmingen for IFN-γ, IL-4, and IL-10 or R and D Systems for TGF-β and IL-13) to each cytokine was applied at a concentration of 1:1000, and plates were incubated for 2 h at R.T. (TGF-β, IL-13), 1 h at R.T. (IFN-γ), or 45 min at R.T. (IL-4, IL-10). Following washing, an HRP-labeled anti-biotin antibody (Vector Laboratories) was applied at a concentration of 1:1000 for 1

h at R.T. Plates were then washed and 1X peroxidase substrate (Moss, Inc.) developer was added for 40 min. Absorbances were measured at 415 nm on a VERSAmax Ext. microplate reader (Molecular Devices, Sunnyvale, CA).

Experimental Autoimmune Encephalomyelitis (EAE) Induction

MOG-dependent EAE was induced in 6- to 8-week old female C57BL/6 mice via a single peptide dose. MOG_{35-55} (Bio-Synthesis, Inc. Lewisville, TX) peptide was emulsified in complete Freund's adjuvant (CFA) with 4 mg/mL of killed *Mycobacterium tuberculosis,* which was subcutaneously (s.c.) injected in 100 µL into the flank of anesthetized mice on Day 0. Mice also received 200 ng of pertussis toxin (PT; List Biological Laboratories, Inc., Campbell, CA) intraperitoneally (i.p.) on Day 0 and Day 2. Mice were evaluated daily for clinical scores which were evaluated as described in Table 1.

Table 1: Clinical Scores for EAE

Score	Clinical Observation
0	Normal
1	Limp Tail
2	Limp Tail; Hind end Dysfunction
3	Limp Tail; Hind end Paralysis
4	Limp Tail; Hind end Paralysis; Partial Front end Paralysis
5	Moribund

Intramuscular and/or Intranasal Administration of Proteins in EAE-Induced Mice

Mice received 80 µg of either purified CFA/I fimbriae, cfaB protein, cfaE or 80 µg each of both proteins. Mice receiving intranasal (i.n.) proteins received 80 µg of protein in a maximum of 35 µL per dose under light anesthesia. Mice received protein either on day-7, Day +1, or day +6 respective to EAE induction. Mice receiving intramuscular (i.m.) proteins received 80 µg of protein in a maximum of 40 µL of protein in the flank under anesthesia. PBS control mice received an equal volume of PBS administered via the same route also under anesthesia.

Proliferation Assay

$CD4^+$ T cells and feeder cells were prepared, as described for T cell ELISPOTs. $CD4^+$ T cells were cultured in 96-well plates (BD Falcon, Sparks, MD) at $2x10^5$cells/well with feeders at a ratio of 1:1, 10 units/mL of human IL-2, and peptides or proteins at 1 µg/mL or 10 µg/mL, respectively. Cells were cultured in triplicate for 72 h at 37˚C, 5% CO_2 followed by pulsing with ^{3}H-TdR for 18 h (0.5 µCi/well). Thymidine incorporation was measured using a liquid scintillation counter, and proliferation was expressed as a stimulation index (S.I.).

Fluorescence Activated Cell Sorter (FACS) Analysis and Intracellular Staining

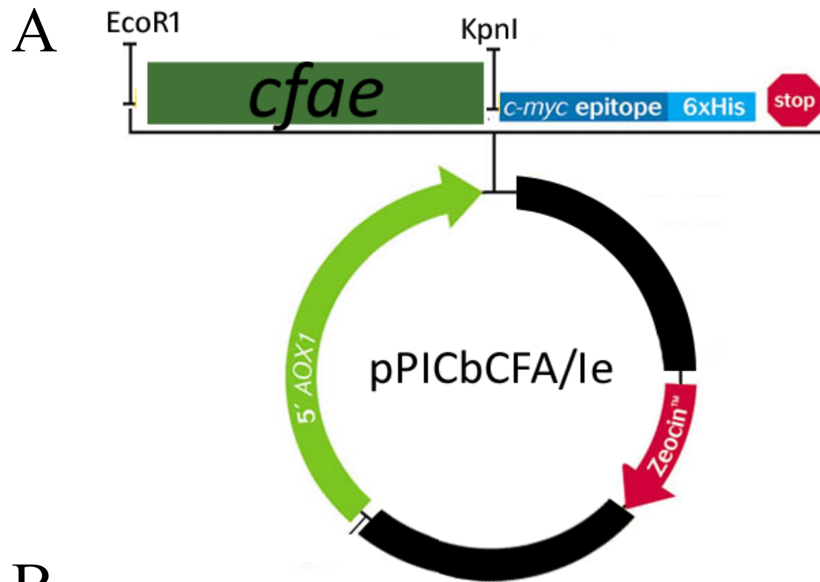
$CD4^+$ T cells were sorted from spleens and MLNs two weeks post-H696 immunization and prepared, as described above, for T cell ELISPOTs. Feeder cells were

also prepared, as described above. $CD4^+$ T cells were resuspended in cRPMI and cultured at either 2 $x10^6$ cells/mL in the presence of irradiated feeder cells at a concentration of 1:1 and peptides at 5 μg/mL (peptide 25) or 20 μg/mL (all other peptides) and proteins at 10 μg/mL at indicated concentrations for two days in 24-well BD Falcon plates at 37˚C and 5% CO_2. Cells were then stained for FACS analysis with mAbs to the following mouse cell surface markers: CD4 (PerCp-Cy5.5 or FITC, BD Pharmingen), CD25 (PE-Cy7, eBioscience), and TGF-β-biotin (R and D Systems) followed by streptavidin-APC-Cy7 (eBioscience). Cells were then fixed with 2% paraformaldehyde in PBS and permeabilized with 0.2% saponin in PBS according to standard protocols prior to staining with the following intracellular stains to mouse cytokines: EBI3 (LifeSpan Biosciences, Seattle, WA) conjugated in-house to Alexa Fluor (AF)-488, per manufacturer's instructions), IL-12p35-AF647 (eBiosciences), IL-27p28-PE (Biolegend, San Diego, CA), IL-4-APC, IL-10-PE, and IFN-γ-PerCp-Cy5.5 (eBiosciences). Samples were read on a BD Biosciences LSR II FACS machine, and 200,000 events were collected for each sample.

RESULTS

Our previous work has demonstrated that *Salmonella*-CFA/I (H696) stimulates a Th2 cell response within the first two weeks post-immunization.[29,39] Additionally, H696-treatment also induces regulatory T (T_{reg}) cells.[43,48] For this reason, in an effort to determine the responsible peptide or peptides from CFA/I fimbriae capable of inducing $CD4^+$ T cells from immunized mice to generate a response equivalent to that of whole CFA/I fimbrial antigen, MHC II restricted epitopes were examined. To evaluate the contributions of the major and minor fimbrial subunits of CFA/I fimbriae individually, *cfaE* and *cfaB* were also cloned into individual expression vectors (Figure 4A, 5A). *cfaE* was modified to contain restriction sites ECORI and KPNI and was then inserted into the pPICZB vector for expression in *Pichia pastoris.* cfaE protein was purified via its carboxyl terminal histidine tag. Protein quality of cfaE was determined via Coomassie Blue staining and detection in Western blot via anti-His tag mAb (Figure 4B). *cfaB*, modified to contain HINDIII and XHOI, was inserted into the pET32a vector and was expressed in *E. coli* (Figure 5A). Purified cfaB protein quality was verified via Coomassie Blue staining (data not shown) and detection in Western blot by using pAb against whole CFA/I fimbriae (made in-house; Figure 5B).

To determine the optimal mouse strain for future studies, we first evaluated cytokine profiles from mice immunized with H696. C57BL/6, BALB/c, and SJL mice were selected for testing due to previous use in experiments with measurable responses to CFA/I fimbriae.[29,39,40,43,44,55,56] These mice were immunized with *Salmonella*-CFA/I orally and were sacrificed two weeks post-immunization prior to the previously



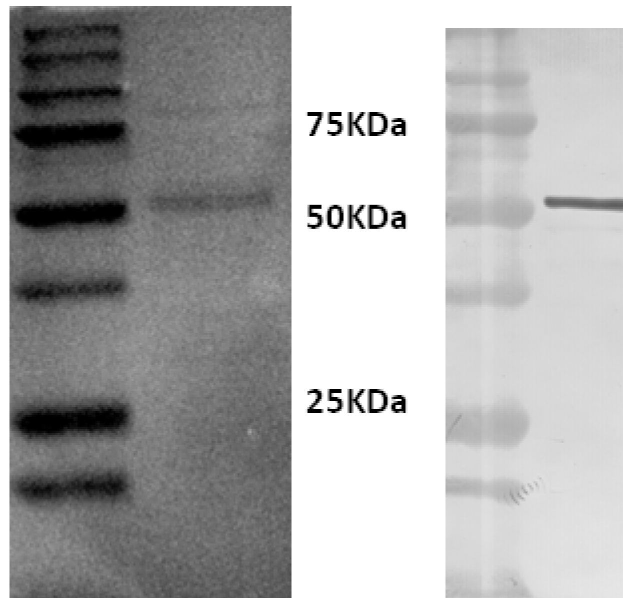


Figure 4: Plasmid map and protein expression of cfaE in pPICBZ vector. *cfaE* was inserted into the multiple cloning site of pPICZB. C-terminal of cfaE is a c-myc epitope followed by a 6 histidine tag used for purification (A). SDS-PAGE (left, B) and Western blot (right, B) of cfaE. Western blot was probed with anti-histidine monoclonal antibodies. pPICBCFA/Ie plasmid map adapted from invitrogen.com.

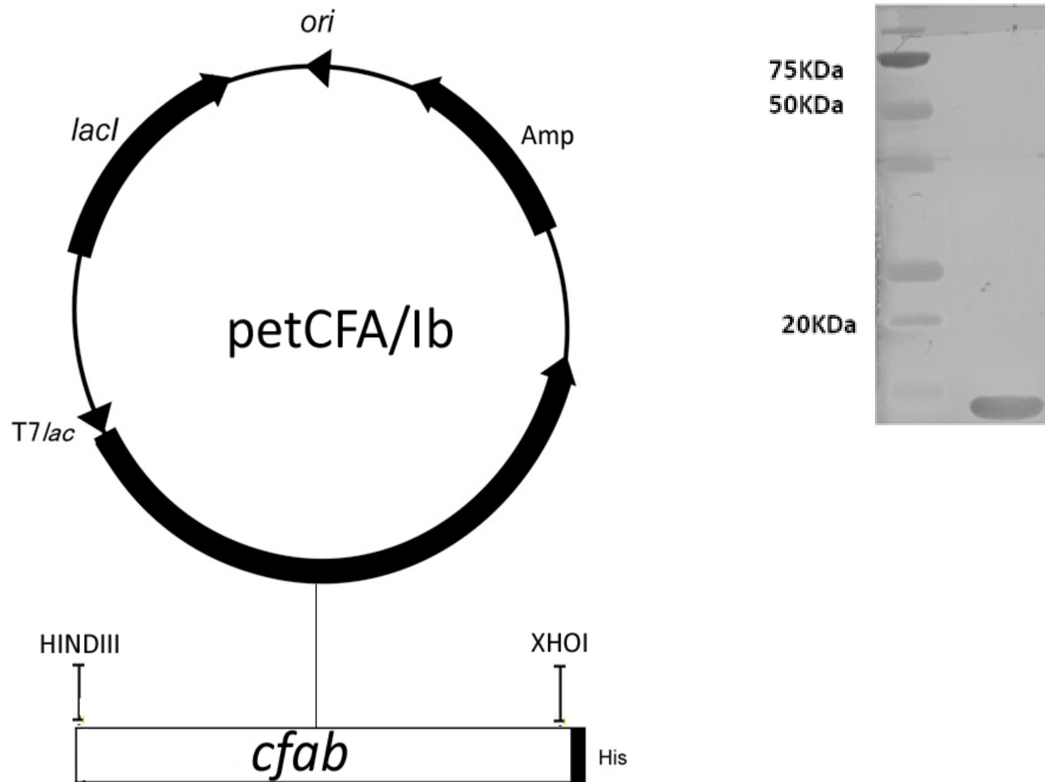


Figure 5: Plasmid map and protein expression of cfaB in the pET-32a.
cfaB was inserted into the multiple cloning site of pET-32a. It was modified to contain only a C-terminal 6-histidine tag, which was used for purification(A). Western blot of cfaB probed with anti-CFA/I polyclonal fimbriae antibody (B).

described shift to Th1 cell responses and after development of T_{reg} cells. Sorted CD4^{+} T cells were restimulated with purified CFA/I fimbriae, cfaE, or cfaB and evaluated for cytokine expression via T cell ELISPOT. Although sorted cells from all mouse strains generated cytokines following restimulation, C57BL/6 mice were selected for future experiments due to robust responses of IFN-γ, IL-4, and IL-10 from CFA/I fimbriae and cfaB (Figure 6). Cytokine production from splenic CD4^{+} T cells is shown and was similar to results seen in C57BL/6 MLN CD4^{+} T cells (data not shown).

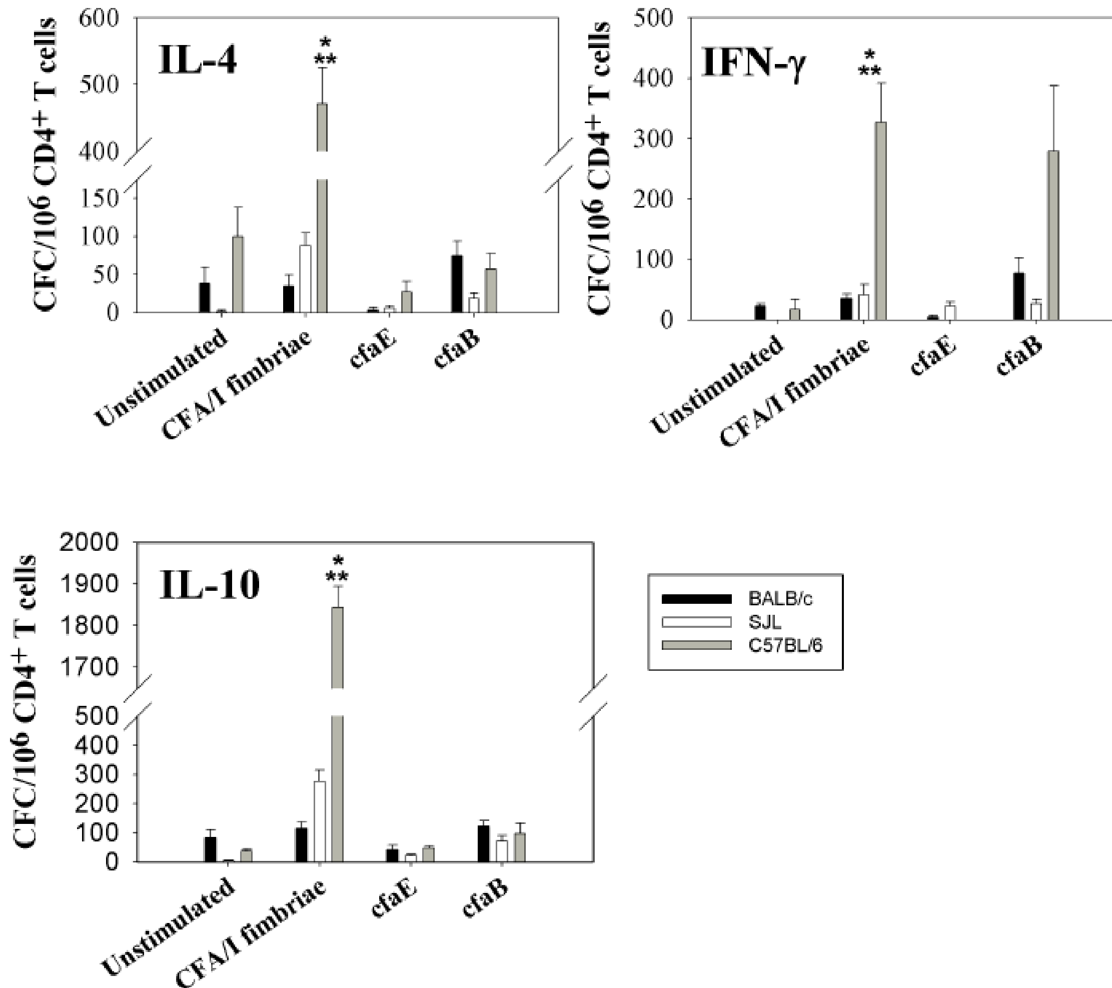


Figure 6: C57BL/6 mice selected for future studies based on robust cytokine response. Sorted splenic $CD4^+$ T cells from H696-treated mice were cultured with IL-2 (10 units/mL) and CFA/I fimbriae (10 µg/mL), cfaE (10 µg/mL), cfaB (10 µg/mL), or media alone (Unstimulated) and then evaluated for IL-4 (A), IFN-γ (B), or IL-10 (C) production via T cell ELISPOT. Data represent the average number of CFC/10^6 $CD4^+$ T cells from triplicate cultures +/- S.E.M. Statistics were calculated using ANOVA/ Tukey test. * $p < 0.001$ versus control; ** $p < 0.001$ versus other mouse strains subjected to identical culture.

Splenic $CD4^+$ T cells cultured with cfaB were responsible for the majority of observed IFN-γ production (Figures 6A, 7). Culture with cfaE generated more IL-4 producing $CD4^+$ T cells than medium alone, CFA/I fimbriae, or cfaB in both the spleen

and MLN (Figure 7). Of note, only CFA/I fimbriae produced a significant level of IL-10, suggesting the necessity of both subunits for the production of this cytokine (Figure 7).

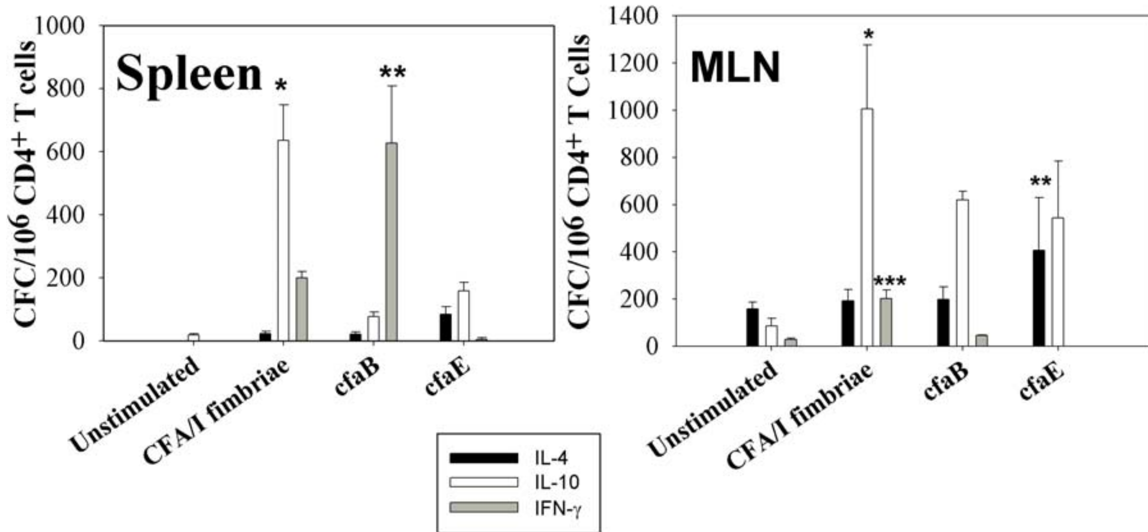


Figure 7: Key Th1 and Th2 cell cytokines evaluated following culture with CFA/I fimbriae or independently expressed cfaB or cfaE.
Sorted splenic (A) or MLN (B) CD4$^+$ T cells from H696-treated C57BL/6 mice were cultured with IL-2 (10 units/mL) and CFA/I fimbriae (10 μg/mL), cfaE (10 μg/mL), cfaB (10 μg/mL), or media alone (Unstimulated) and then evaluated for IL-4, IL-10, or IFN-γ production via T cell ELISPOT. Data represent the average number of CFC/10^6 CD4$^+$ T cells from triplicate cultures +/- S.E.M and are representative of 2 independent experiments. Statistics were calculated using ANOVA/ Tukey test. * $p < 0.01$ or **; $p < 0.008$; *** $p < 0.001$ versus control.

Each subunit was also compared *in vivo* for its ability to ameliorate EAE clinical disease, as previously observed with H696[29,43,55] (Figure 8). C57BL/6 mice were treated intramuscularly (i.m.) with 100 μg of CFA/I fimbriae, cfaE, cfaB, or PBS seven days prior to EAE challenge. Only mice receiving CFA/I fimbriae showed significant reduction in average clinical scores or weight loss when compared to the PBS control group (Figure 8A), reiterating the importance of both subunits in the generation of maximally therapeutic CD4$^+$ T cells.

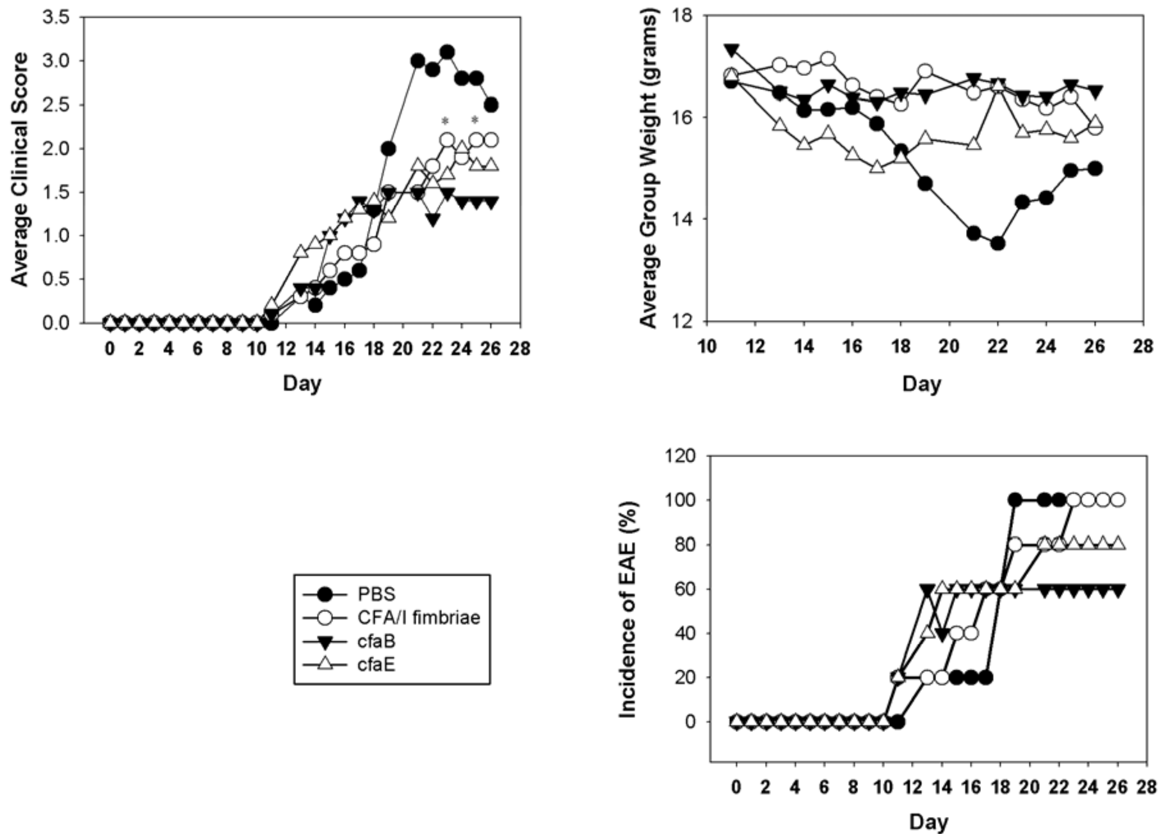


Figure 8: EAE challenge in C57BL/6 mice receiving CFA/I fimbriae, cfaB, cfaE, or PBS i.m. 1 week prior to challenge.
C57BL/6 mice (n = 5 mice/group) were immunized i.m. with 100 µg of indicated protein 1 week prior to EAE challenge. Graphs represent Average Clinical Scores (A), Average Weight (B), or Incidence of Disease (C). *, $p < 0.05$, ANOVA/ Mann-Whitney test versus PBS-treated mice from the same day.

Since none of the three treatment groups were able to protect mice as previously observed with H696,[29,43,55] nor was individually administered cfaE or cfaB able to reduce disease as well as CFA/I fimbriae (Figure 8), mice received cfaE and cfaB in tandem. Specifically, C57BL/6 mice received 80 µg of cfaE and 80 µg of cfaB on Days +1 and +6 of EAE induction (Figure 9). To determine the potential effects of administration route, treatments were either i.m., i.n., or a combination of one i.m. dose followed by one i.n.

dose. Mice receiving proteins first by i.m. (Day +1) and then by i.n. (Day +6) displayed significantly lessened disease (Figure 9), demonstrating the necessity of each protein's presence for disease therapy. These data also show that an administration route bias exists, with the combination of i.m. and i.n. dosing being the most effective compared with i.n. alone or i.m. alone (Figure 9).

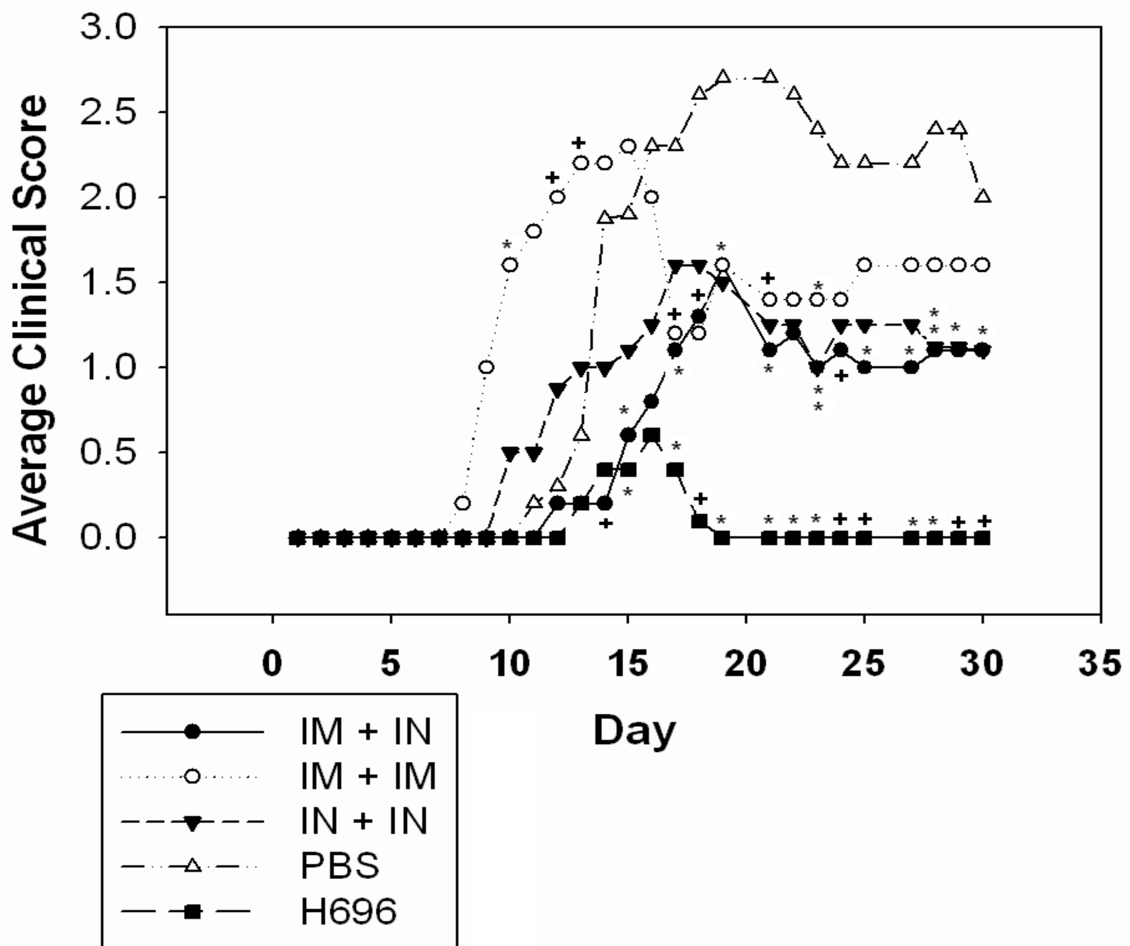


Figure 9: EAE challenge in mice receiving cfaE and cfaB i.n. and/or i.m. on Days +1 and +6 of EAE challenge.
C57BL/6 mice (n = 5 mice/group) were immunized i.n. and/or i.m. with 80 μg of both cfaE and cfaB on days +1 and +6 of EAE challenge. Graph represents Average Clinical Scores of mice from day 1 to day 30. *$p < 0.05$; + $p \leq 0.01$ ANOVA/ Mann-Whitney or Student's *t*-test.

In order to determine the region of each fimbrial subunit responsible for the observed cytokine responses and reduced EAE, peptide libraries were generated to both cfaE and cfaB (Tables 2-4), which spanned the length of each protein. Synthesized peptides were 15 amino acids long and overlapped by either 10 amino acids (cfaB library) or 12 amino acids (cfaE libraries). Peptide libraries were titrated by pooling all peptides and testing restimulation responses of $CD4^+$ T cells at increasing concentrations, ranging from 0.1 μg/mL to 100 μg/mL, in order to determine the optimal concentrations for culture. For cfaB peptides, the optimal concentration was 5 μg/mL (data not shown). For screening pools, total peptide concentrations were 5 μg/mL. For individual peptide cultures, each peptide was cultured at 5 μg/mL.

Table 2: cfaB Peptide Library Pools and Response

Pool #	Peptides Included	Observed Response
1	1-4	
2	5-8	
3	9-12	
4	13-16	
5	17-20	
6	21-24	
7	25-28	IFN-γ Production, Proliferation
8	29-32	
7a	25,26,27	IFN-γ Production
7b	25,26,28	IFN-γ Production
7c	26,27,28	

Table 3: C-terminal cfaE Peptide Library Pools and Response

Pool #	Peptides Included	Observed Response
1	1-6	IL-4 and IL-10 production
2	7-11	
3	12-16	
4	17-21	
5	22-26	
6	27-31	
7	32-36	
8	37-41	
9	42-46	
10	47-51	
11	52-56	

Table 4: N-terminal cfaE Peptide Library Pools and Response

Pool #	**Peptides Included**	**Observed Response**
1	1-5	
2	6-10	
3	11-15	
4	16-20	
5	21-25	
6	26-30	
7	31-35	
8	36-40	
9	41-45	
10	46-50	
11	51-55	
12	56-60	

To confirm the peptide restimulation assay could efficiently measure T cell responses, restimulation of $CD4^+$ T cells with peptide was verified by utilizing DO11.10 OVA transgenic mice (H2-d). These mice express a TCR from the T cell hybridoma, DO11.10, which recognizes chicken ovalbumin ($OVA_{323-339}$).[12,57] $CD4^+$ T cells from DO11.10 transgenic mice were sorted and cultured in the presence of irradiated feeder cells with either medium alone, OVA protein, or $OVA_{323-339}$. $CD4^+$ T cells were evaluated for both proliferation and cytokine production via Carboxyfluorescein Succinimidyl Ester (CFSE) staining and intracellular staining, respectively. $CD4^+$ T cells cultured with both OVA protein and $OVA_{323-339}$ displayed proliferation and IFN-γ and IL-4 production (data not shown), indicating exogenous peptide is able to stimulate $CD4^+$ T cells under the culture conditions used herein.

To map immunodominant epitopes from cfaB, $CD4^+$ T cells from H696-immunized C57BL/6 mice were cultured with one of eight cfaB peptide pools (Table 2) and were monitored for cytokine production. Unstimulated $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice, as well as $CD4^+$ T cells from naïve mice, were cultured either with media alone or with the immunodominant pool from each peptide library, respectively, as controls. In initial experiments, $CD4^+$ T cells were further subdivided into either $CD25^+$ or $CD25^-$ cells, but the best cytokine response was generated by utilizing whole $CD4^+$ T cell populations (data not shown). Culture of whole $CD4^+$ T cells with cfaB peptide pools revealed that the active fraction was within pool 7, which consistently generated IFN-γ devrived from cells of both the spleen and MLN

(Figure 10). Culture with individual peptides from pool 7 indicated peptide 25 was responsible for the majority of the IFN-γ production activity (Figure 10B and 10D).

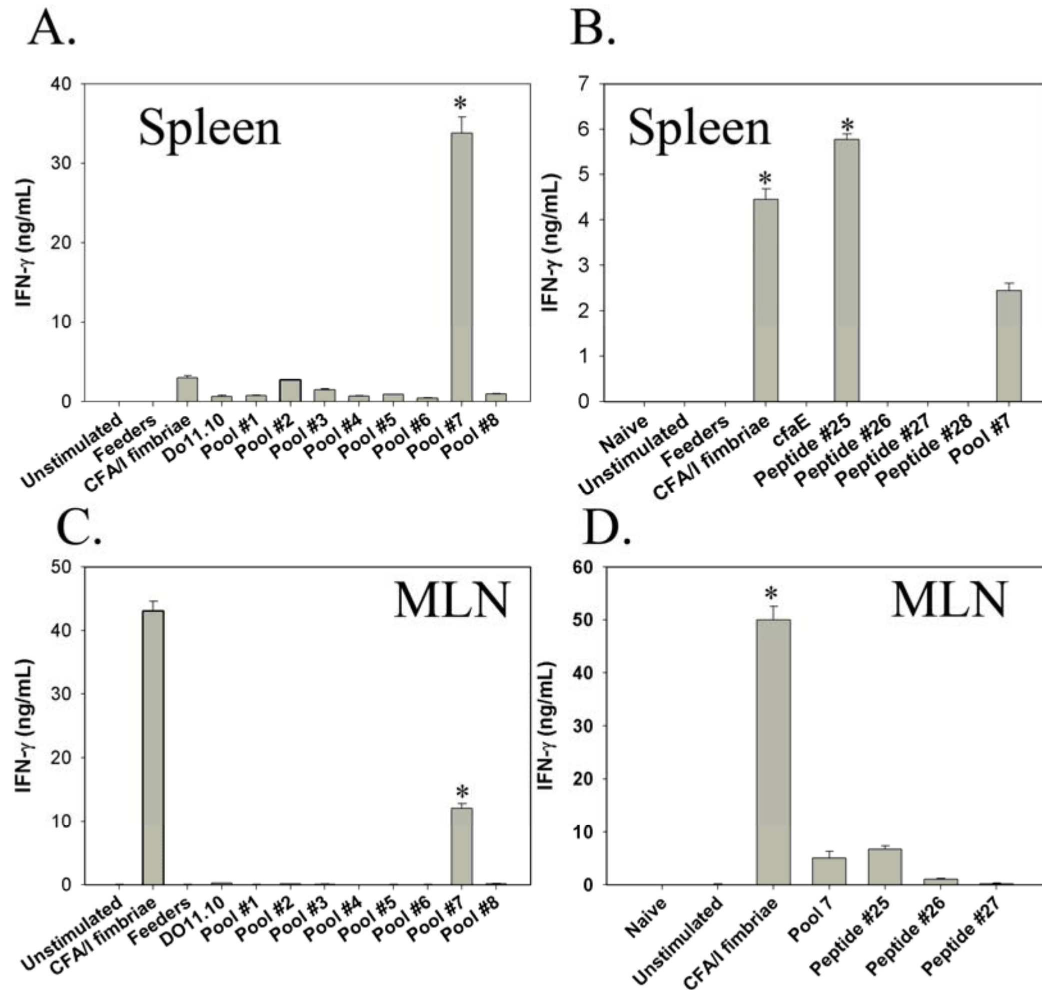


Figure 10: IFN-γ production by $CD4^+$ T cells from H696-treated C57BL/6 mice cultured with cfaB peptide pools.
Sorted $CD4^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I fimbriae (10 μg/mL) or with cfaB peptide pools (1 μg/mL per peptide) (A and C) or individual peptides (5 μg/mL) (B and D) and monitored for IFN-γ production via cytokine ELISA in the spleen (A and B) or MLN (C and D). $CD4^+$ T cells from naïve mice were cultured with pool #7 as a negative control. $CD4^+$ T cells from immunized mice were also cultured with DO11.10 peptide $OVA_{323\text{-}339}$ or media alone as a negative control. Data shown are the mean +/- S.E.M., n=3, and are representative of 3 independent experiments. Statistics were calculated using ANOVA/ Tukey test where $*p < 0.001$.

Although other cytokines (IL-4, TGF-β, IL-13) were also generated following culture with pool 7, these amounts were low and reproducibility was inconsistent (Figure 11). Neither unstimulated $CD4^+$ T cells nor naïve $CD4^+$ T cells cultured with pool #7 induced detectable cytokines (Figure 10), indicating that observed responses are indeed due to restimulation of $CD4^+$ T cells from immunized mice. Additionally, $CD4^+$ T cells from immunized mice failed to generate cytokines when restimulated with the DO11.10, peptide $OVA_{323-339}$, again indicating that the observed restimulation response was specific to CFA/I fimbriae peptides (Figure 10). To address the kinetics of the biphasic shift, mice were sacrificed one week earlier (7 days) or one week later (21 days) and evaluated for cytokine production. Peptide 25 was still the immunodominant peptide at either time point based on cytokine expression although sacrificing mice at 14 days post-H696 immunization yielded the optimal cytokine response (data not shown).

To confirm that peptide 25 was the immunodominant region within pool 7 with regards to cytokine production, subtraction assays were conducted using permutations of pool 7 (Table 2, pool 7a-7c). Culture with pool 7c, in which peptide 25 was absent, resulted in significantly less production of IFN-γ and TGF-β (Figure 12). This trend was also seen with regard to IL-13 production although the reduction was not significant. Eliminating any other peptide from pool 7 did not result in loss of cytokine production. Additionally, culture with individual peptides revealed that only peptide 25 was capable of generating IFN-γ (data not shown). These results confirmed that peptide 25 was the immunodominant region within cfaB, and the $CD4^+$ T cells, which recognized this peptide, generated significant levels of IFN-γ (Figure 13).

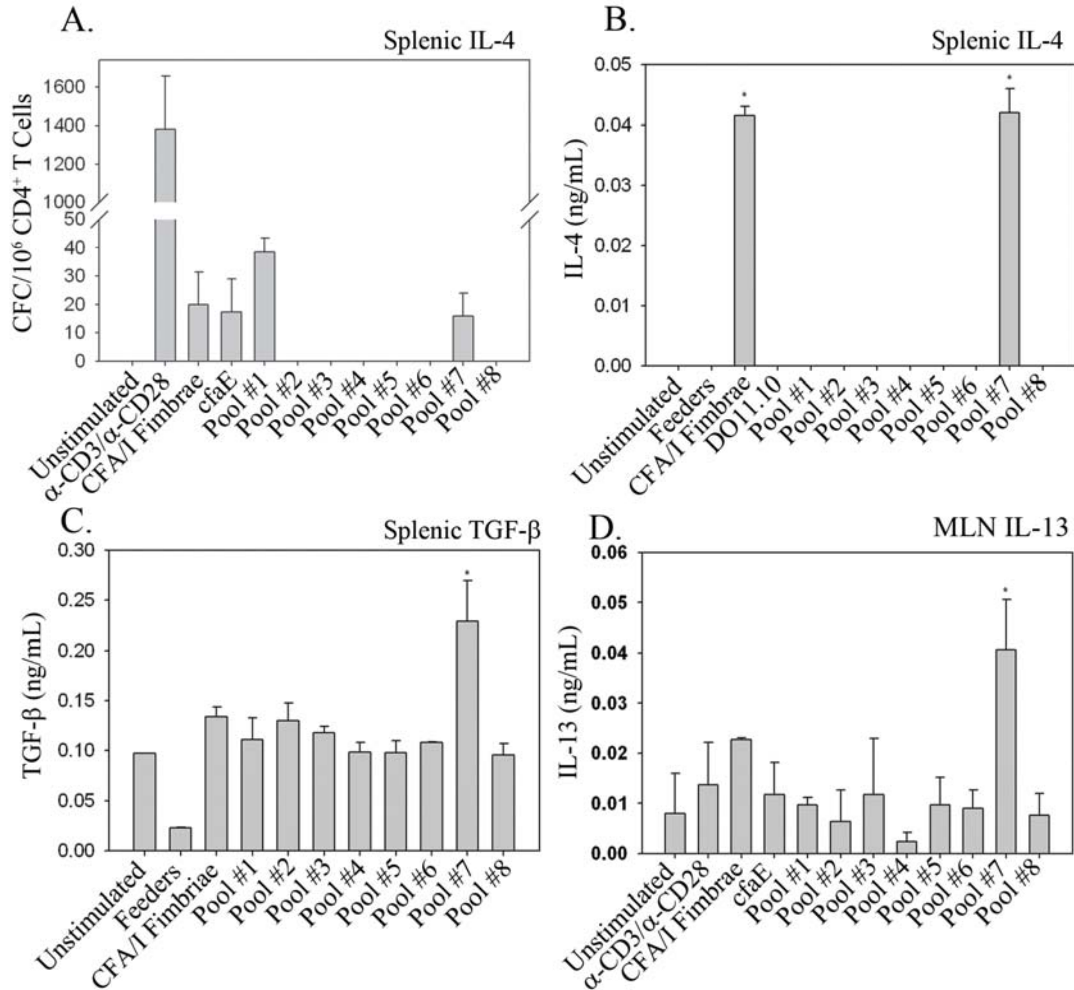


Figure 11: Generation of other cytokines following culture with cfaB peptide pools. Sorted CD4$^+$ T cells from H696-treated C57BL/6 mice were cultured with cfaB peptide pools (1 μg/mL per peptide) and monitored for cytokine production via T cell ELISPOT (A) or cytokine ELISA (B,C,D) in the spleen (A,B,C) or MLN (D). CD4$^+$ T cells from immunized mice were stimulated with α-CD3/α-CD28 as a positive control. Negative controls included restimulation with media alone (Unstimulated) or culture of feeder cells alone (Feeders). Data shown are the mean +/- S.E.M., n=3, and are representative of 3 independent experiments. Statistics were calculated using ANOVA/Tukey Test where * $p < 0.001$.

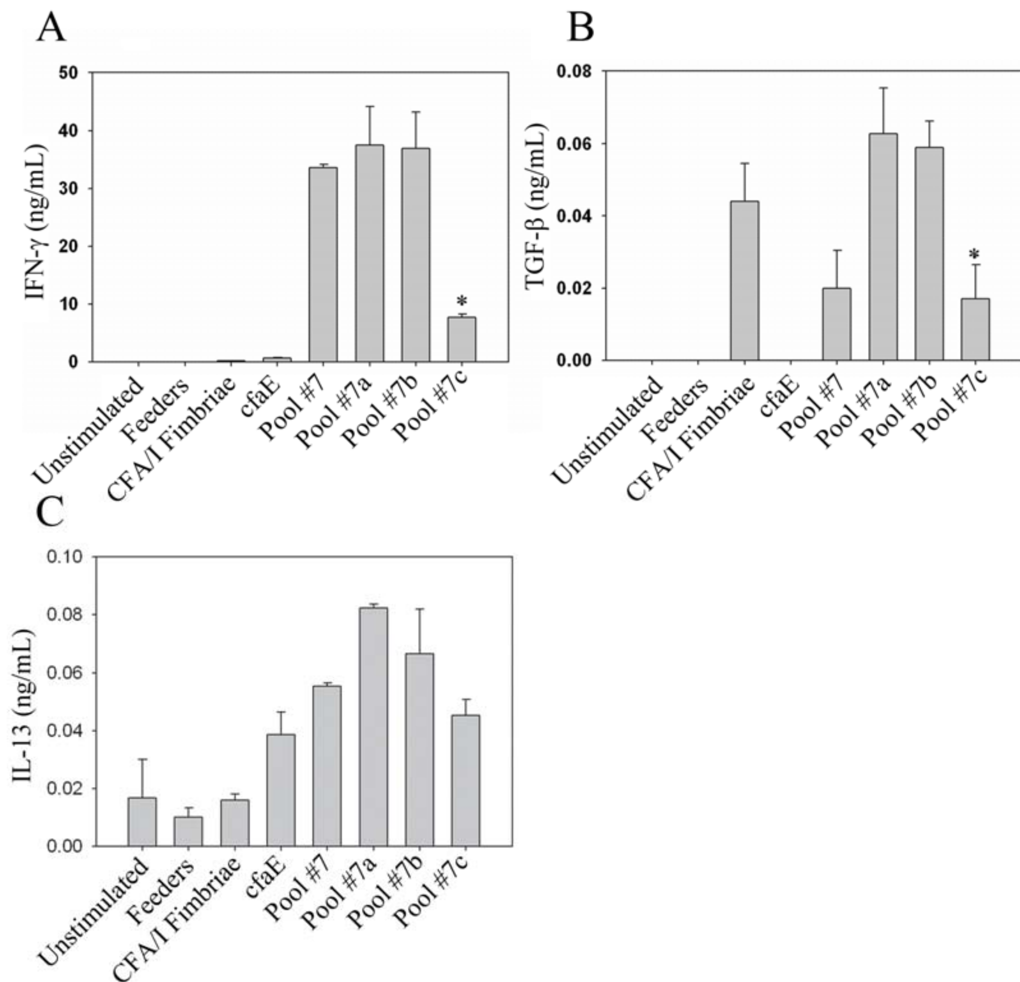


Figure 12 : Subtraction assay determining if a single peptide within pool #7 generates cytokines.
Sorted $CD4^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I fimbriae (10 μg/mL), cfaE (10 μg/mL), or cfaB peptide pools (1 μg/mL per peptide) and monitored for cytokine production via cytokine ELISA. Feeder cells (Feeders) or $CD4^+$ T cells from immunized mice (Unstimulated) were also cultured in media as a negative control. Data shown are the mean +/- S.E.M, n=3, and are representative of 2 independent experiments. Statistics were calculated comparing subtraction groups using ANOVA/ Tukey Test where * $p < 0.001$.

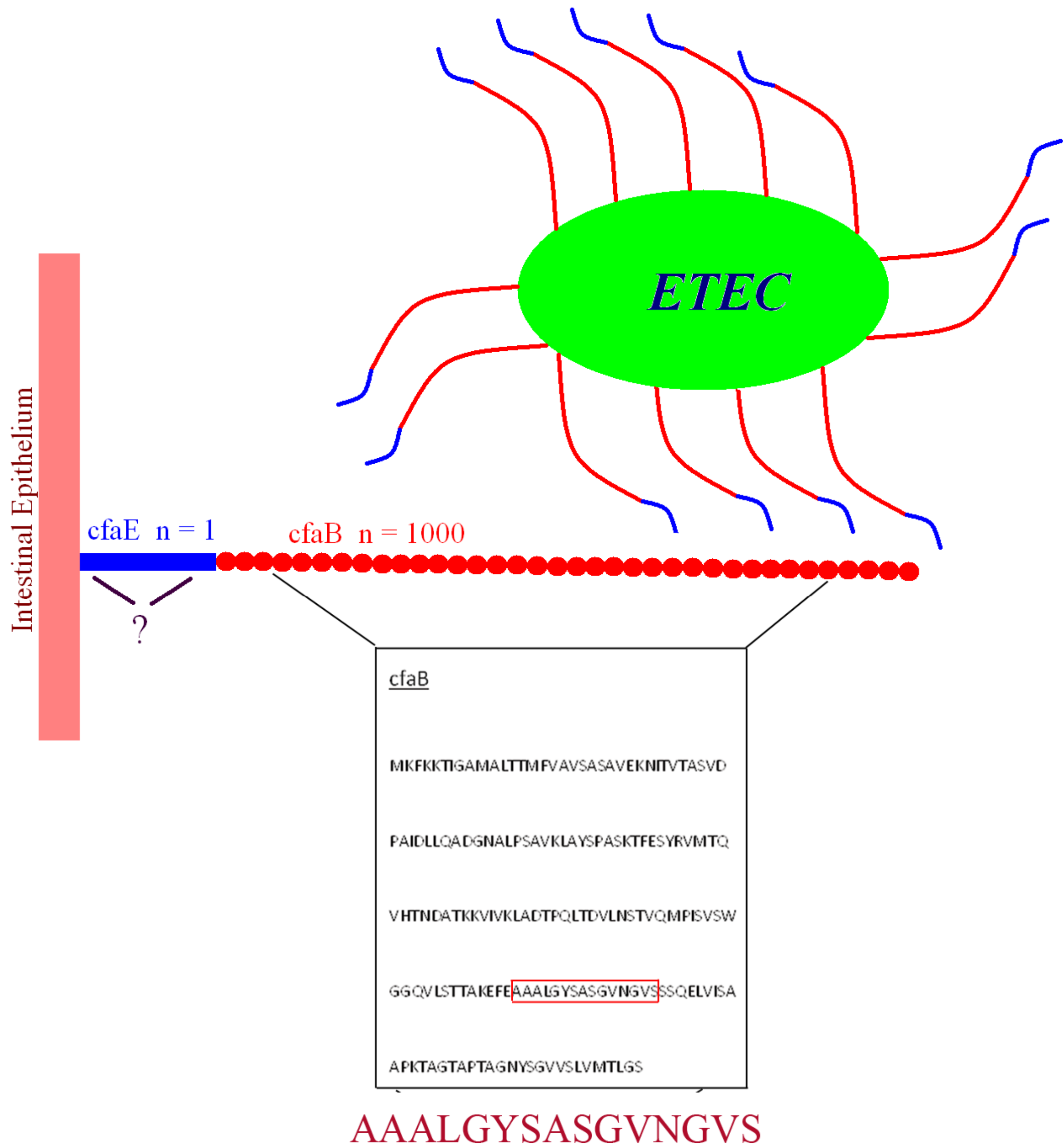


Figure 13 : Immunodominant region from major fimbrial subunit, cfaB.
The immunodominant region from cfaB is indicated in red, AAALGYSASGVNGVS.
Culture with this peptide resulted in IFN-γ production by $CD4^+$ T cells.

We also attempted to map immunodominant regions in cfaB based on $CD4^+$ T cell proliferation. Specifically, H696-immunized mice were sacrificed 2 weeks post-immunization, and $CD4^+$ T cells were cultured with individual cfaB peptides in the presence of feeder cells. After 3 days of culture, ^{3}H-thymidine was added to cell cultures, and 18 hours later, proliferation was calculated as counts per minute (cpm) of scintillation (Figure 14). A stimulation index was generated to normalize data by comparing observed proliferation resulting from restimulation with a single peptide to proliferation observed following restimulation with whole CFA/I fimbriae. The stimulation index generated from $CD4^+$ T cells cultured with peptide 25 was decreased after normalization when compared with CFA/I fimbriae stimulation, indicating that proliferation of $CD4^+$ T cells cultured with peptide 25 was below average (Figure 14). Since ^{3}H-thymidine uptake was measured in the total $CD4^+$ T cell population, it is possible that subsets within the $CD4^+$ population were proliferating in response to peptide 25, but remained undetected due to limitations from experimental design. Since we were interested in mapping epitopes that are responsible for generating the cytokine responses seen after H696 immunization, including Th1, Th2, and T_{reg} cells, we continued further analysis based on cytokine production rather than on proliferation.

Since cfaB overwhelmingly generated the Th1-type cytokine, IFN-γ, and immunization with *Salmonella*-CFA/I resulted in a biphasic T cell response preceded by a robust Th2 response, we hypothesized that the Th2-promoting region could be found in the minor fimbrial subunit, cfaE (Figure 13). Therefore, cfaE was next investigated for potential immunodominant regions that could stimulate Th2-type cytokines. The web-

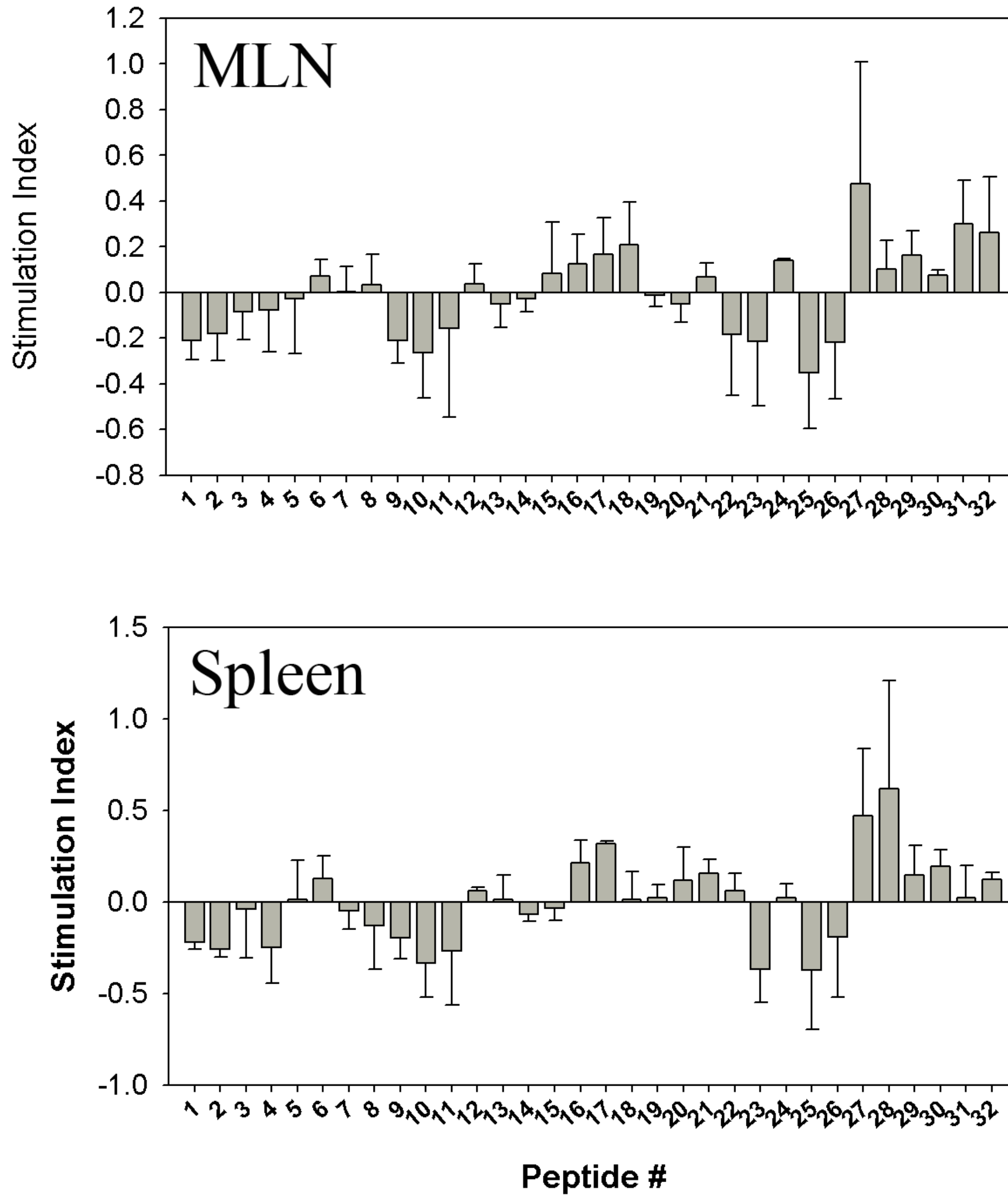


Figure 14: Proliferation of CD4$^+$ T cells from H696-treated C57BL/6 mice cultured with peptides from cfaB.

Proliferation of sorted CD4$^+$ T cells from spleens (top) or MLN (bottom) from H696-treated C57BL/6 mice. Sorted cells were cultured for 3 days with the indicated peptide (5 μg/mL) from cfaB along with feeder cells. Proliferation was determined by ^{3}H-thymidine uptake measured 18 hours after addition. Stimulation Index = [Average Peptide 'n' – Average Proliferation of all Peptides] / Average Proliferation of all Peptides. Data shown are cumulative proliferation from 3 independent experiments.

based tool NetMHCII[58] was utilized to predict regions of cfaE likely to have MHC II-restricted epitopes. Since this neural network predictor highlighted regions in the C-terminal half of cfaE as most likely to bind H2-b (C57BL/6) restricted MHC II, a peptide library spanning the C-terminal region of cfaE was first examined. Using the same experimental protocol used to evaluate cfaB peptides, the peptide library spanning the C-terminal region of cfaE (Table 3) was grouped into 11 pools and evaluated for cytokine production when cultured with $CD4^+$ T cells from H696-treated mice. Titration of C-terminal peptides was performed as described for cfaB peptides and indicated that the optimal culture concentration was 20 µg/mL (data not shown). For screening pools, total peptide concentrations were 20 µg/mL. For individual peptide cultures, each peptide was cultured at 20 µg/mL.

Overwhelmingly, pool 1 was responsible for significant levels of IL-4 produced by splenic $CD4^+$ T cells (Figure 15). A similar trend was also noted in MLN $CD4^+$ T cells (data not shown). Although pool 1 also appeared to generate robust levels of IL-10 in some experiments, this level was not significantly different from all other pools (Figure 15). These data suggest pool 1 is able to generate increased levels of IL-4, indicative of Th2 $CD4^+$ cells that recognize some of the peptide members; however, this pool is not recognized by T_{reg} cells capable of producing IL-10 or TGF-β, which are generated following H696 immunization.[43,48,55]

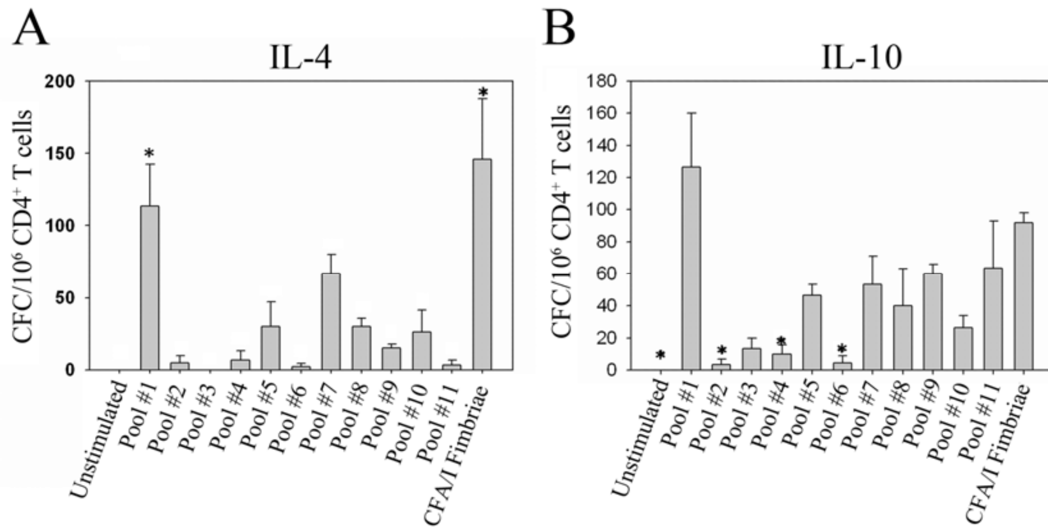


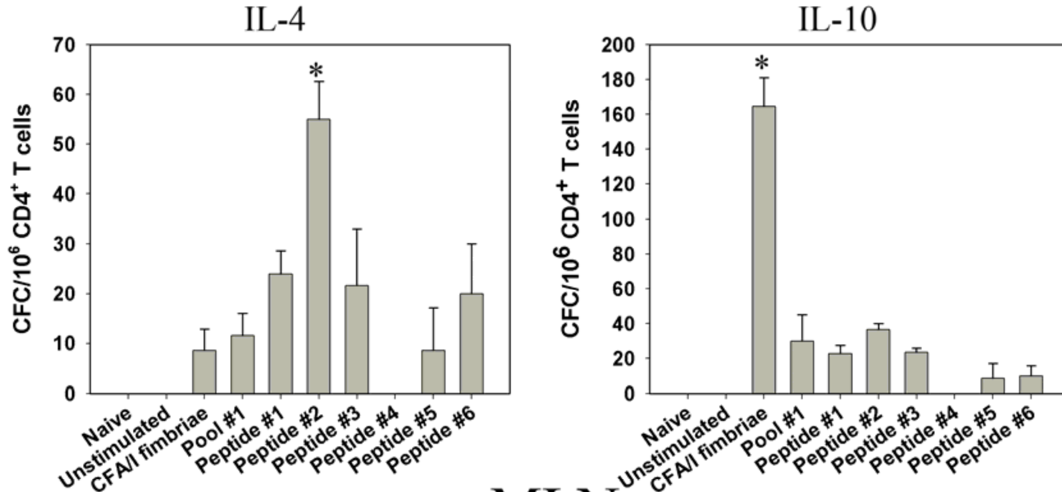
Figure 15: Cytokine production following culture with C-terminal cfaE peptide pools. Sorted CD4$^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I fimbriae (10 µg/mL) or with C-terminal cfaE peptide pools (5 µg/mL per peptide) and monitored for cytokine production via T cell ELISPOT. As a negative control, CD4$^+$ T cells from immunized mice were cultured in media alone (Unstimulated). Data shown are the mean +/- S.E.M, n=3, and are representative of 3 independent experiments. Statistics were calculated using ANOVA/ Tukey test when *$p < 0.001$ comparing pools to CFA/I.

Additionally, subtraction assays were also unable to discern the peptides within pool 1 responsible for IL-4 production (data not shown). Although culture with individual peptides from pool 1 indicates that peptide 2 generates the most IL-4, all peptides within the pool appear to contribute to some degree (Figure 16A and 16C). Individual peptides from pool 1 were also examined for their ability to generate IL-10 independently to control for inhibition of IL-10 production by other members of the pool. In a manner similar to IL-4, all peptides appeared to generate low levels of IL-10, but the amounts detected were not significant (Figure 16B and 16D). Also, there was not a single peptide within this pool that produced a significant level of this cytokine alone,

suggesting these individual peptides are not responsible for the generation of IL-10 producing T_{reg} cells. Of note, C-terminal cfaE peptides generated only low levels of IFN-γ, if any was detected at all (data not shown). Additionally, $CD4^+$ T cells from naïve mice restimulated with pool #1 failed to generate cytokine indicating that the observed cytokine production is due to restimulation of $CD4^+$ T cells by cfaE peptides (Figure 16). Collectively, these data suggest a region within the C-terminal region of cfaE is recognized by Th2 $CD4^+$ T cells that generate IL-4, but is not recognized by $CD4^+$ T cells, which produce cytokines associated with T_{reg} cell function (Figure 17).

Since an immunodominant region recognized by T_{reg} cells was not identified in the C-terminal half of cfaE, the peptide library spanning the N-terminal half of cfaE was examined. The N-terminal half was divided into 60 peptides grouped into 12 pools of 5 for initial experiments (Table 4). Titration of N-terminal peptides was performed as described for cfaB and C-terminal cfaE peptides and indicated that the optimal culture concentration was 20 μg/mL, just as for C-terminal cfaE peptides (data not shown). For screening pools, total peptide concentration was 20 μg/mL. For individual peptide cultures, each peptide was cultured at 20 μg/mL.

Preliminary screening of N-terminal cfaE peptides indicated that immunodominant regions might lie within pool 5 or pool 11 (data not shown). However, culture of sorted $CD4^+$ T cells from H696-treated mice with individual peptides from each of these pools revealed the absence of peptides within this group consistently generating any $CD4^+$ Th2 or T_{reg} cell cytokines (data not shown). Despite the fact that N-terminal cfaE pool 12 did not appear to render further investigation, due to its close



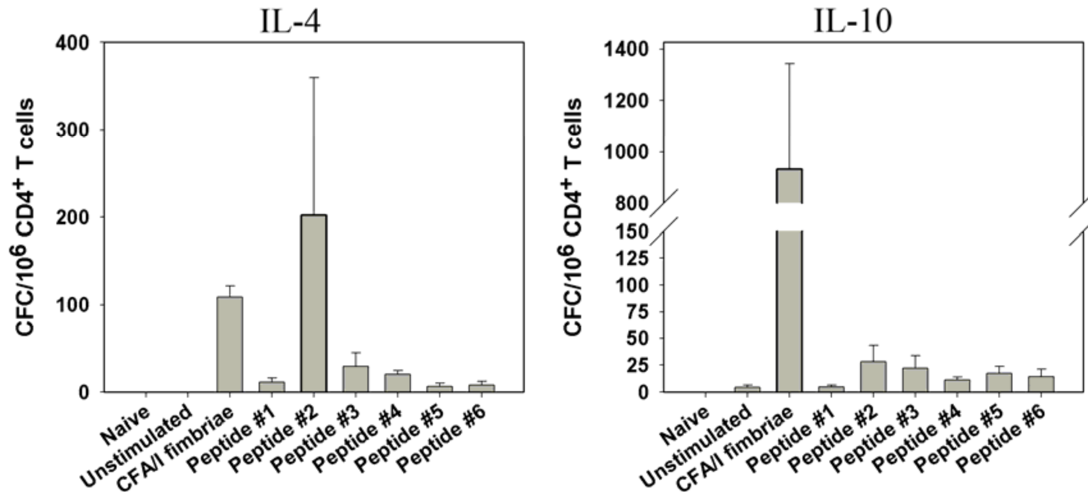


Figure 16: Determination of cytokine-producing peptide within C-terminal cfaE pool. Sorted $CD4^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I fimbriae (10 µg/mL) or with peptides from C-terminal cfaE pool 1 (20 µg/mL per peptide) and monitored for cytokine production via T cell ELISPOT. As controls, $CD4^+$ T cells from immunized mice were cultured with media alone (Unstimulated) or $CD4^+$ T cells from naïve mice were cultured with pool #1. Data shown are the mean +/- S.E.M, n=3, and are representative of 3 independent experiments (A,B) or are compiled data from 3 experiments each with triplicate cultures (C,D). Statistics were generated using ANOVA/ Tukey test comparing samples to CFA/I fimbriae. * $p < 0.001$.

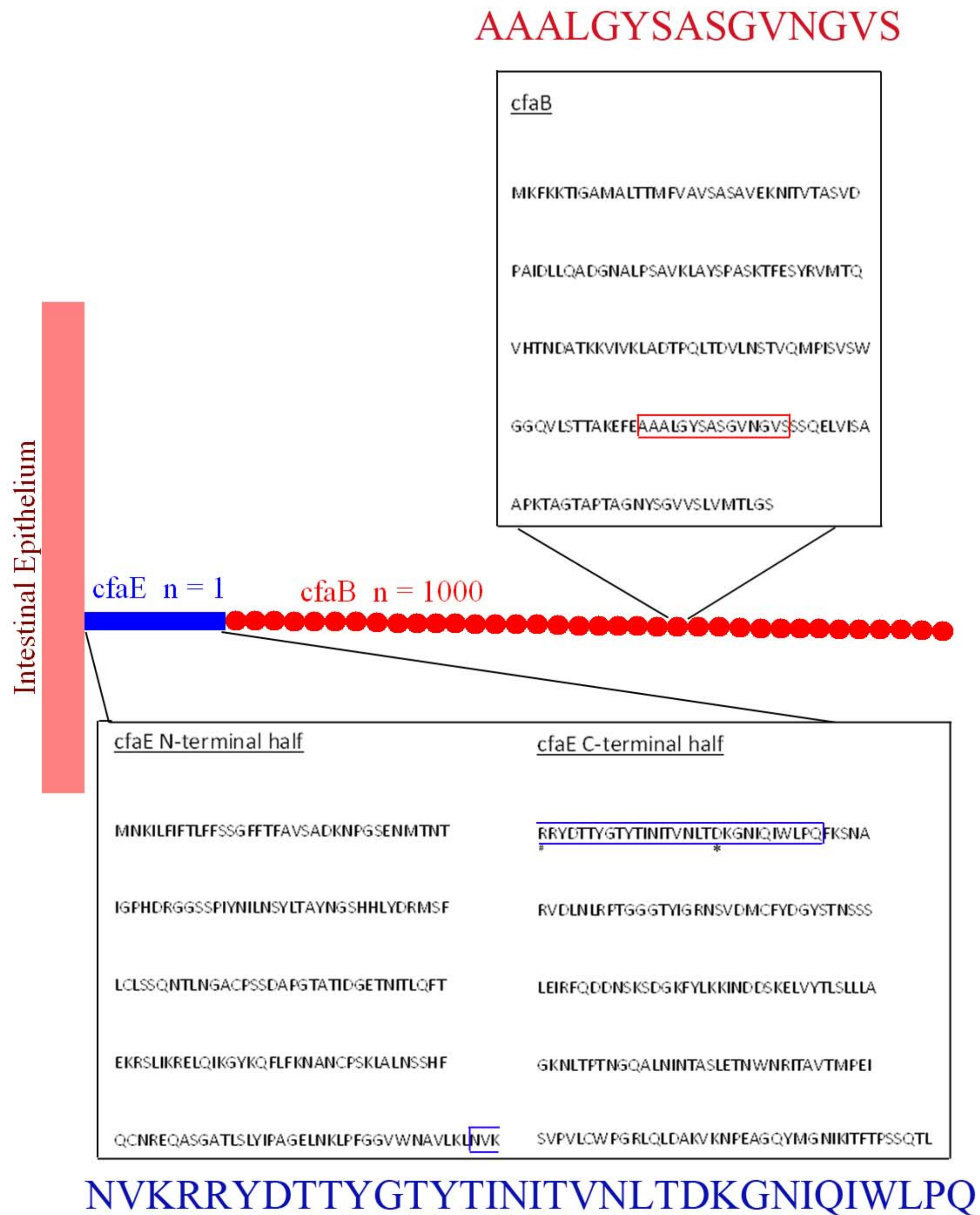


Figure 17: Immunodominant region from CFA/I fimbriae minor subunit, cfaE
The immunodominant epitope from cfaE is outlined in blue and spans the division between the N-terminal and C-terminal halves of cfaE. The 33 a.a. region is listed in blue.

proximity to C-terminal cfaE pool 1 (Figure 3) and N-terminal pool 11, it was included in further experiments.

To address the earlier *in vivo* observation that both cfaB and cfaE are required for optimal responses, we hypothesized that a similar response may be required for the generation of T_{reg} cell-associated cytokines following culture with peptides. To this end, the Th1-producing peptide from cfaB, peptide 25, was added to co-culture experiments with peptides from immunodominant regions of C-terminal cfaE and N-terminal cfaE. Specifically, sorted $CD4^+$ T cells from H696-immunized mice were cultured with cfaB peptide 25 paired with individual peptides from pool 1 from C-terminal cfaE (Table 2 and Figure 18) and individual peptides from pool 11 and pool 12 from N-terminal cfaE (Table 3 and Table 4; Figure 19;data not shown).

Interestingly, pairing peptide 25 with pool 1 peptide 2 resulted in appreciable levels of the T_{reg}-associated cytokines IL-10 and TGF-β not previously generated by any pool 1 peptides (Figure 18D and 18E). The amount of IL-10 generated by co-culture of cfaE peptide 2 with cfaB peptide 25 was significantly more than IL-10 generated by restimulation with CFA/I fimbriae. TGF-β levels generated following co-culture with both cfaE peptide 2 and cfaB peptide 25 was significantly more than with either peptide alone (Figure 18E). With respect to IFN-γ production, the addition of peptide 25 resulted in increased IFN-γ levels when added to cfaE peptides 3,4, and 6, but not when added to cfaE peptides 1 or 2 (Figure 18A). These data indicate that immunodominant regions from both cfaE and cfaB are necessary to generate IL-10- and TGF-β-producing regulatory T cells.

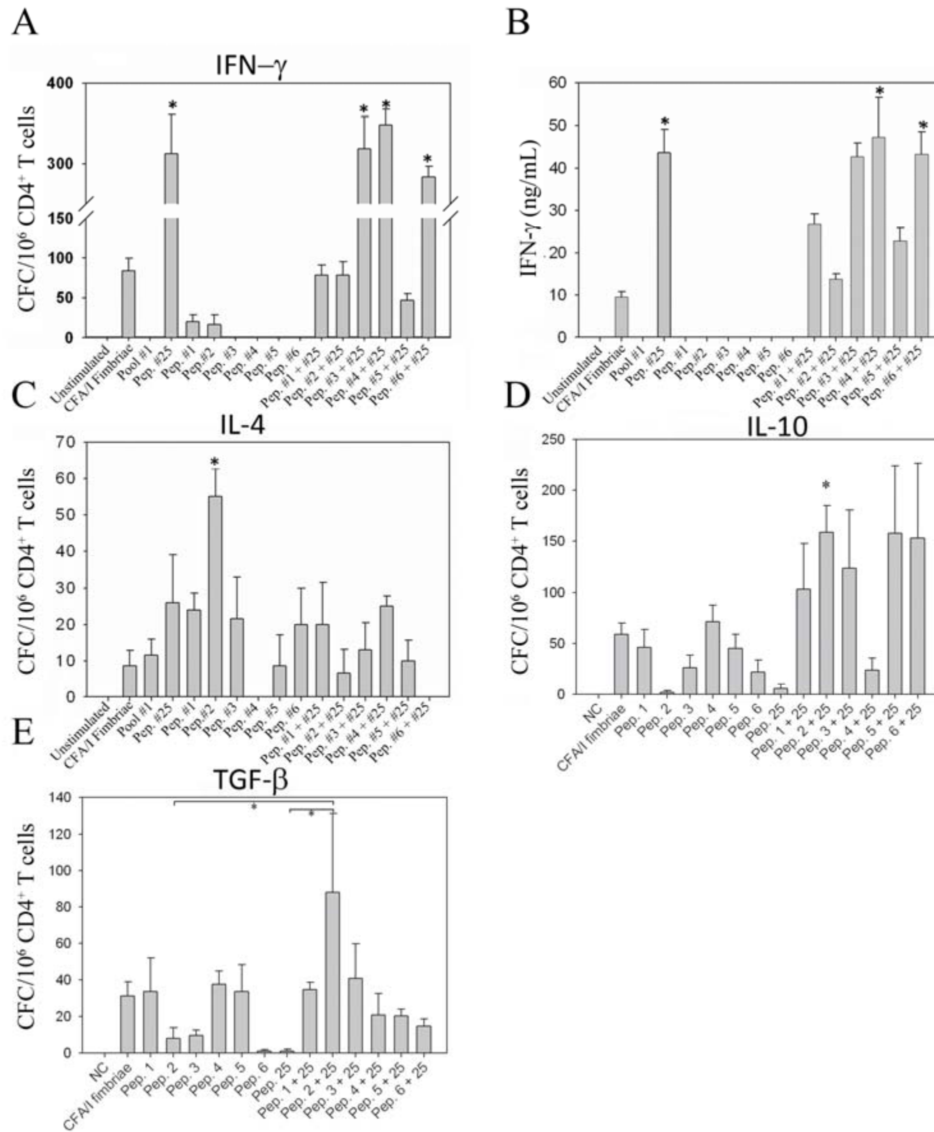


Figure 18: Cytokine production generated from C-terminal cfaE peptides cultured with cfaB peptide 25.
Sorted splenic CD4$^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I fimbriae (10mg/mL) or with peptides from C-terminal cfaE pool 1 (20 µg/mL per peptide) with or without cfaB peptide 25 (5 µg/mL) and monitored for cytokine production via T cell ELISPOT (A,C,D,E) or cytokine ELISA (B). CD4+ cells from immunized mice were also cultured in media alone (Unstimulated). Data shown are the mean +/- S.E.M, n=3, and are representative of 2 independent experiments (A,B,C) or are compiled data from 3 experiments each with triplicate cultures (D,E). Statistics for A-D were generated using ANOVA/ Tukey test comparing samples to CFA/I fimbriae in which * $p < 0.001$. C was also represented in Figure 11. Statistics for E were generated using the student's *t* test.

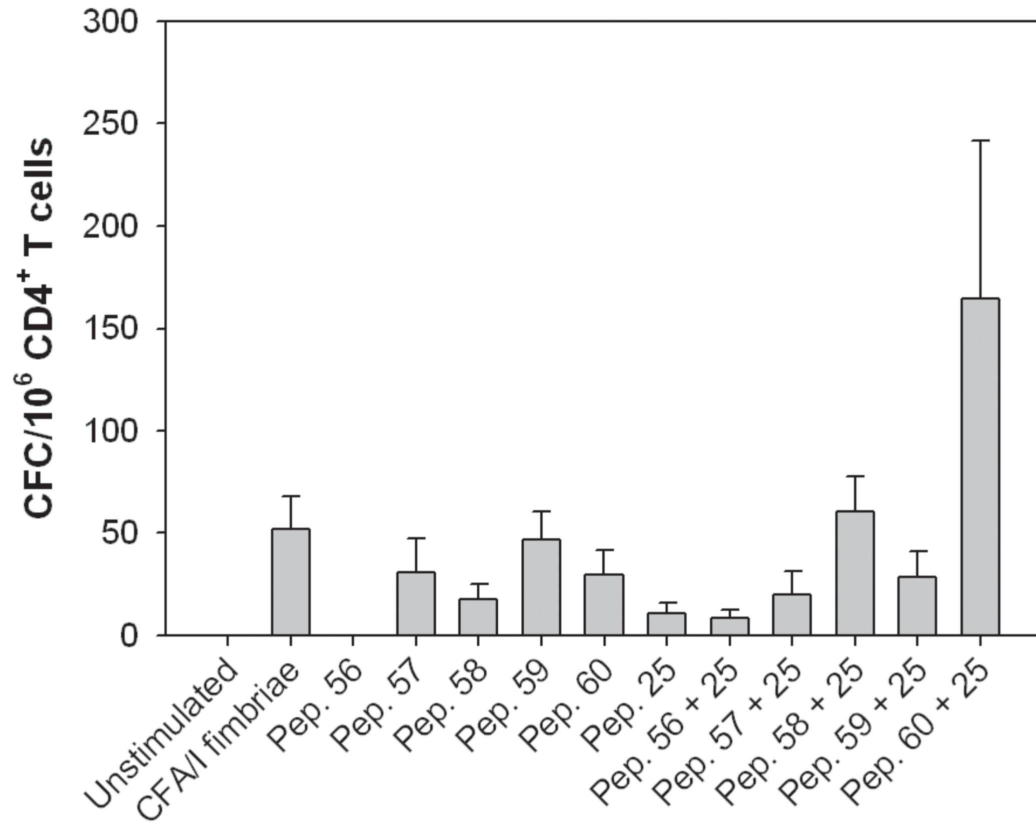


Figure 19: IL-10 production following culture with N-terminal cfaE pool 12 with or without cfaB peptide 25.
Sorted splenic $CD4^+$ T cells from H696-treated C57BL/6 mice were cultured with purified CFA/I (10 µg/mL) or with peptides from N-terminal cfaE pool 12 (20 µg/mL per peptide) with or without cfaB peptide 25 (5 µg/mL) and monitored for cytokine production via T cell ELISPOT. $CD4^+$ T cells from immunized mice were also cultured with media alone (Unstimulated). Data shown are compiled data from three independent experiments, each of triplicate cultures +/- S.E.M.

Restimulation with individual peptides from pool 11 or pool 12, each from N-terminal cfaE (Table 4), was unable to generate measurable cytokine production alone via ELISPOT or cytokine ELISA (data not shown). However, each of these peptides was also paired with cfaB peptide 25 and tested for cytokine production (data not shown;

Figure 19). cfaE peptide 60, in combination with cfaB peptide 25, was able to generate a T_{reg}-associated cytokine, specifically IL-10 (Figure 19). Preliminary intracellular staining also indicated that this combination of peptides was capable of generating TGF-β; however, this result was not confirmed in ELISPOT (data not shown). The close proximity of N-terminal cfaE peptide 60 to C-terminal cfaE pool 1 peptides suggests that this peptide may be part of a larger immunodominant region encompassing regions from C-terminal cfaE (Figure 3and Figure 17).

Unpublished observations indicate that oral immunization with *Salmonella*-CFA/I yields a population of IL-35-producing T cells. We next attempted to determine whether a combination of peptides from CFA/I fimbriae could induce this cytokine. Figure 20 shows intracellular staining for each subunit of IL-35, p35 and EBI3, as well as a control stain for p28, since EBI3 can alternately pair with p28 resulting in proinflammatory IL-27 rather than suppressive IL-35 when paired with p35. Panel A from Figure 20 shows that when MLN $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice are co-cultured with C-terminal cfaE peptide 1 and cfaB peptide 25, increased expression of p35 and EBI3 results, indicating the generation of suppressive IL-35. Of note, an up-regulation of p35 and EBI3 was also noted in these cells upon co-culture of C-terminal cfaE peptide 5 with cfaB peptide 25 (data not shown). Collectively, these data suggest that an immunodominant region, 33 amino acids in length, exists at the division between the N-terminal half of cfaE (pili domain) and the C-terminal half of cfaE (adhesion domain; Figure 3). This region is highlighted in Figure 17. Contained within this region is C-terminal cfaE pool 1 (peptides 1-6) as well as N-terminal cfaE peptide 60. IL-4 is

generated in response to an individual peptide from this region presented in the context of MHC II (Figure 16 and Table 5). The combination of multiple peptides from this immunodominant region paired with the immunodominant region from cfaB results in the production of the T_{reg}-associated cytokines IL-10, TGF-β, and IL-35 (Table 5).

Crystal structure images of cfaE folded with cfaB (PDB ID: 3F83;[32,59,60] Figure 21A) and of cfaB folded with two additional subunits of cfaB (PDB ID: 3F84;[59-61] [32] Figure 21B) were generated using software from www.pdb.org.[32,59,60] Each image is color-coded to discern cfaE structure from cfaB structure. Also labeled on each image is the immunodominant region from cfaB, peptide 25, highlighted in green and the immunodominant region from cfaE, highlighted in yellow (Figure 21). Of note is that there are approximately 1000 copies of cfaB per single copy of cfaE.[32] Extrapolating this fact when viewing Figure 21A and envisioning hundreds of additional cfaB subunits all bearing the same immunodominant peptide 25, one would expect that infection with ETEC would result in a robust immune response of $CD4^+$ T cells recognizing and responding to peptide 25 thus generating IFN-γ as demonstrated in the *in vitro* experiments herein (Figure 10 and Figure 12).

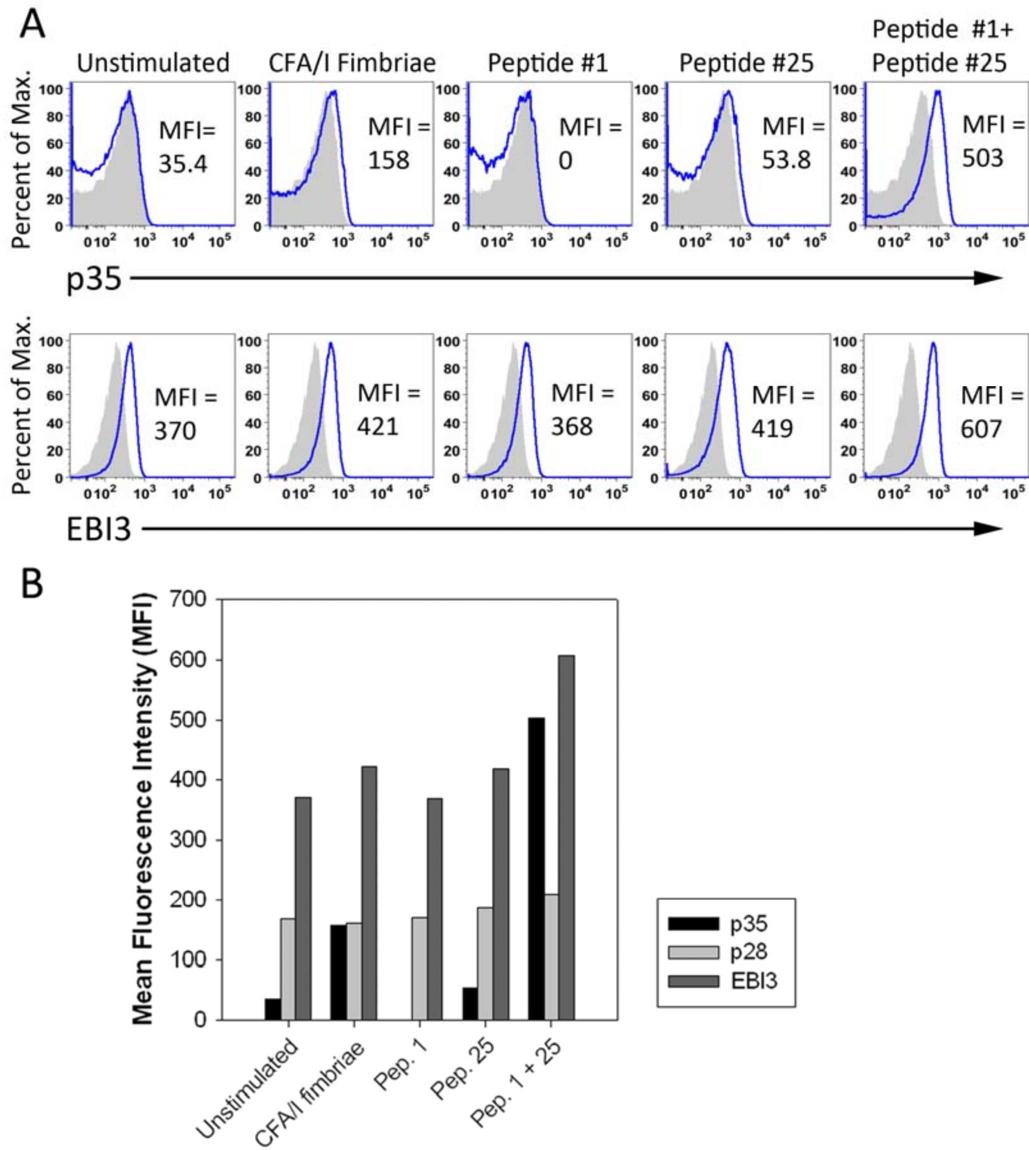


Figure 20: p35 and EBI3 production following culture with C-terminal cfaE peptide 1 and cfaB peptide 25.

Sorted MLN CD4+ T cells from H696-treated C57BL/6 mice (n =15) were cultured with individual peptides from C-terminal cfaE pool 1 (20 µg/mL per peptide) and N-terminal cfaE pool 12 (20 µg/mL per peptide) with or without cfaB peptide 25 (5 µg/mL), and were monitored for production of IL-35 subunits via intracellular staining. Shown are p35, p28, and EBI3 production following culture with unstimulated, CFA/I fimbriae, or cfaE peptide 1 (C-terminal) and cfaB peptide 25. Data shown are representative of single cultures from two independent experiments.

Table 5: Immunodominant Peptides and Associated Response

CFA/I fimbrial region/ Peptide #	**Peptide sequence**	**Observed Response**
N-terminal cfaE #60	NVKRRYDTTYGTYTI	IL-10 and TGF-β(?) when paired with cfaB Peptide 25
C-terminal cfaE #1	RRYDTTYGTYTINIT	IL-35 when paired with cfaB Peptide 25
C-terminal cfaE #2	DTTYGTYTINITVNL	IL-4 alone; IL-10 and TGF-β when paired with cfaB Peptide 25
C-terminal cfaE #3	YGTYTINITVNLTDK	
C-terminal cfaE #4	YTINITVNLTDKGNI	
C-terminal cfaE #5	NITVNLTDKGNIQIW	IL-35 when paired with cfaB Peptide 25
C-terminal cfaE #6	VNLTDKGNIQIWLPQ	
cfaB #25	AAALGYSASGVNGVS	IFN-γ alone; Multiple T_{reg} cell cytokines when paired with cfaE peptides

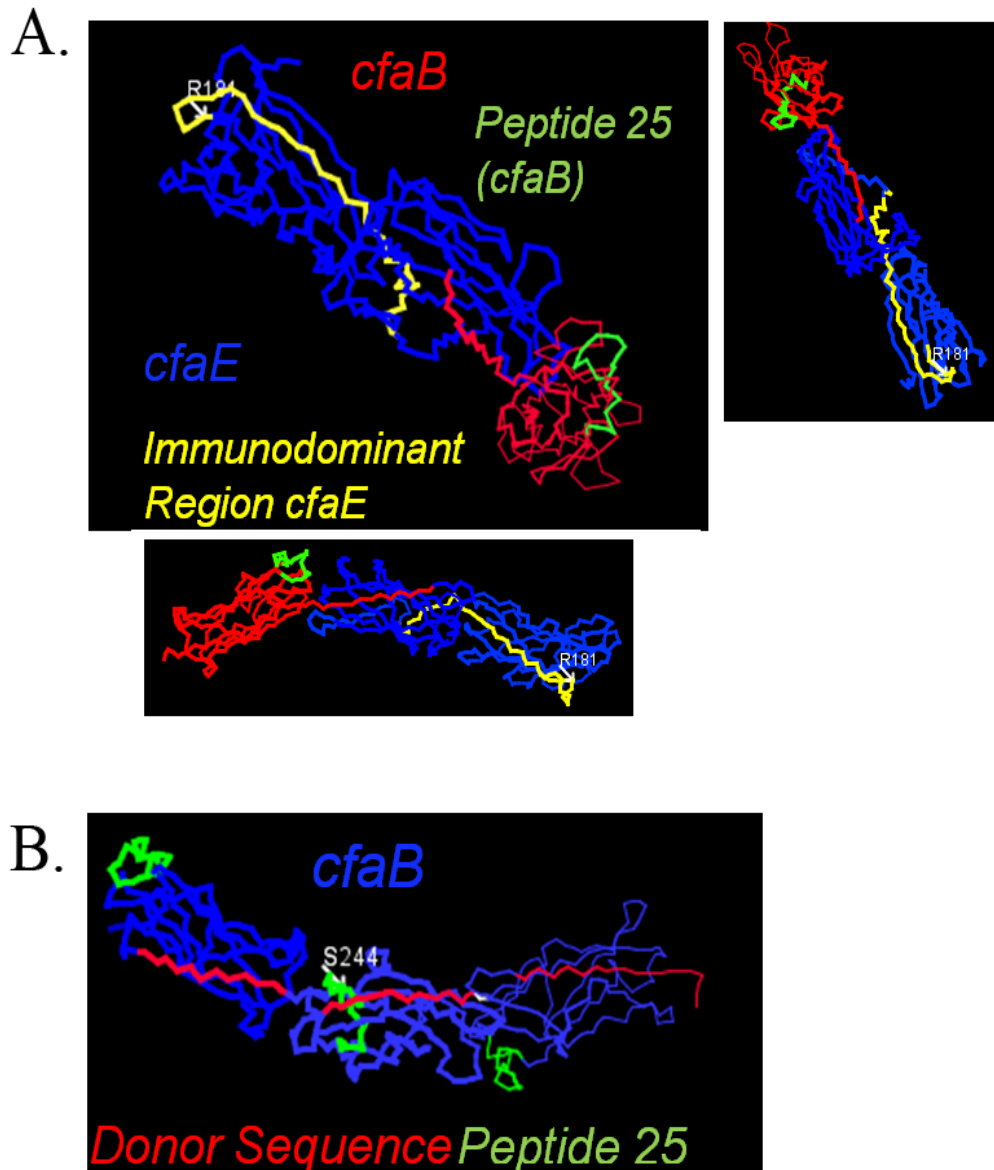


Figure 21: Crystal structure of cfaE and cfaB.
Crystal structure of cfaE assembled with cfaB (A) or cfaB assembled with two additional cfaB subunits (B). In A, cfaE is blue while cfaB is red. The immunodominant region from cfaE is labeled in yellow while the immunodominant region from cfaB is labeled in green. R181 is also labeled. This crystal structure is available from three separate angles. In (B), all three cfaB subunits are labeled in blue. The donor sequence is labeled in red while the immunodominant region from cfaB (peptide 25) is labeled in green. S244 from peptide 25 is also labeled.

DISCUSSION

Immunodominant Epitopes from CFA/I Fimbriae

Previously, we reported the generation of a biphasic Th2/Th1 cell response in mice treated with an attenuated *Salmonella* vector expressing ETEC CFA/I fimbriae. Herein, these results expand upon this finding to characterize the immunodominant regions of these fimbriae that generate the observed responses. Collectively, these data suggest an immunodominant region exists within cfaB, which results in the robust generation of IFN-γ by $CD4^+$ T cells (Figure 13 and Table 5). An immunodominant region also exists in the C-terminal region of cfaE that generates the classic Th2-type cytokine, IL-4 (Figures 15, 16, and 17). However, a single immunodominant region within either fimbrial subunit could not be identified that stimulated $CD4^+$ T cells to produce T_{reg}-associated cytokines shown to be associated with *Salmonella*-CFA/I immunization.[43,44,48,55]

Studies here indicate that both cfaB and cfaE are necessary for therapeutic treatment of EAE (Figure 8 and Figure 9), and since we have previously shown that T_{reg} cells are critical to effective treatment of EAE,[43,55] it is possible that immunodominant regions from both fimbrial subunits are necessary to generate T_{reg} cell-associated cytokines *in vitro*. To this end, immunodominant peptides from cfaB and cfaE were co-cultured and monitored for production of T_{reg} cell-associated cytokines (Figure 18, 19, and 20). Co-culture of several different 15 a.a. peptides from an immunodominant region of cfaE (Figure 17) with the immunodominant region from cfaB (Figure 17) resulted in

the generation of T_{reg}-associated cytokines IL-10, TGF-β, and IL-35 (Table 5 and Figure 22).

NVKRRYDTTYGTYTINITVNLTDKGNIQIWLPQ

Peptide 60 (N-term. cfaE) + Peptide 25 (cfaB) = IL-10

Peptide 1 (C-term. cfaE) + Peptide 25 (cfaB) = IL-35

Peptide 2 (C-term. cfaE) = IL-4;
Peptide 2 + Peptide 25 (cfaB) =IL-10 and TGF-β

Peptide 5 (C-term. cfaE) + Peptide 25 (cfaB) = IL-35

Figure 22: Immunodominant peptides from cfaE.
Determined immunodominant region from cfaE; individual peptides are highlighted along with the cytokines produced by each either alone or in conjunction with peptide 25 from cfaB.

Critical Amino Acid Arginine 181

The observed immunodominant region of cfaE, which is centered at the division between the pili and adhesion domains (Figures 3, 17, 21A, 22, and Table 5) of cfaE, contains a region known to be critical for CFA/I fimbrial function. Specifically, arginine 181 (R181) is critical for adhesion of CFA/I fimbriae to their receptor(s) in the gut, and mutation of R181A causes a cessation of CFA/I fimbriae's ability to bind bovine erythrocytes.[30] R181 lies at the boundary (Figures 3, 21A, and 22) between the N-terminal and C-terminal halves of cfaE and is included in peptide 60 (N-terminal cfaE) and peptide 1 (C-terminal cfaE). In addition to being critical for CFA/I fimbriae binding,

this amino acid is also present within the cfaE immunodominant region responsible for the generation of IL-10, TGF-β, and IL-35 (Figures 17 and 22). In order to fully determine the contribution of this amino acid to MHC II binding and immunodominance, the core immunodominant epitope contained within the immunodominant region, already determined through this work, must be elucidated. This is accomplished through the addition/elimination of single amino acids within the immunodominant region in order to determine the set of amino acids which best bind MHC II.[61,62]

Immune Evasion by ETEC

Attempts to develop a vaccine against ETEC, which would harness the predicted robust population of IFN-γ-producing $CD4^+$ T cells, has fallen short of expectations.[39,63,64] The development of a vaccine against this bacteria is vital because enterotoxigenic *Escherichia coli* (ETEC) affects approximately 400,000,000 children less than 5 years old annually.[64] Approximately 2,000,000 people in developing countries will expire due to gastroenteritis every year. The primary bacterial cause of gastroenteritis is ETEC. The demographic for the majority of these deaths is children under 12 months old.[64] *E. coli* becomes enterotoxigenic after acquiring plasmid(s) that allow for the production of heat-stable enterotoxin and/or the heat-labile enterotoxin.[29] Additionally, ETEC also acquires a plasmid that produces pili or fimbriae, which bind surface receptors in small intestine.[30,31] Colonization factor antigen I (CFA/I) is one such surface fimbriae of ETEC.[32] ETEC utilizes these fimbriae to adhere to the host intestine

during the early phases of infection.[31,32] After adherence to the small intestine, ETEC secretes the heat-labile or heat-stable enterotoxins, resulting in diarrhea.[33]

Since colonization factors and the heat-labile enterotoxins are highly conserved, they have been actively targeted as vaccine candidates.[64] Of the colonization factor antigens associated with ETEC isolates, CFA/I is the most common found on approximately 33% of isolates, and thus, it follows that CFA/I fimbriae were targeted as a potential vaccine candidate.[64] An effective vaccine against ETEC is attainable since children can receive protection via passive immunity either by the passage of antibodies across the placenta to the developing fetus[65] or by ingesting their mother's colostrums and milk, which contains antibodies directed against ETEC.[64,65] This passive immunity has also been observed in our laboratory following immunization of adult CD-1 female mice with *Salmonella* expressing the bovine ETEC fimbriae, K99 (*Salmonella*-K99).[66,67] Following immunization with *Salmonella*-K99, mice were mated and subsequent pups were allowed to receive colostrum and milk for 24 h, after which neonatal mice were challenged with bovine ETEC ($F5^+$ Bovine ETEC).[67] Neonatal mice from immunized dams displayed significantly increased survival following challenge.[67] This protection was mediated by IgA and IgG antibody responses directed against K99 fimbriae.[66,67] Further support for the feasibility of an effective ETEC vaccine stems from the observed natural inverse relationship between the incidence of ETEC and age, implying the acquisition of natural immunity.[64]

Previous studies have administered ETEC colonization factor antigens as whole proteins, but these have failed to result in elevated antibody titers even when

encapsulated microparticles were used to minimize degradation by stomach acids.[39,63] Thus, in an effort to develop an effective vaccine against human ETEC, CFA/I was expressed on the surface of an attenuated *Salmonella* vaccine vector, ie, *Salmonella*-CFA/I.[39] Classically, *Salmonella* vaccine vectors result in a robust Th1 cell response, categorized by elevated IFN-γ and associated IgG2a levels directed at both *Salmonella* and any passenger antigens. However, immunization with *Salmonella*-CFA/I resulted in an initial Th2 cell response characterized by robust amounts of IL-4, IL-5, and IgG1.[29,39] Further experiments in our laboratory determined that in addition to Th2 cells, various subsets of T_{reg} cells were also induced.[39,43,44,48,55] The results presented herein suggest that multiple epitopes are necessary to generate the observed response to *Salmonella*-CFA/I.

The secluded location of the observed epitopes from cfaE (Figure 21A) might suggest the mechanism of immune evasion by ETEC that has previously prevented an effective vaccine. R181, labeled on all crystal structures in Figure 21A, is the key residue implicated in CFA/I fimbriae binding to the intestinal epithelium. When bound to the epithelium, this region of cfaE is unlikely to be readily accessible to cells of the immune system. In addition, the very structure of CFA/I fimbrae would suggest that likely immunodominant epitopes lie within cfaB based on the frequency of this major fimbrial subunit when compared with the minor fimbrial subunit (1,000 copies of cfaB per single copy of cfaE[32]). For this reason, much emphasis has been placed on cfaB when attempting to discern potential B and T cell epitopes. It is possible that the immune

system itself also focuses on cfaB during an infection both due to the increased frequency of this fimbrial subunit as well as the inaccessibility of cfaE (Figures 2 and 21).

Our results suggest that while immunodominant regions do exist within cfaB, they only account for the production of the Th1 cell-associated cytokine, IFN-γ. However, the impact of immunization with *Salmonella*-CFA/I is more complicated than IFN-γ alone. The work described here demonstrates that epitopes within cfaE promote IL-4 producing CD4 T cells. Other cytokines such as IL-10, TGF-β, and IL-35, require the presence of immunodominant regions from both cfaE and cfaB. It is possible that, in nature, cfaB acts as a decoy to the immune system, while the minor fimbrial subunit, cfaE, whose immunodominant epitopes remain predominantly buried and inaccessible to immune cells (Figure 21A), results in the generation of T_{reg} cells when coupled with cfaB epitopes, which could dampen the immune response associated with ETEC infection allowing disease to perpetuate. It is possible that ETEC has evolved to utilize the generation of T_{reg} cells by the host in response to epitopes from cfaE paired with epitopes from cfaB as a mechanism for immune evasion by dampening the resulting immune response following infection and thus allowing disease to persist.

The generation of T_{reg} cells by CFA/I fimbriae epitopes could account for obstacles encountered when attempting to develop an effective vaccine against ETEC. Although passive immunity has been observed in the human population with regard to human ETEC[64] and in mice with regard to F5$^+$ bovine ETEC,[66,67] passive immunity does not generate memory because the immune system is not actively stimulated, but rather specific antibodies are passed from one individual to another.[65] An ideal vaccine would

be protective against future antigen encounters and would incorporate both the humoral and adaptive immune systems and generate memory.[68] Such activation of both the humoral and cell-mediated branches of the immune system could be hindered by the induction of T_{reg} cells.

Specifically, Th cell-dependent activation of B cells is dependent upon the production of IL-4, IL-5, and IL-2 by Th cells following initial antigen uptake by the B cell.[65] The production of these cytokines is facilitated by the up-regulation of costimulatory molecules, such as CD40 on B cells, as well as CD40L on Th cells. The activated B cell then up-regulates receptors for IL-4, IL-5, and IL-2 and engagement of these receptors drives B cell proliferation and class switching.[65] However, the induction of T_{reg} cells could negatively impact this process in several ways.

First, T_{reg} cells often express CD25, the alpha receptor for IL-2, and are able to regulate other immune cells within their environment by sequestering free IL-2, thus, preventing it from acting upon other cells. Secondly, the T_{reg} cell-associated transcription factor Foxp3 has recently been shown to be a negative regulator of IL-2 by several mechanisms. Foxp3 can independently downregulate IL-2 expression through NFAT, or alternatively Foxp3 can couple with the gene SIVA to quench IL-2 expression via NFκB.[69] Additionally, Foxp3 can also regulate IL-2 expression via chromatin rearrangement,[70] resulting in diminished production of the cytokine IL-2. Lastly, T_{reg} cells can negatively impact the proliferation of B cells by preventing the production of B cell promoting cytokines by Th cells in the surrounding environment. Thus, the generation of T_{reg} cells by CFA/I fimbriae could dampen the associated humoral

response, allowing for suboptimal immune responses following ETEC vaccination, as well as perpetuating disease with regard to ETEC in nature.

Induction of a Biphasic Response

The observed polarization of Th2 and Th1 cell-associated epitopes to cfaE and cfaB, respectively, could also account for the observed biphasic shift observed following immunization with *Salmonella*-CFA/I.[39] If cfaE immunodominant epitopes form a stronger complex with MHC II, then it follows that early immune responses would be driven by responses induced by these epitopes, ie, Th2-type responses (Table 5) despite cfaE is being less abundant than cfaB. By 4 weeks following immunization however, the frequency of cfaB would dictate that the overwhelming response would shift toward a Th1-type response (Table 5) since the sheer number of loaded cfaB epitopes would overcome any minor deficiencies in MHC II binding. Additionally, T_{reg} cell numbers associated with *Salmonella*-CFA/I immunization peak at approximately 10 days post-immunization (Javier Ochoa-Repáraz, Montana State University, personal communication). The work performed herein shows that immunodominant epitopes from both cfaB and cfaE are necessary to drive T_{reg} cell-associated cytokine responses following restimulation. Initial preferential loading of cfaE epitopes would fit with this timeline of T_{reg} cell development. In order to explore these hypotheses, the dissociation rates between cfaE immunodominant epitopes and cfaB immunodominant epitopes must be compared. Additionally, immunization with individual immunodominant regions could also aid in determining the relationship between cytokine production and peptide

loading kinetics. Specifically, if the above hypothesis were true, one would expect that immunization with cfaE epitopes alone would result in the production of IL-4, while immunization with cfaB epitopes would result in the sole production of IFN-γ.

Salmonella-CFA/I as an Anti-Inflammatory Vaccine and T_{reg} Cell Inducer

While the generation of T_{reg} cells following immunization with *Salmonella*-CFA/I is undesirable with regard to a vaccine against ETEC, it does provide the opportunity for a suppressive vaccine utilizing the bystander effect. This vaccine is effective at treating both EAE and CIA[29,43,44,48,55] by generating specific subsets of T_{reg} cells. However, the types of T_{reg} cells generated vary depending on disease model. In EAE, the T_{reg} cells generated are $Foxp3^+CD25^+CD4^+$, generate IL-10 and TGF-β, and are functional at both preventing and treating EAE.[43] In mice immunized with *Salmonella*-CFA/I and induced with CIA, however, a different subset of T_{reg} cells dominates. Although mice are protected from CIA following *Salmonella*-CFA/I immunization, adoptive transfer of the $CD25^+CD4^+$ or the $CD25^-CD4^+$ T cell subset fails to be completely protective.[44] However, adoptive transfer of total $CD4^+$ T cells is protective in CIA in a TGF-β dependent manner.[44] Examination revealed a T_{reg} cell subset defined by CD39 expression rather than by CD25 expression. Further analysis of the phenotypes associated with CD39 expression revealed two phenotypically separate populations exist, $Foxp3^+CD39^+CD4^+$ and $Foxp3^-CD39^+CD4^+$ T cells, which produce IL-10 and TGF-β, respectively. While adoptive transfer of either aforementioned subset alone was not fully

protective against CIA, adoptive transfer of the whole $CD39^+CD4^+$ T cell population was, suggesting interdependence between the two subsets.[48]

CD4$^+$ Regulatory T cell Subsets

Currently, there are two primary categories of T_{reg} cells, innate or natural T_{reg} cells (nT_{reg}) and inducible T_{reg} cells (iT_{reg}). nT_{reg} cells are thymus derived $Foxp3^+CD25^+CD4^+$ and protect against autoimmune reactions, as well as moderate inflammation to minimize tissue and cell damage.[43,47,48,50,55,71-75] Inducible T_{reg} cells are defined by the cytokines that induce them and are generated from naïve $CD4^+$ T cells by exposure to the surrounding cytokine environment.[47,48,50,71,73,75] T_{reg} cells modulate the immune environment by the following three methods: secretion of soluble molecules, such as IL-10, TGF-β, and IL-35[73]; direct cell-cell contact, such as galectin-1 binding on T_{eff} cells or DC, resulting in cell cycle arrest and/or apoptosis;[75] and competition for growth factors, such as IL-2.[75]

Two classic groups of inducible T_{reg} cells exist, one induced by IL-10 and the second induced by TGF-β. Cells induced by IL-10, termed Tr1 or IL-10 inducible T_{reg} cells, are $Foxp3^-CD25^+CD4^+$ and produce large quantities of IL-10, although they can also produce TGF-β.[73,75] T_{reg} cells generated by TGF-β are termed Th3 cells and are defined as $Foxp3^+CD25^+CD4^+$ T cells and produce TGF-β, but can also produce IL-10.[73,75]

In addition to the two aforementioned classic groups of inducible T_{reg} cells, a new type has recently been defined by Vignali et al.[50,71-74] These cells are induced by IL-35,

are Foxp3$^-$, and exclusively produce IL-35 (iT_{reg}35), making them unique from Tr1 and Th3 subsets.[73] This subset of IL-35-producing T_{reg} cells will be discussed in more detail below. In addition to iT_{reg}35, Kochetkova *et al.* describe two additional groups of T_{reg} cells unique from those already discussed.[48] First is a population of induced TGF-β-producing T_{reg} cells, which are Foxp3$^-$CD39$^+$CD4$^+$, and second is an IL-10-producing Foxp3$^+$CD39$^+$CD4$^+$ T_{reg} cell, neither of which correlate to CD25 expression.[48] The variability of T_{reg} cell subsets generated under various conditions and in varying disease models highlights the plasticity of this important immune cell.

IL-10, TGF-β, IL-35, and Their Production by CFA/I Fimbriae Peptides

IL-10 is produced primarily by monocytes, macrophages, and Th cells, [76] but can also be produced by B cells, eosinophils, and mast cells, as well as others.[77] IL-10 is an anti-inflammatory cytokine that plays a key role in prevention of autoimmune diseases, as well as in preventing immunopathology during infection[78] by limiting the function of dendritic cells and macrophages.[77] IL-10 is also down-regulated by MHC class II transactivator (CIITA), which is responsible for the regulation of MHC II proteins.[5,77,78]

Since IL-10 expression occurs in many different cell types[77] and is influenced by multiple transcription factors, signaling molecules, and/or extracellular cytokines,[77,78] it follows that multiple immunodominant epitopes might be required in order to generate IL-10 *in vitro* from *Salmonella*-CFA/I-immunized mice. For example, Th1-type CD4$^+$ cells (IFN-γ producing) can produce IL-10 under proinflammatory conditions via the Notch pathway coupled with STAT4 and ERK.[76,78] The key to the production of IL-10

by these Th1-polarized cells is high-dose antigen and endogenous IL-12.[78] Phenotypically, these cells are $CD25^-$ and $Foxp3^-$.[77] Since significant IL-10 production was only noted in our *in vitro* system when $CD4^+$ T cells were cultured in the presence of two peptides, one being the IFN-γ-producing cfaB peptide 25, it is possible IFN-γ is needed to drive IL-10 production within this system. Such a phenomenon has been noted in cells infected with respiratory syncytial virus (RSV) and cells infected with *Rickettsia conorii*[79], wherein a population of cells exists that produces both IFN-γ and IL-10.[80] Additionally, Th1 cells are also the primary producer of IL-10 in infection models, such as influenza A virus, *Leishmania* species, *Borrelia burgdorferi* infections, and mycobacterial infections.[80] This suggests that, under certain conditions, IFN-γ and IL-10 can be co-produced by the same cell. It is possible that in our system, $CD4^+$ T cells recognizing cfaB peptide 25, might be driven to also produce the suppressive cytokine IL-10.

Other cell types responsible for IL-10 production are the natural and inducible T_{reg} cells. In this scenario, IL-10 production is dependent upon the Foxp3 transcription factor and TGF-β.[78] IL-10 also results in the generation of a population of inducible T_{reg} cells that perpetuates IL-10 production by likewise producing IL-10.[50] Although all the transcription factors involved in the IL-10 pathway are not yet defined, TGF-β's presence in this instance also promotes IL-10 production.[78]

Herein we show IL-10 is produced by culturing $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice with epitopes from fimbrial subunits, cfaB and cfaE. Having identified the combination of peptides responsible for the production of this cytokine

(Table 5 and Figure 22), determining the cell-type responsible for its production is the next task. It is possible IL-10 is produced by all three cell types, Th1, natural T_{reg} cells, and inducible T_{reg} cells or a combination thereof. To determine this, $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice can be co-cultured with peptides from the immunodominant region of cfaE and peptide 25 from cfaB (Table 5 and Figure 22) and tested for co-production of IFN-γ and IL-10, as well as for expression of the transcription factor, Foxp3, via flow cytometry analysis.

Another T_{reg} cell-associated cytokine is TGF-β, which is primarily produced by $Foxp3^+CD25^+CD4^+$ T_{reg} cells,[50,72,74] although it can also be produced by DCs and macrophages.[81] In the *in vitro* system discussed herein, TGF-β cytokine can be detected following co-culture with C-terminal cfaE peptide 2 and cfaB peptide 25 (Figure 18), as well as in culture with N-terminal cfaE peptide 60 with cfaB peptide 25 (data not shown). However, low reproducibility of these experiments suggests further analyses of the kinetics of TGF-β production, in addition to an evaluation of the phenotype of the responsible cell(s), are necessary.

The co-culture of $CD4^+$ T cells with immunodominant regions from each fimbrial subunit uniquely generates yet another cytokine. Specifically, C-terminal cfaE peptide 1 co-cultured with cfaB peptide 25 results in the production of IL-35 (Figure 20), a newly described cytokine produced by both natural and inducible T_{reg} cells.[50,72,74] IL-35-producing T_{reg} cells are of particular interest because, unlike IL-10 and TGF-β, IL-35 is produced only by T_{reg} cells and not by conventional $CD4^+$ T cells.[74] These IL-35-producing T_{reg} cells are able to suppress conventional T cells by limiting proliferation and

inflammatory cytokine production and can induce conversion of naïve $CD4^+$ T cells into IL-35-producing T_{reg} cells.[72] Additionally, secretion of IL-35 is able to generate IL-35 production by other surrounding T cells in a process termed "infectious tolerance,"[73] which will be discussed in more detail below.

Number of Responding $CD4^+$ T Cells and Infectious Tolerance

Approximately 15 – 40% of white blood cells are lymphocytes with the majority of these being T cell lymphocytes.[82,83] Of these T cell lymphocytes, $CD4^+$ T cells outnumber $CD8^+$ T cells approximately 2 : 1.[65] Of these $CD4^+$ T cells, approximately 5 – 10% are T_{reg} cells.[73,84] When a complex antigen is encountered by the immune system, only a limited number of epitopes are recognized by responding $CD4^+$ T cells. As such, these epitopes are referred to as immunodominant. There is some debate regarding what percentage of $CD4^+$ T cells actually respond to a T cell epitope upon restimulation. Some suggest that a cryptic epitope, an epitope generally not recognized by $CD4^+$ T cells, will be recognized by less than 3% of responding T cells following restimulation with antigen.[3] On the contrary, they suggest that an immunodominant epitope, capable of forming a stable association with MHC II ($t_{1/2} > 80$ h), can be recognized by as much as 20% of responding $CD4^+$ T cells following restimulation.[3] However, others suggest this number is actually much lower, with only 0.1% of T cells specific for a given antigen[82] based on *in vitro* restimulation assays. The discrepancy is likely attributable to complicated *in vitro* protocols where not all cells survive/expand equally.[82] This

response may also be attributed to mechanical limitations regarding accurate detection of antigen-specific T cells via many methods such as tetramer labeling.[82]

However, whether one adopts a high estimate (20%) or low estimate (0.1%) of $CD4^+$ T cells responding to a given antigen upon restiumulation, the observation that all $CD4^+$ T cells examined in Figure 20 appear to be producing IL-35 is at first glance confounding. However, even $CD4^+$ T cells that do not recognize and respond directly to a particular epitope can contribute to the associated immune response by reacting to cytokines secreted by responding $CD4^+$ T cells. One such example is the process termed infectious tolerance.[73] This is the process by which the secretion of a suppressive cytokine by a responding T cell can stimulate T cells in the surrounding environment to also initiate secretion of the suppressive cytokine, resulting in a population of suppressive T_{reg} cells where initially only a limited number of cells respond to the antigen in question[73]. TGF-β has long been believed to drive infectious tolerance,[73] and now IL-35 has been shown to do the same.[73] Thus, the observation in Figure 20 that restimulation with peptide 1 and peptide 25 (Table 5 and Figure 22) of $CD4^+$ T cells from *Salmonella*-CFA/I immunized mice causes an up-regulation of IL-35 production by all cells can be attributable to the induction of infectious tolerance. To our knowledge, this is the first evidence of IL-35 generation by peptide culture, and future experiments can be performed to determine CD25 and Foxp3 expression in these IL-35-producing T_{reg} cells. Understanding the mechanisms by which T_{reg} cells are induced and thus, how infectious tolerance is stimulated, is critical. Enhancing this process could prove beneficial to therapies for autoimmune disorders including, but not limited to, M.S. and R.A., but

could also prove beneficial in limiting the induction of T_{reg} cells under certain situations such as in certain tumor models, could also prove therapeutic.

CREB Regulation/Contribution

Immunization with *Salmonella*-CFA/I is responsible for the generation of diverse T_{reg} cell populations. First, *Salmonella*-CFA/I generates a population of $Foxp3^{+}CD25^{+}CD4^{+}$ T_{reg} cells that produce IL-10 and TGF-β, which are protective in EAE.[43] *Salmonella*-CFA/I also generates a population of $Foxp3^{+}CD39^{+}CD4^{+}$ T_{reg} cells and a population of $Foxp3^{-}CD39^{+}CD4^{+}$ T_{reg} cells that produce IL-10 and TGF-β, respectively.[44,48] The transcription factor, cAMP response element binding protein (CREB), induces the up-regulation of CD39.[85] TGF-β phosphorylates CREB, enabling CD39 expression.[86] Since neutralization of *Salmonella*-CFA/I results in loss of TGF-β dependent protection against CIA, which is dependent upon the T_{reg} subset $CD39^{+}CD4^{+}$, it is suggestive that TGF-β neutralization results in the downregulation of $CD39^{+}CD4^{+}$ cells.[48]

Additionally, we have previously shown recombinant IL-35 (rIL-35) results in an increase in IL-10 coupled with an increase in $CD39^{+}CD4^{+}$ cells with elevated Foxp3 expression that are protective in CIA. Others have recently detected IL-35 following immunization with *Salmonella*-CFA/I (unpublished results). Since CREB controls transcription of IL-10,[76] it is possible there is a direct relationship between the suppressive cytokine IL-35 and the transcription factor CREB. A review of current literature indicates this relationship has not yet been evaluated. Thus, future studies

could include an evaluation of the relationship between IL-35 and CREB, specifically, if IL-35, like TGF-β, activates CREB, resulting in increased IL-10 expression.

Additional Future Experiments

The hypothesis that regulatory T cells are generated from epitopes within cfaE centered around the receptor binding domain suggests potential mechanisms by which an effective ETEC vaccine could be developed from the vaccine system defined herein. Deletion of cfaE entirely would likely result in inefficient intestinal epithelial binding by CFA/I fimbriae since cfaE presence is required for receptor binding.[30] However, if a core immunodominant region were determined, then amino acids central to efficient MHC II binding could be determined and mutated, thus, eliminating the generation of a T_{reg} cell population following *Salmonella*-CFA/I immunization. This could potentially result in the development of an ETEC vaccine whereby only a Th1-type response would occur likely in response to cfaB peptide 25.

Conversely, *Salmonella*-CFA/I is also of particular interest because of its ability to generate Th2 cells and T_{reg} cells that are effective in treating and/or preventing certain autoimmune disorders.[29,36,43,44,55] Previous studies in our lab show this protection is mediated via $CD4^+$ T cells since adoptive transfer of these cells from *Salmonella*-CFA/I immunized mice provides protection in EAE and CIA models.[43,44] Having determined the immunodominant regions responsible for the cytokines observed within these disease models, the opportunity for further research into the mechanisms of the immune response to *Salmonella*-CFA/I now exists.

One result of identifying the immunodominant regions is now mice can be directly immunized with immunodominant peptides, and $CD4^+$ T cells can then be examined *ex vivo* for cytokine production, thus, eliminating the complication of *in vitro* stimulation. Direct peptide delivery would allow for a more accurate determination of cytokine amounts generated from these specific regions in an *in vivo* situation, as well as provide the opportunity to study the kinetics of cytokine production following various immunization routes. Additionally, other mouse strains could be tested to evaluate whether the determined immunodominant regions from CFA/I fimbriae are restricted to a single MHC II allele (haplotype b from C57BL/6 mice) or if multiple haplotypes preferentially load the same peptide regions. Immunodominant epitopes from other antigens indicate that some epitopes are haplotype-specific,[87-89] while others span multiple MHC II configurations.[12] If the identified immunodominant regions of CFA/I are able to preferentially bind multiple haplotypes of laboratory mice, then potentially these peptides could also preferentially bind multiple human MHC II configurations.

Others have previously identified immunodominant epitopes that utilize a heterogeneous population of TCRβ chains, despite having a single immunodominant epitope.[62] Future work could determine if multiple TCRβ chains are being induced in our system. Preliminary work indicates multiple TCRβ chains are generated following immunization with *Salmonella*-CFA/I (data not shown). Immunization with specific immunodominant peptides followed by evaluation of induced TCRβ chain configurations would determine if such a phenomenon is also occurring following *Salmonella*-CFA/I immunization. If a heterogeneous population of TCRβ chains is in fact generated, we

could evaluate if particular cytokines, for example IL-35, correlate with specific chain usage using flow cytometry analysis.

Other future experiments include the possibility of creating a transgenic mouse. Specifically, immunodominant peptides would be utilized to generate a transgenic mouse expressing a modified TCR that would only recognize a certain immunodominant epitope. Such a mouse would allow for critical evaluation of the kinetics of CFA/I fimbriae-specific $CD4^+$ T cells, including the number of specific cell phenotypes, the induction of cell-specific phenotypes, the longevity of key cytokine producing cells, and the tissue dispersion.

The observations that CFA/I fimbriae impact disease has therapeutic potential; however, the use of an attenuated *Salmonella*-vaccine vector could prohibit clinical use. To address this possibility, the determined immunodominant regions of CFA/I could be administered as peptides to mice in conjunction with EAE or CIA induction to determine if peptides alone could mimic the therapeutic effects of CFA/I fimbriae seen *in vivo*. If so, the administration of a peptide or peptides would be easier than producing purified CFA/I fimbriae and would bypass the current attenuated *Salmonella*-vaccine vector and thus providing a potential therapeutic that could be used in humans.

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