Immunogenicity characterization of enterotoxigenic *Escherichia coli* (ETEC) toxoid fusion and adhesin MEFA antigens in intradermally or intramuscularly immunized mice

by

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) strains are the most common bacterial cause of diarrhea. ETEC bacterial adherence to the small intestinal epithelial cells and delivery of enterotoxins cause diarrhea in children living in developing countries and international travelers. Currently, there are no vaccines licensed for ETEC associated children's diarrhea and travelers' diarrhea. Recently, toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (toxoid fusion), adhesin MEFA (multiepitope fusion antigen) CFA/I/II/IV (CFA MEFA), and toxoid-adhesin MEFA CFA/I/II/IV -3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (CFA-toxoid MEFA) are demonstrated to induce neutralizing antitoxin and/or anti-adhesin antibodies in intraperitoneal (IP) or subcutaneous (SC) immunized mice, suggesting these antigens are potential candidates for ETEC subunit vaccines. However, these antigens have not been examined for immunogenicity using intradermal (ID) or intramuscular (IM) routes, the routes perhaps are more suitable for human vaccine administration. In this study, toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$, CFA/I/II/IV MEFA, alone or combined, or toxoid-adhesin MEFA CFA-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ were ID or IM immunized to mice (8 mice per group) induced antigen-specific antibodies were titrated, and antibody neutralization activities were assessed in vitro. Data showed that mice ID or IM immunized with the toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ antigen developed anti-LT and anti-STa antibodies and mice immunized with the CFA/I/II/IV MEFA developed antibody responses to all seven adhesins (CFA/I, CS1-CS6). In addition, mice co-administered ID or IM with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ and CFA/I/II/IV MEFA, or with toxoid-adhesin MEFA CFA-3xST_{aN12S}-$mnLT_{R192G/L211A}$ developed antibodies to both toxins and all seven adhesins. Antibody neutralization studies of the serum samples of the immunized mice showed that the induced antibodies neutralized enterotoxicity of LT and STa and/or inhibited adherence of ETEC or E. coli

bacteria producing any of these seven adhesins. These data confirmed immunogenicity of these ETEC subunit vaccine target antigens and provide useful information for vaccine development against ETEC diarrhea.

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Acknowledgements

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Chapter 1 - Literature Review

Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) is a gram-negative rod- shaped bacterium that resides in human or animal intestinal tract. ETEC is one of the main causes of diarrhea in children under the age of 5 years living in developing countries including Africa, South America, and South Asia [1]. The bacterium also targets adults traveling from developed countries into the endemic countries or regions [1]. One of the first cases of ETEC occurred during the mid-1900s in Kolkata, India [3]. Soon after the initial discovery De and his colleges took different *E. coli* isolates from both children and adults whom displayed cholera-like sickness and injected live strains into isolated ileal loops of rabbits where they discovered that large amounts of fluid accumulated in the loops [3]. Later De and his colleges ligated ileal loops from rabbits and determined the changes in the loop inoculated with *E.coli* isolates were very similar to the ones induced by *Vibrio cholera* [3]. The new findings suggested similar pathogenesis between *cholera* and *E.coli* during diarrheal induction. Studies from Taylor and Bettelheim reported that gut-dilating *E.coli* isolate culture fluids, sterilized using chloroform were able to induce fluid secretion in the intestines, which led to the discovery of *E.coli* enterotoxins and enterotoxigenic characterizations [4]. In 1967 Smith and Halls reported that ETEC could also be induced in cell-free culture fluids [4]. Later Smith and Gyles discovered that ETEC had two different classes of enterotoxins: one was called heat- labile toxin (LT) because it was inactivated at 60°C for 30 minutes and the other was called heat- stabile (ST) because it retained its functional stability after being exposed to boiling temperatures for 30-minutes due to disulfide bonds [4].

Other than ETEC ST and LT enterotoxins there are adhesins that play a major role in diarrheal initiation. ETEC colonization in the small intestine is mediated by adhesins called

colonization factors (CFs), which are located on the surface of individual bacterium. There are various types of CFs; they differ in genetic make-up, structure, immunological function, and morphology. CF morphology is grouped into 4 types: fimbrial, fibrillar, helical, and non- fimbrial [5]. Fimbrial or fibrillar colonization factors are composed of pili, which are often called fimbriae, whereas some adhesins are proteins that are located on the outer membrane of the bacteria thus they lack macromolecular structure [5]. In the mid-1900s a nomenclature for CFs designated them as coli surface antigens (CSs) and colonization factor antigens (CFAs). Most CFs changed designation except CFA/I- CFA/IV: CFA/I, CFA/II (CS1-3), and CFA/IV (CS4-6) [2]. Currently, there are more than 25 colonization factors among human ETEC that have been identified [8].

ETEC Enterotoxins

Enterotoxin ST was one of the first enterotoxin detected in *E. coli* isolated from other species besides humans [7]. That led to subdivision of heat stable enterotoxins into STa and STb. STa is a low molecular weight peptide that consist of 18-19 amino acids and is produced by ETEC bacteria causing diarrhea in animals and humans. STa is nonantigenic and it has a molecular size of 2 kilodaltons (kDa) [7]. Two host-specific STa toxins were identified: STp or pSTa for ST causing diarrhea in pigs and other animals, whereas STh or hSTa for the ST causing diarrhea in humans. Even though STp and STh are different, they can both be found in human ETEC strains [7]. Both STp and STh have identical invasive mechanisms [2]. ST toxins are encoded with *estA* gene, which resembles to the hormone guanylin or uroguanylin in humans [6]. Guanylate cyclase C(GC-C) is activated upon STa binding, which lead to elevated levels of intercellular cyclic guanosine monophosphate (cGMP). Elevated intercellular cGMP levels activate protein kinase II and protein kinase A, which phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR) anion channels. The intercellular disruptions open the Cl- anion channels and

inhibit the NaCl transporters [6]. The disruption of the cell homeostasis leads to hypersecretion of water known as watery diarrhea [6].

Enterotoxin heat-labile (LT) is considered one of the most important virulence factors for ETEC [6]. It has been demonstrated that heat-labile production and secretion is influenced by extracellular pH of 7 or above, so when ETEC bacteria reach and colonized in the small intestine, LT is quickly secreted [57]. A study indicated that 17,205 ETEC isolates, which accounts for 60% of the global isolates were tested for the presence of either LT or STa enterotoxins. Out of the 60% isolates, 27% of the isolates expressed LT alone and 33% were in a combination with ST [58]. Therefore, production of anyone of these enterotoxins is enough to cause diarrheal illness [16]. LT toxin is 80% similar structurally, functionally, and antigenically to cholera toxin (CT). LT has a molecular mass of 84 kilodaltons composed of one 28-kDa A subunit and five identical 11.5-kDa B subunits [16]. Subunit B pentamer is responsible for the binding and colonization of the small intestine [2]. After ETEC colonization and secretion of LT, LT-B subunits bind irreversibly to ganglioside (GM1) on the outer surface of the epithelial cell. After GM1 binding LT is endocytosed and transported to the endoplasmic reticulum (ER) [60]. Next the internalized subunit A, which has two domains A1 and A2 is cleaved and secreted through the vacuolar membrane. A1 polypeptide is responsible for the observed toxic effects it also acts on the α subunit of the GTP-binding protein (Gs-α) located on the basolateral membrane of intestinal epithelial cells by transferring an ADP-ribosyl moiety from NAD to Gs-α [59;60] . The activation of Gs protein activates adenylate cyclase, that leads to elevated intercellular cyclic AMP (cAMP) levels. Elevated levels of cAMP activate protein kinas, which then stimulate Cl secretion through the cyclase activates cystic fibrosis transmembrane regulator (CFTR) causing inhibition of sodium

chloride in the villus tips [16]. Finally, there is a large amount of salt and water transported into the intestinal lumen, when the bowel reaches its absorbance capacity leads to watery diarrhea [2].

Aside from LT being a toxin, it has been demonstrated that both cholera and heat labile toxins display strong adjuvant capabilities [16]. Subunit B demonstrates adjuvant activity, but it reaches it optimal activity when subunit A is retained. When using LT as an adjuvant, many challenges rise, one of the most significant one is its toxicity, but to overcome this challenge, the construction of a mutant LT was designed specifically for adjuvant use [16]. The double mutant LT (dmLT) contain mutations in the A subunit (R192G, L211A), that prevent the activation of LT into catalytically active form [16].

ETEC Bacterial Adhesins

There are more than 25 colonization factors detected from various ETEC strains. Among these adhesins, CFA/1, CS1, CS2, CS3, CS4, CS5, and CS6 adhesins have been characterized to be the most prevalent and expressed by more virulent ETEC strains. These seven CFs account for 50-70% of ETEC isolates associated with human diarrhea [8]. In addition, CFs have been identified on ST and LT- or ST-producing ETEC strains. Studies have shown that the relationship between the presence of CFs and the effect of ETEC induction differ based on regional settings [2]. In community-based studies diarrhea increased with the presence of CFs [2]. In Mexico there was a reduction of severity in infants that have been re-infected with ETEC containing different CFs. Volunteer challenge studies indicated protection against ETEC with a vaccine containing present CFs. The results indicate that creating a vaccine that targets the most potent CFs and enterotoxins STa and LT would induce a broad- spectrum protection [2].

After the initial ETEC discovery, scientist found similar ETEC strains in animals such as: rabbits, pigs, cattle, and other farm animals [5]. Although, ETEC pathogenesis and enterotoxin ST

and LT used in the process for the stimulation of water hyper-secretion causing diarrhea in animals are similar to the one in humans, there are different CFs [5].

ETEC is the leading cause of diarrhea in the developing world such as Africa, South Asia, and South America. Annually there are 280-400 million cases of diarrhea in children younger than 5 years and 100 million cases in children older than 5 years old [10]. The illness usually last 3-5 days and the symptoms range from mild diarrhea with no dehydration to sever cholera like symptoms. Although, the symptoms have different adversities, the yearly deaths due to ETEC are 300,000-500,000 [10]. Studies show that each year children under the age of 5 living in the epidemic areas experience a median of 3.2 episodes of diarrhea within their first 2 years of life [10;12]. This suggest that immunogenicity to ETEC comes with age. Furthermore, episodes of diarrhea have often led to malnutrition and immune deficiencies. Long term effects of severe and repeated episodes in children lead to stunting and cognitive impairments [13].

Although, ETEC is most likely to occur in infants, naïve adults living in industrialized countries are susceptible to infection. Studies suggest that 20-60% of adult travelers get diarrhea and out of that percentage ETEC is responsible for 20-40% of the diarrhea [7]. That confirms that ETEC is the most common cause of diarrhea in adult travelers. Annually 42% of traveler's experience diarrhea in Latin America, 36% in Africa, and 16% in South Asia [11]. Yearly there are an estimate of 400 million cases of diarrhea in adult travelers [10]. Most of the ETEC bacterial infections are naturally eliminated by our body due to natural immunity, but it is still a significant danger to children, pregnant women, and old people due to weak immune system [10].

ETEC Infection

ETEC is a food and waterborne pathogen. It is mostly found in impoverished areas due to the lack of sanitation and limited access to clean drinking water [13]. ETEC infection is initiated

by three main ways: direct contact to animal or human feces (oral- fecal transmission), intake of large quantities of ETEC due to contaminated water or food, and person-to-person contact. When individual's intake contaminated water, an intake up of 10^9 bacteria per milliliter (mL) immediately leads to diarrhea [7]. Water contamination comes from ETEC-containing feces from wildlife, livestock animals, and humans get into water sources and soil. Published data suggest that when fecal bacteria is discharged into toilets or other water sources the bacterial is suddenly exposed to colder and less nutritional environments. Due to the instant change the bacterial growth halts, but can remain viable for extended time periods which poses a huge threat to disease distribution(Lothigius *et al*., 2010),. One study showed that families in Bangladesh, who use cow dung as cooking fuel have ETEC isolates present in their stool due to the cross contamination of ETEC and food.

ETEC is very good at adapting to harsh conditions for survival, but its optimal place for growth is in areas that are warm and humid. A study in Mexico suggested diarrhea was significantly higher during the summer season than during the winter season. The study also demonstrated an association between warmer and wetter summers to an increase in prevalence of diarrhea, suggesting ETEC infection is seasonal [15].

Diarrhea cause an extreme loss of fluid and electrolytes, which leads to rapid dehydration. Therefore, providing fluids intravenously (IV), orally, or through a nasogastric tube (NG) is necessary for rehydration [62]. Antibiotics are an alternative for bacterial reduction. Although, antibiotics are available it is not advisable to use them, especially in children [2]. Childhood diarrheas are not only caused by ETEC, it could also be caused by other bacteria or viral agents. Antibiotics are not advisable to be used in adults due to risks of antibiotic resistance buildup in ETEC isolates, which might lead to an ETEC with stronger survival mechanisms and virulence

[13]. Therefore, antimicrobial resistance is a significant problem when trying to fight ETEC. A study in Vietnam tested 162 diarrheagenic *E. coli* strains including ETEC strains, for antibiotic resistance. The results suggested that ETEC had a 78% resistance build up against 8 classes of antibiotics [63]. Furthermore, studies have shown that rehydration and antibiotics do not pose stable and long-term treatment against ETEC [2]. The most practical and beneficial way of presenting ETEC diarrheal disease is through vaccination [7].

Vaccines and Vaccination

Vaccine development has been one of the greatest achievements in modern medicine. A vaccine is a biological preparation used to stimulate immunity against any toxic or unwanted pathogenic trying to invade the human body. Vaccines are significant because the exposure of low doses of the pathogen to the immune system can generate an immunological memory against the pathogen [31]. Vaccines act as an antigen but does not induce diseases. In the late 1700s Edward Jenner discovered that dairymaids had immunity against smallpox after being exposed to cowpox [32]. In addition, Edward concluded that cowpox transmitted from human to human and that could lead to a protective mechanism against small pox. He then injected small doses of cowpox agents into the arms and legs of uninfected individuals and later saw that they developed immunity to small pox, and due to his discovery small pox was eradicated [32]. Since Edwards vaccination discovery, many scientists have focused on vaccine development to prevent, control, or eradicate life threatening infectious diseases.

Vaccine against ETEC

Since the beginning of vaccine development, most vaccines have been delivered through injections. Injection is suitable because the antigen is delivered directly into the immune system.

However, most pathogens including ETEC enter the body through the mucosal surface. Oral vaccines are complex to design because there are three barrier inferences that can degrade the vaccine. First, the vaccines have to overcome all the obstacles in GI tract such as: pH, digestive enzymes, and bile salts. Second, the vaccine has to make it to the intestine to mimic natural infection. Finally, the vaccine needs to cross the epithelial layer to enter the mucosal tissue to elicit mucosal immune responses [22]. Current ETEC pathogenesis and immunology indicated that ETEC vaccine should be administered orally and target CFs and enterotoxins to stimulate anti-colonization and antitoxin response in the intestine [24].

Whole-cell Inactivated ETEC Vaccine

In 1969 Gyles and Barnum discovered that enterotoxigenic *E. coli* and *Vibrio cholera* enterotoxins shared a similar immunological relationship [25]. In 1974 Carlton Gyles confirmed that there are antigens common to *E. coli* LT and *V. cholera* LT enterotoxins that induce antibodies that allow *E.coli* enterotoxin LT to be neutralized by anti-cholera toxins at high dilutions. Although *V. cholera* enterotoxin can neutralize the *E.coli* LT, *V. cholera* LT cannot be neutralized by anti-*E.coli* LT [26]. *Vibrio cholera* enterotoxin contains two different subunits: subunit A which is responsible for the toxin diverse biological effect and subunit B, which is a protein called choleragenoid and binds to the ganglioside receptor on the host cell membrane [25]. In 1974, Heyningen discovered that intact *V. cholera* toxins contain 4 fragments subunit B plus all of subunit A. Choleragenoid is nontoxic and it is entirely made up of fragment B [27]. In 1974, Gyles also demonstrated that there was a human *E coli* enterotoxin LT that had a reaction against choleragenoid (subunit B) and anticholera toxins [26]. In 1978 Clements and Finkelstien discovered that *E.coli* LT and *V. cholera* are antigenically related to cholera subunit A and B [28].

In addition, they also discovered that a portion of enterotoxin *E.coli* LT was antigenically similar to cholera subunit B [26].

Due to the immunological similarities between cholera toxin (CT) and enterotoxigenic *E.coli* LT in 1988, Clemens and Stack developed an oral vaccine composed of a combined cholera toxin B subunit/ whole cell (BS-WC) vaccine to create protection against enterotoxigenic *E.coli* targeting LT [29]. They hypothesized that the vaccine composition would elicit antibodies that cross-react with LT and prevent diarrhea in humans. Their results determine a 70% short term protection rate against LT-producing ETEC diarrhea in both adult travelers and children span of only three months [29].

In 1993, Jertborn and Ahren were the first to develop an oral vaccine that targeted not only ETEC LT, but STs for broaden and long-lasting protection. The vaccine was composed of recombinant cholera B subunit (rCTB) as well as formalin killed ETEC bacteria expressing CFA/I and II [30]. Results showed that the vaccine was safe to use for adults, and it elicited high IgA response against CTB and major CFAs. In addition, the vaccine had no adverse effects, but the vaccine had little to no protection in children from an endemic country [30]. Scientists are still trying to develop oral inactivated vaccines that not only protect adults, but also children with little to no side effects.

Parenteral Immunization Routes

There are different administration or injection routes to be considered during vaccine development. For every injection used there are different absorbance rates and dosages that influence efficacy of the vaccine. Four parenteral injections: Intradermal (ID), Intraperitoneal (IP), Intramuscular (IM), and Subcutaneous (SC) are commonly used in vaccine development. These injection routes are very distinct from each other. Although, they all share similar immune

response mechanisms, some routes are better than the others based on the level of antigen presenting cells that are available. In 1868, Paul Langerhans identified dendritically shaped cells in the epidermis that he later named Langerhans cells). He also identified dermal dendritic cells, which were phenotypically distinct from Langerhans cells [42]. Langerhans cells and dermal dendritic cells guard the host and they play a crucial role in the induction of the adaptive immune response when pathogens try to invade. The overall immunogenicity of administered vaccines are dependent upon antigen presentation and processing by lymph node and dendritic cells in the injected area [35]. Dendritic cells are multivariant cells with multiple functionalities such as: antigen processing and presentation to the MHCII complex, they are primary initiators of T cell responses, they mediate cross talk between C-type lectin/Fc receptors, and they signal danger signals to toll-like receptors, which mediate effective immune presentation [35].

Nowadays most vaccines are administered using subcutaneous and intramuscular injections [51]. Intradermal injections are less likely to be used due to the thought of them being difficult to inject, require special training, and they are unreliable to perform. Lately, researchers have developed a liquid jet injector that administers vaccines intradermally [61]. Intramuscular, subcutaneous, and intradermal routes require the presence of DC in the tissue so that the vaccine can be taken up, processed, transported, and presented to T lymphocytes in the draining lymphoid organs [51]. Intraperitoneal injections are not used in humans or mammals, they are mostly used for preclinical studies to determine antigen immunogenicity on rodents [53]. Therefore, the type of injection used for experimental vaccines are extremely critical for the induction of immune response [42].

Intraperitoneal Immunization (IP)

Intraperitoneal (IP) injections are rarely used on humans due to practicality, rather this route is a common technique in lab rodents, but not even then should this injection be used on rodents because this technique is unreliable due to accidentally injecting some substance into the gut, abdominal fat, or subcutaneous tissue [53;54]. When the injection is used for rodents for which intravenous injections are inaccessible and IP injections can deposit large quantities of volume. Absorption rate using IP injections are much slower than interventions injections. The injection causes little to no pain on the rodents, but to reduce further discomfort, the injection should be given at room temperature [54].

Subcutaneous Immunization (SC)

Subcutaneous immunization is to administer vaccine products under the skin. This type of injection requires a short needle to ensure that the injection lies between the skin and the muscle. Subcutaneous route is a slower route because the injection lies where there is less blood flow. Subcutaneous contents are absorbed over a 24-hour span and the total volume that can be used ranges from 1-5ml [4]. This route is used when other methods of administration are not effective [33]. The first case of subcutaneous immunization in humans was performed by Edward Jenner in the late 1700s when he administrated the vaccine product on human legs and arms to eradicate small pox [32]. Subcutaneous injections usually have little to no pain and they are easy to administer [32].

Intradermal Immunization (ID)

Intradermal (ID) immunization is to deliver vaccine product into the dermis, which is right under the epidermis. The dermis and the epidermis of human skin contains the highest amount of antigen presenting cells when compared to all other parenteral injections. ID is the slowest antigen

absorbing route when compared to all other parenteral routes. This route has an advantage, which is vaccine administration can be confirmed at the right injection site by the formation of a bleb. The amount of antigen administered ranged from 0.1ml-0.5ml [39]. In 2008 Lambert suggested that the large amount of antigen presenting cells leads to an efficient induction of immune response, while using smaller doses of antigen [40]. Antigen dose sparing is beneficial especially for vaccine development because it reduces cost, increase vaccine coverage, and it aids in stretching the availability [41]. Intradermal injections have shown a 20% volume decrease in inactivated poliovirus vaccine when compared to intramuscular route [43]. An experiment done by Laurent indicated that intradermal route is a dose sparing route, in his rabies vaccine he only used 25% of the vaccine when compared to a full does using intramuscular route [44].

Intramuscular Immunization (IM)

Intramuscular (IM) injections have been commonly used since the 1960s [38]. This immunization route is to inject vaccine products right into the muscle fascia, which consist of a rich bloodstream that allow antigens to be absorbed at a much quicker manner than other immunization routes [38]. The antigen absorbance time ranges from 15-20-minutes [36]. In addition to the fast absorbance rate, IM injection is also demonstrated with a prolonged antigen presentation duration. Due to the accessibility to blood supply, IM injections are able to administrate large volumes of antigen in adults ranging anywhere from 0.5 to 5 ml depending on the muscle. Although 5ml volume can be injected, most clinicians do not use any more than 3ml due to the unknown side effects and efficacy [36]. Furthermore, IM injection is suitable for research use and human administration due to large antigen intake as well as antigen stability [37]. Finally, IM injection has little to no pain, due to the muscle tissue being less sensitive when

compared to subcutaneous tissue, but inappropriate injection site and poor technique can cause injury and discomfort [3].

Study National and Significance

Enterotoxigenic *E.coli* (ETEC) is found in the intestine of both humans and animals [1]. ETEC causes alarming diarrheal episodes in children whom live in undeveloped countries [2]. Furthermore, associated morbidity is extremely high in many endemic countries as children experience 1-2 diarrheal episodes per child during their first 2 years of life [19]. Sanitation facility can reduce the ETEC diarrheal incidence in the affected countries, but vaccination is the most practical prevention method of stopping colonization of ETEC. ETEC virulent factors are highly heterogeneous, so it poses a huge challenge in developing an effective ETEC vaccine [5]. One of the latest vaccine development technologies is of a multiepitope fusion (MEFA) vaccine [45]. Different epitopes from various virulence factors were used and fused together, creating a broad epitope vaccine. Such a vaccine product needs to be tested for stability, safety, immunogenicity, and protective efficacy against targeted virulence factors. In recent studies the MEFA vaccine was developed and targeted the most potent adhesins based on computational analyses, it also incorporated the two enterotoxins STa and LT, which disrupt host cell homeostasis to cause watery diarrhea by ETEC [45]. Previous studies suggest that different immunization routes induce better protection in humans when vaccinated against diseases [56]. Therefore, the route of administration is the second biggest factor after vaccine development

Chapter 2 - Research Design & Experiment

Introduction

Enterotoxigenic *E. coli* (ETEC) is a leading cause of diarrhea in children from developing countries such as South Asia, South America, and Africa [10]. ETEC bacteria produce adhesins and enterotoxins to attach to host small intestine and to cause diarrhea [5]. Currently, there are no effective methods of preventing diarrheal diseases. Vaccines inducing protective antibodies would be the most effective and most practical way to prevent ETEC infection. In order to develop vaccines, *in vitro* studies have to be conducted before *in vivo* or human subject studies. Because bacterial adhesins and enterotoxins are the virulence determinants of ETEC in diarrhea, they are often targeted in ETEC vaccine development [6.8]. ETEC adhesins, including colonization factor antigens (CFAs) and coli surface antigens (CSs), mediate ETEC bacteria initial attachment to host epithelial cells and promote bacteria colonization in host small intestines [5]. Therefore, preventing bacterial adherence is the first line of defense against ETEC infection, and developing anti-adhesin vaccines becomes a priority for prevention of ETEC diarrhea. In this study, we intradermally and intramuscularly immunized mice with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (toxoid fusion), the CFA/I/II/IV MEFA (CFA MEFA), alone or combined, or toxoid-adhesin CFA-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (CFA-toxoid MEFA), and characterized antigenicity of each protein and their candidacy for ETEC vaccine development.

Methods and Material

Bacterial Strains and Plasmids. *E.coli* recombinant strain 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (toxoid fusion) was derived from 6x-His tag 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (Ruan et al., 2014, IAI) by removing the 6x-Histidine tag and expressing as a tag-less toxoid fusion protein in vector pET28

[46]. The CFA/I/II/IV MEFA gene was constructed by using CFA/I major subunit CfaB backbone to present epitopes in silico identified from the major subunits of the 7 most important ETEC adhesins CFA/I, CS1-CS6. The identified epitopes were incorporated in the CFA/I/II/IV MEFA gene [47]. For the gene construction of CFA/I/II/VI-STa-toxoid-dmLT (CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, 9419) two PCR fragment products: CFA/I/II/IV and STa-toxoid-dmLT toxoid fusion were overlapped for a single open-reading-frame to form a CFA/I/II/IV-STa-toxoid-mnLTR192G/L211A chimeric gene [48].

Protein Expression and Detection. CFA/I/II/IV MEFA (9472) was streaked on an LB agar plate. One colony was taken selected to ensure purity. Bacteria was then grown in 5ml LB medium containing 5µl kanamycin (30 g/ml) shaking (220 rpm) overnight at 37°C. Four ml overnight culture was then added to 200ml 2xYT broth (2x yeast extract and tryptone) (Fisher Scientific, MA) containing 200µl kanamycin. Bacteria was incubated for 2-2.5-hours in an incubator- shaker (220rpm) until the optical density at 600nm (OD_{600}) reached 0.4-0.7, then induced with isopropyl-1-thio-D-galactoside (IPTG; 1 mM) (Sigma, MO) for 4 additional hours. The bacteria was centrifuged for 15-minutes at 12,000rmp at 10°C. Pellets was suspended in 10 ml bacterial protein extraction reagent (B-PER, Thermo Fisher Scientific, MA), and suspension was shaken for 30-minutes at room temperature (RT) and then centrifuged at 12,000rpm for 15-minutes at 10°C. Resultant pellets were suspended in B-PER, mixed with lysosome (200µg/ml) for 40-minutes at RT to lyse the bacterial cell, followed by centrifugation. Afterward, pellets were suspended with 1:10 diluted B-PER (in PBS) and centrifuged again. Finally, protein pellets were washed with 100ml PBS and centrifuged 2 more times. After final wash, PBS was used to suspend the pellet to obtain total insoluble protein (inclusion body). The protein was moved to the refolding process using a protein refolding kit following Novagen manufacture protocol., Solubilized proteins were

transferred into a molecular porous membrane tubing (Spectra/Por Dialysis Membrane) (Spectrum Laboratories, Inc., Rancho Dominquez, CA) and refolded using dialyzed buffer (20 mM Tris-HCl) containing 0.1mM 1,4-Dithiothreitol (DTT) at 4°C, with refolding buffer change every 4-hours. The steps were followed once more but with no DTT added. Dialysis buffer was changed at least 2 times every 4-hours. Finally, protein concentration was measured using lowery protein assay, aliquoted into 1.5ml tubes, and stored immediately at -80ºC.

Refolded proteins were examined in SDS-PAGE. Ten µl refolded protein was electrophoresed using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). toxoid fusion protein 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (9471) and CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (9419) were extracted, refolded, and analyzed.

Mouse Intradermal Immunization with CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$. Eight-week old female BALB/c mice (Charles River Laboratories International, Inc., Wilmington, MA) were used for the experiment. Five groups, 8 mice/ group, were used in intradermal (ID) immunization. Holotoxin-structured double mutant heat-labile toxin, dmLT (mnLT$_{R192G/L211A}$) provided by Walter Reed Army Institute of Research (Silver Spring, MD) was used as adjuvant for the immunizations. Each mouse in the immunization group was immunized with 25µg of antigen and 0.2µg dmLT adjuvant. Mice in the first group were immunized with PBS served as the control group; mice in the second group were immunized with CFA/I/II/IV MEFA (9472); mice in the third group were immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (9471); mice in the fourth group were immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (9419);, ,mice in the fifth group was immunized with a combination of CFA/I/II/IV (12.5µg) and toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (12.5 ug). Two boosters followed the primary immunization, with a two-week time interval between

immunizations. All mice were monitored for behavioral and physical abnormalities. Blood and fecal samples were collected from individual mice before the priming and two weeks after the second boost. Mouse serum samples were collected and stored at -20°C until further use. Mice immunizations complied with the 1996 National Research Council guidelines. Mouse immunization protocols were approved by Kansas State University Institutional Animal Care and Use Committee (IACUC).

Mouse Serum Anti-adhesin and Antitoxin Antibody Titration. Since CFA fimbriae adhesin contain thousands of copies of the adhesin major structural subunit, extracted fimbriae were used as coating antigens for Enzyme-linked immunosorbent assay (ELISA) to titrate mouse antibody responses specific to each target ETEC adhesin [49]. In this study, ELISA 96- well plates were coated with different antigens and were used to titrate anti-adhesin IgG antibodies from the serum samples of mice ID immunized CFA/I/II/IV MEFA, CFA-3xSTa_{N12S}-$mnLT_{R192G/L211A}$, and CFA/I/II/IV combined with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$. CFA/I, CFA/II (CS1, CS2, CS3) and CFA/IV adhesins, (CS4, CS5) were extracted from recombinant *E.coli* stains using a heat- extracted adhesin fimbriae method as previously described [45]. CS6 is a non-fimbrial outer membrane protein, thus recombinant CS6 structural subunit CssB was used for anti-CS6 antibody titration. Extracted adhesins CFA/I , CS1, CS2, CS3, CS4, or CS5 adhesin, or CS6 recombinant protein was coated, 500 ng (in 100 ul) per well using coating antigen buffer made from 0.015MNa2CO3 and 0.035MNaHCO3, to each well of Immulon 2HB plates (Thermo Scientific, Rochester, NY) to titrate antibodies that were specific to each of the seven adhesins. Coated plates were incubated at 37°C for 1-hour, then they were transferred to 4°C overnight. Coated plates were washed three times with PBS containing 0.05% Tween 20 (PBST), blocked with 200µl of 10% nonfat instant dry milk (each well) at 37°C for 1-hour. Wells were washed three times with PBST.

Mouse serum samples from each immunized group and control group (in duplicate) two-fold diluted (1:800 - 1:526.000 dilutions) using 2.5% milk + PBST and was added to wells and incubated at 37°C for 1-hour. Incubated wells were washed with PBST and incubated with 100µl diluted (1:3000) horseradish peroxidase (HRP)- conjugated goat anti-mouse IgG (Bethyl) at 37°C for 1-hour. After PBST washes, 100µl with 3,3-,5,5- tetramethylbenzidine (TMB) Microwell peroxidase substrate system (2-C) (KPL, Gaithersburg, MD) was added to each well. Plates were stored in a dark place for 5-minutes room temperature. The optical density (OD) was measured using a plate reader with a wavelength set at 650nm. Antibody titers were calculated as the highest dilution that produced OD readings greater than 0.3; they were then presented using log10 scale [50]. The same protocol was used for Intramuscular route with the only exception that immunized, and control serum was initially diluted at1:400.

Mouse serum samples of the groups ID immunized with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$, CFA-3xSTa_{N12S}-$mnLT_{R192G/L211A}$, or CFA/I/II/IV combined with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ were titrated for IgG antibodies specific to heat-labile (LT) and heat-stable (STa), using the same protocol described above. LT specific antibodies were tested using 100ng CT (Sigma, MO) (per well of H2B plates) coating antigen. To titrate antibodies specific to STa, 10 ng STa-ovalbumin conjugates (per well of Costar plates; Corning Inc., Corning, NY) were used as coating antigen. The protocol for STa was the same except there was a 5% nonfat milk block in the first incubation and the serum dilutions were made with 1% milk +PBST mixture. The serum dilutions stayed the same as listed above for ID and IM route.

Mouse serum anti-adhesin antibody adherence inhibition assay. Pooled mouse serum samples from each immunized and control group were studied in vitro. ETEC and *E.coli* recombinant bacterial stains expressing CFA/I, CS1, CS2, CS3, CS 4/CS6, CS5/CS6, or CS6

adhesin ($2x10^5$ CFU with a cell to bacteria ratio of 1:10) were pre-treated with 4% mannose combined with 30µl serum (that was inactivated at 57°C for 30-minutes) and incubated at room temperature on a shaker (80rpm) for 30-minutes. The mixture was then added to a 24-well tissue culture plate containing (7×10^5) Caco-2 cells (HTB-37, ATCC); in 1 ml Dulbecco's modified Eagle's medium (DMEM- F12) (ATCC, VA) and incubated in a CO_2 incubator (5% CO_2) for 1-h at 37°C. After incubation, non- adherent bacteria were washed away using PBS. Caco 2 cells were then dislodged using (500µl of sterile 0.5% TritonX-100). Adherent bacteria were collected by centrifugation (15,000 g for 10-min), re-suspended in 1 ml PBS, serially diluted, and plated on LB plates for 37°C overnight growth. Antibody adherence inhibition activities were reflected by the reduction of the numbers of *E.coli* or ETEC bacteria expressing adhesins adherent to Caco-2 cells (% with the control as of 100%).

Anti-toxin antibody neutralization assay (CT). Pooled mouse serum samples from the group immunized with toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$, CFA-$3xSTa_{N12S}$-$mnLT_{R192G/L211A}$, or toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ combined with CFA/I/II/IV were used for antitoxin antibody neutralization assays using direct cAMP ELISA kit (ENZO Life Sciences Inc., NY). T-84 cells ($1x10^5$) derived from human colon were seeded in a 24 well- tissue culture plate (Falcon, Franklin NJ) with DMEM-F12 medium (ATCC, VA) containing 15% fetal bovine serum (FBS) (Fisher Thermo Scientific, Pittsburg, PA). T-84 cells produce intracellular cAMP, stimulated by LT enterotoxicity, but antibodies specific to LT can neutralize LT toxins thus prevent cAMP elevation. To test the intracellular cAMP levels, 30µl of pooled serum of each group including control group was incubated with 10 ng CT at RT for 30-minutes. The CT and serum mixture was transferred to each well of a 24 well- tissue culture plate (Falcon, Franklin NJ) bringing the final volume for each well to 1ml. The plate was incubated in a CO_2 incubator for 3-

hours. After three PBS washes, cells were lysed using 300µl of 0.1M HCl with 0.5% triton X-100. Supernatant was collected and used to measure cAMP levels by following the manufactures protocol. DMEM media was used as a blank and it served as a baseline for cAMP levels in the T-84 cells. CT toxin was used as a positive control to indicate the cAMP stimulation in T-84 cells.

Anti-toxin antibody neutralization assay (STa). Antitoxin antibody neutralization against STa toxin was measured with a cGMP EIA kit and T-84 cells. This assay protocol is the same one as above, with the only change of 1-hour incubation instead of 3-hour incubation in cAMP assay.

Statistical analysis. Data was analyzed using GraphPad Prism 5. The results were presented as means with standard deviations. Differences between groups were calculated by one-way analysis of variance (ANOVA). A post-test named Turkey was also used, as it was in charge of comparing all pairs of columns. Both one-way ANOVA and Turkey had a confidential interval of 95%. Calculated p values of less than 0.05 were considered as significant difference between groups.

Results

Mouse Intradermal Immunization Study

Antigen characterized in SDS-PAGE and mouse immunization. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was used to demonstrate the purity and concentration of the three antigens used for the experiment **(Fig 2.1)**. SDS-PAGE with Coomassie blue staining detected toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (toxoid fusion) (47kDa), CFA/I/II/IV MEFA (CFA MEFA) (15kDa), and CFA-3xSTa_{N12S}-$mnLT_{R192G/L211A}$ (CFA-toxoid MEFA) (47kDa).

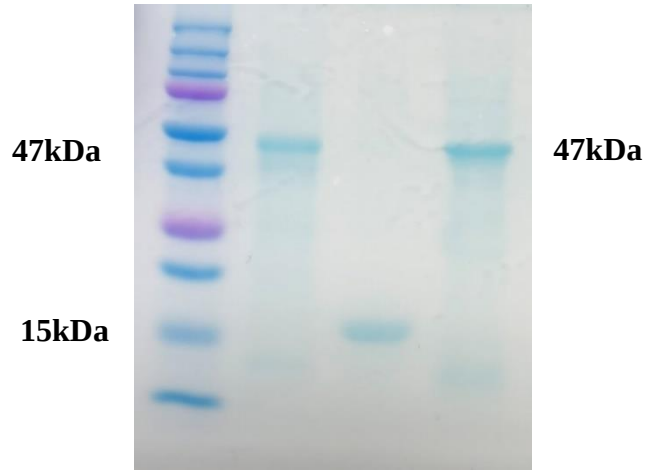

Figure 2.1 Toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, CFA/I/II/IV MEFA, and CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ in SDS-PAGE with Coomassie blue staining. Ten µl of toxoid fusion (47kDa), CFA/I/II/IV MEFA (15kDa), and CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (47kDa) were examined in a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Eight-week old female BALB/c mice, eight per group, were intradermally immunized with 25µg of antigen in adjunction with 0.2µg of double mutant LT adjuvant (dmLT) (**Fig 2.2**). A group of 8 mice immunized with PBS served as the control. Each immunized mouse received one primary immunization followed by two boosters with two-week intervals. Two weeks after the final booster, mice were sacrificed; mouse serum samples were collected and stored at -80°C until use.

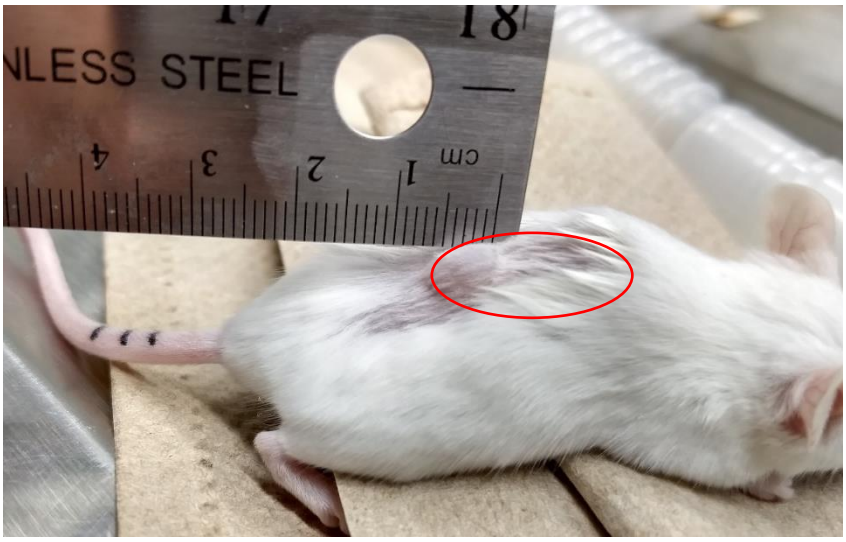

Figure 2.2 Bleb from mouse ID immunized. A diameter of 0.5cm located under the skin of an adult female BALB/c to show a successful ID injection.

ID immunized mice developed anti-adhesin and/or anti-toxin IgG antibodies. ELISA was used to measure serum IgG immune response against 7 adhesins (CFA/1, CS1-CS6) and enterotoxins (STa and LT). Antibody titrations started at 8-fold (1: 800) and serially diluted. Mice ID immunized with CFA/I/II/IV MEFA developed anti-adhesin IgG antibody responses. Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and anti-CS6 IgG titers were detected at 3.79±0.35, 3.14±0.49, 3.55±0.35, 3.08±0.07, 2.81±0.29, 3.32±0.35 and 3.24±0.15 **(Fig. 2.3)**. The group immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, IgG titers were detected at 4.16±0.38, 2.97±0.35, 3.37±0.44, 3.90±0.19, 2.05±0.30, 3.59±0.45, and 3.47±0.55 **(Fig 2.4)**. The group immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had titers of 3.14±0.39, 3.18±0.30, 2.73±0.32, 3.53±0.38, 2.53±0.38, 3.68±0.51, and 2.77±0.33 **(Fig 2.5)**.

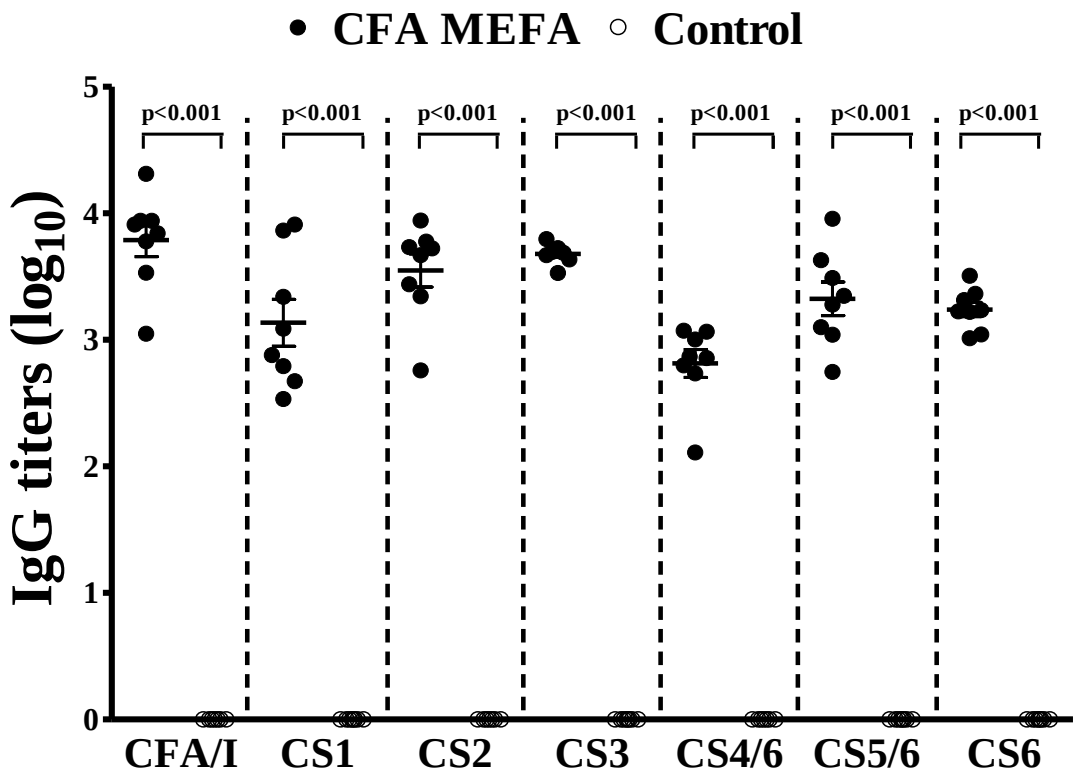

Figure 2.3 Anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from mice intradermally immunized mice with CFA/I/II/IV MEFA (●) and the control mice (○).
Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

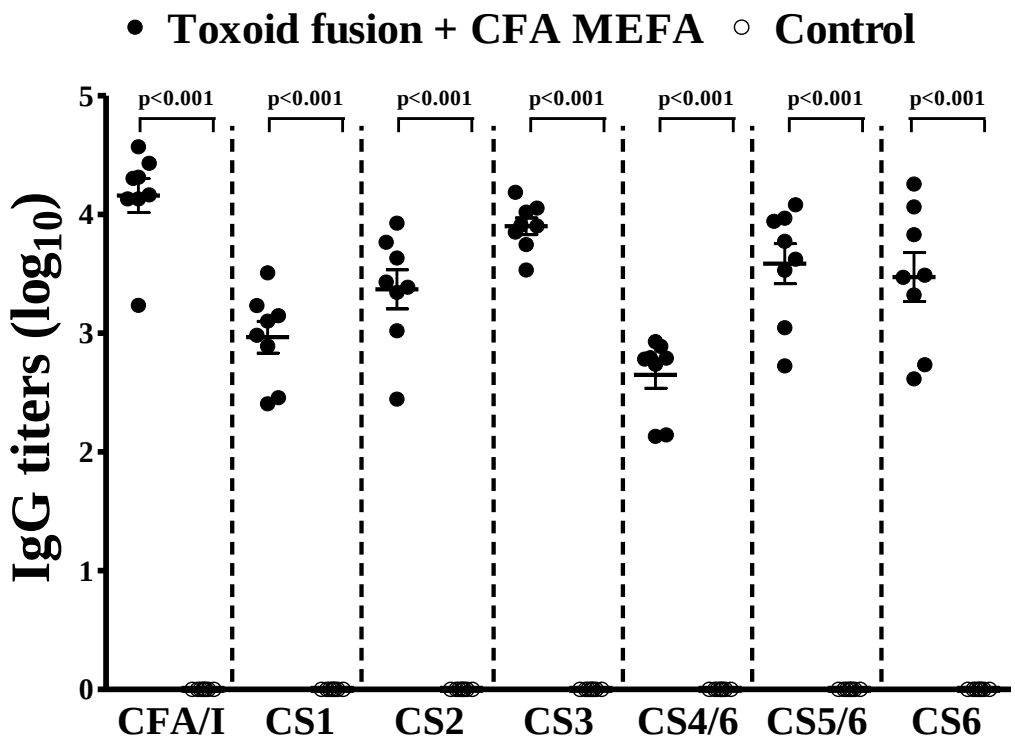

Figure 2.4 Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intradermally immunized mice with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$ + CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

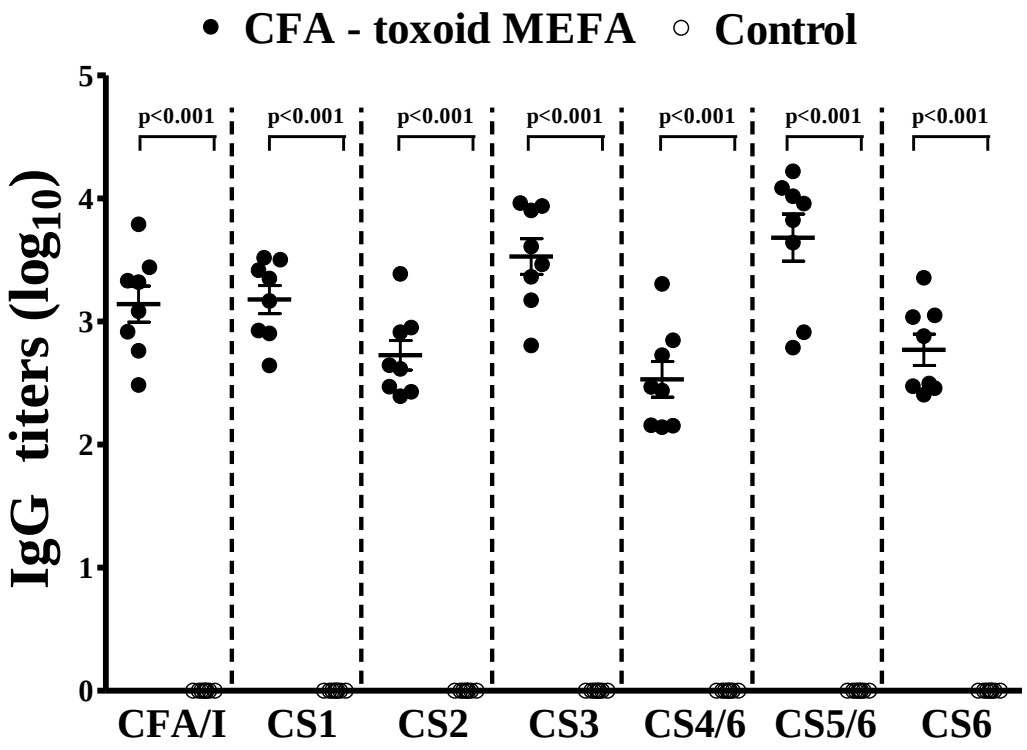

Figure 2.5 Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intradermally immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ developed anti-LT IgG titers at (4.18±0.21). Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA had anti- LT titers detected at (3.93±0.21), and mice immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had LT titers detected at (3.98±0.28) (**Fig 2.6**).

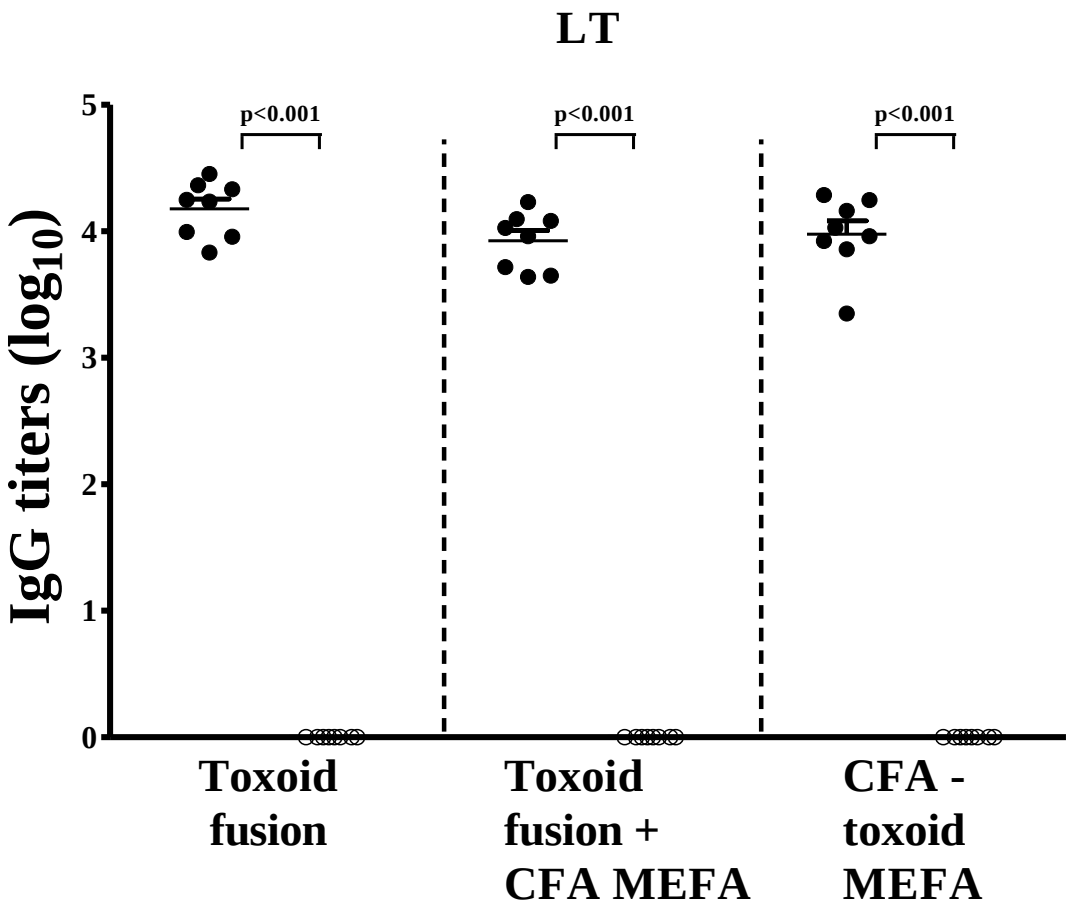

Figure 2.6 Mouse serum anti-LT IgG antibody titers from intradermally immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ developed anti-STa IgG titers at 2.73±1.60, 1.42±0.90, and 3.39±0.50 (log_{10}) respectively (**Fig 2.7**). There were no anti-adhesin or antitoxin IgG antibody response detected in the serum of the control mice immunized with PBS.

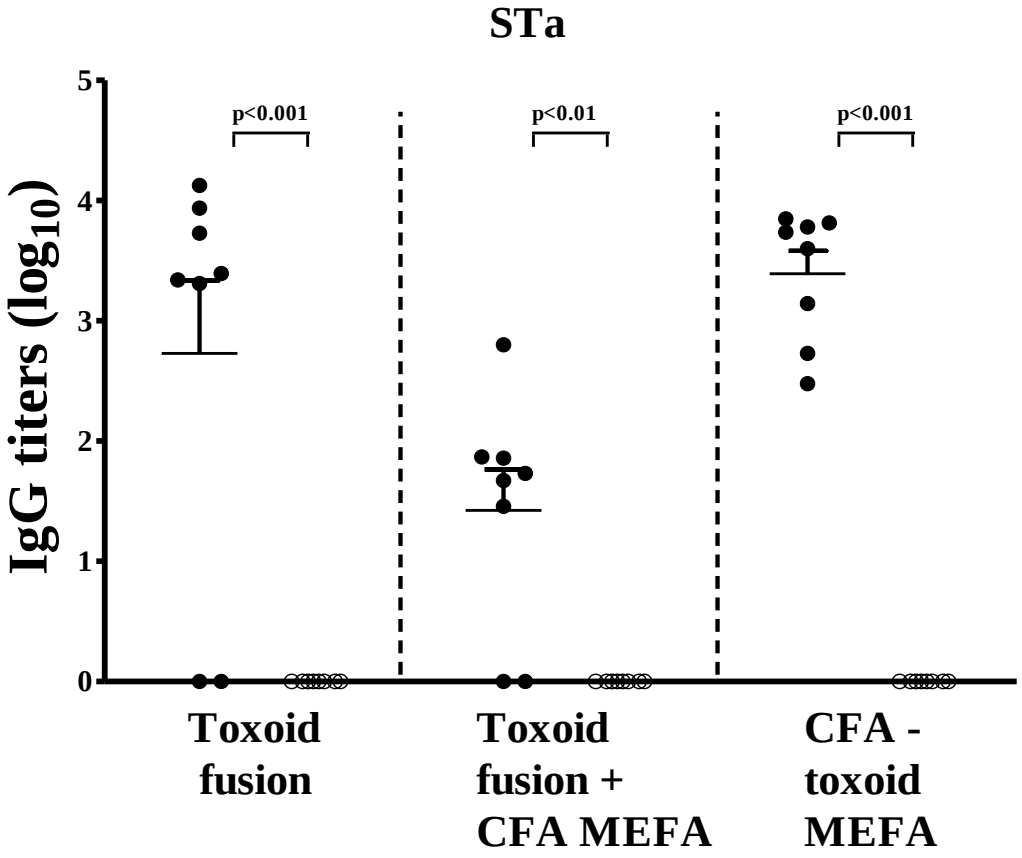

Figure 2.7 Mouse serum anti-STa IgG antibody titers from intradermally immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Immunized mouse serum antibodies inhibited bacterial adherence. Mice immunized with CFA/I/II/IV MEFA had an adherence reduction of *E.coli* or ETEC expressing bacteria (CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6 or CS6) to Caco-2; bacteria adherent to Caco-2 cells (in %) were 39±13%, 61±7%, 59±9%, 52±5%, 59±6%, 41±6%, and 54±4% **(Fig 2.8)**. Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA had bacteria adherent of 31±7%, 52±7%, 49±10%, 51±4%, 47±6%, 33±6%, and 44±5% to CaCo-2 cells **(Fig 2.9)**. Mice immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had a bacteria adherent of 28±10%, 57±5%, 47±11%, 52±3%, 50±4%, 31±8%, and 41±6% **(Fig 2.10)**. Immunized mice with CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed significant reduction at adherence to Caco-2 cells when compared to the control group. When CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ antigens were compared using one-way ANOVA, there were no significant differences among three immunization group **(Fig2.11)**, indicating that all three antigens have characterizations that broadly protection against adhesin colonization.

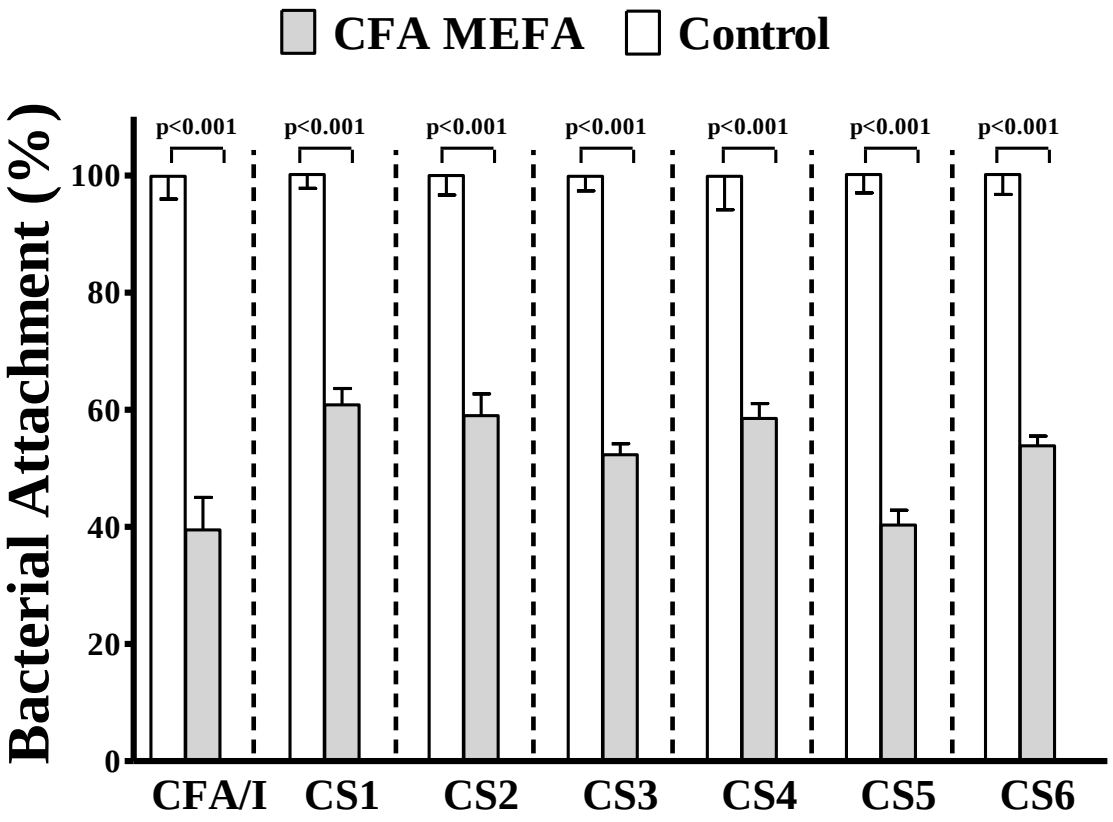

Figure 2.8 Antibody adherence inhibition assay from serum samples of intradermally immunized mice with CFA/I/II/IV MEFA and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

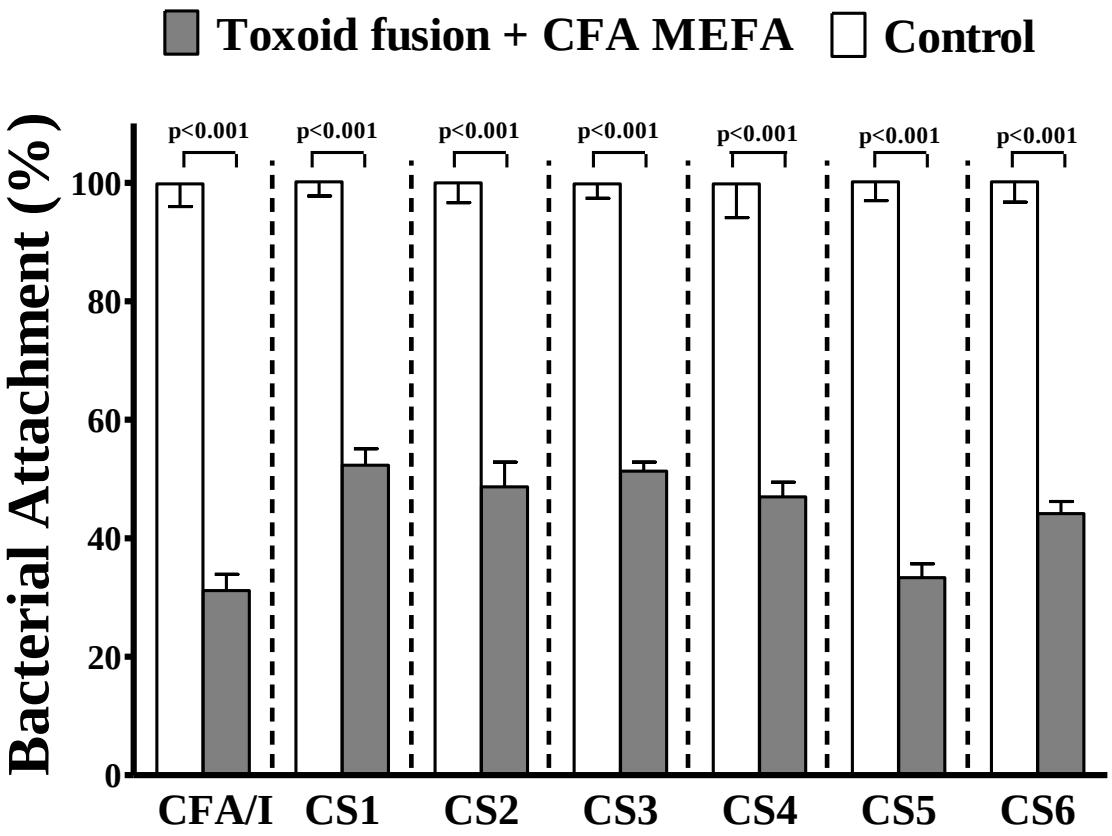

Figure 2.9 Antibody adherence inhibition assay from serum samples of intradermally immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

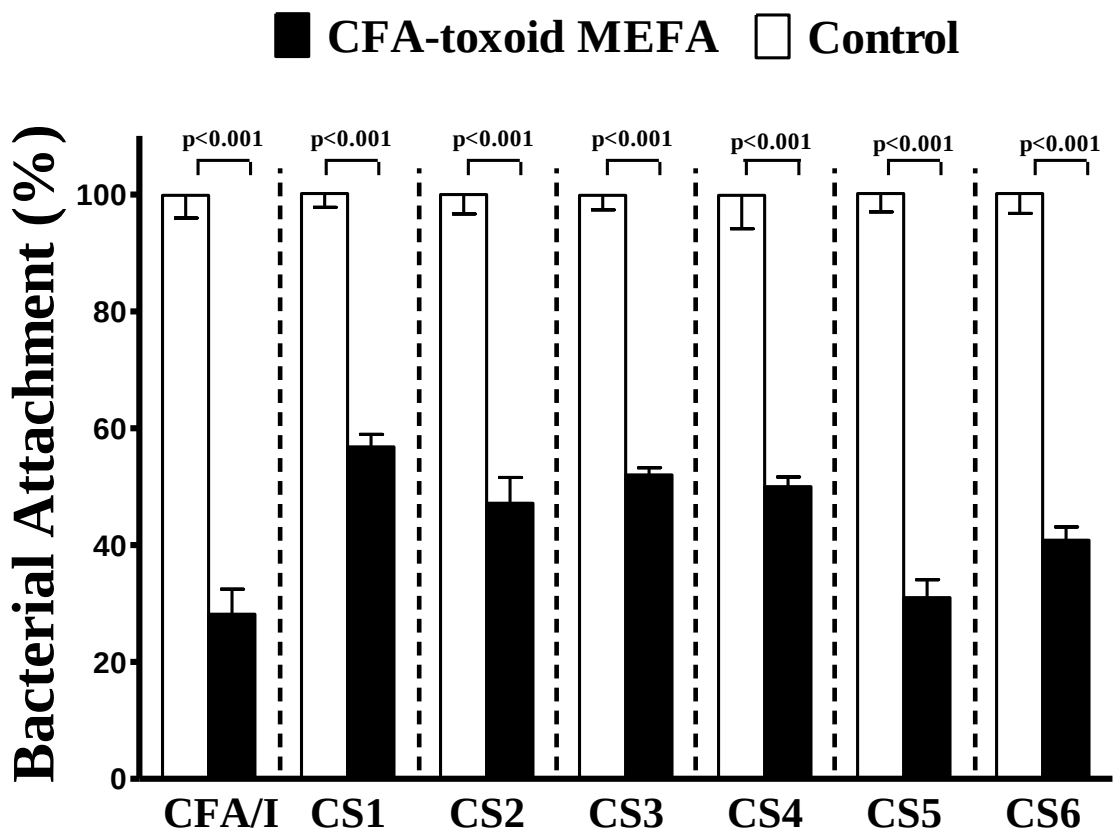

Figure 2.10 Antibody adherence inhibition assay from serum samples of intradermally immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

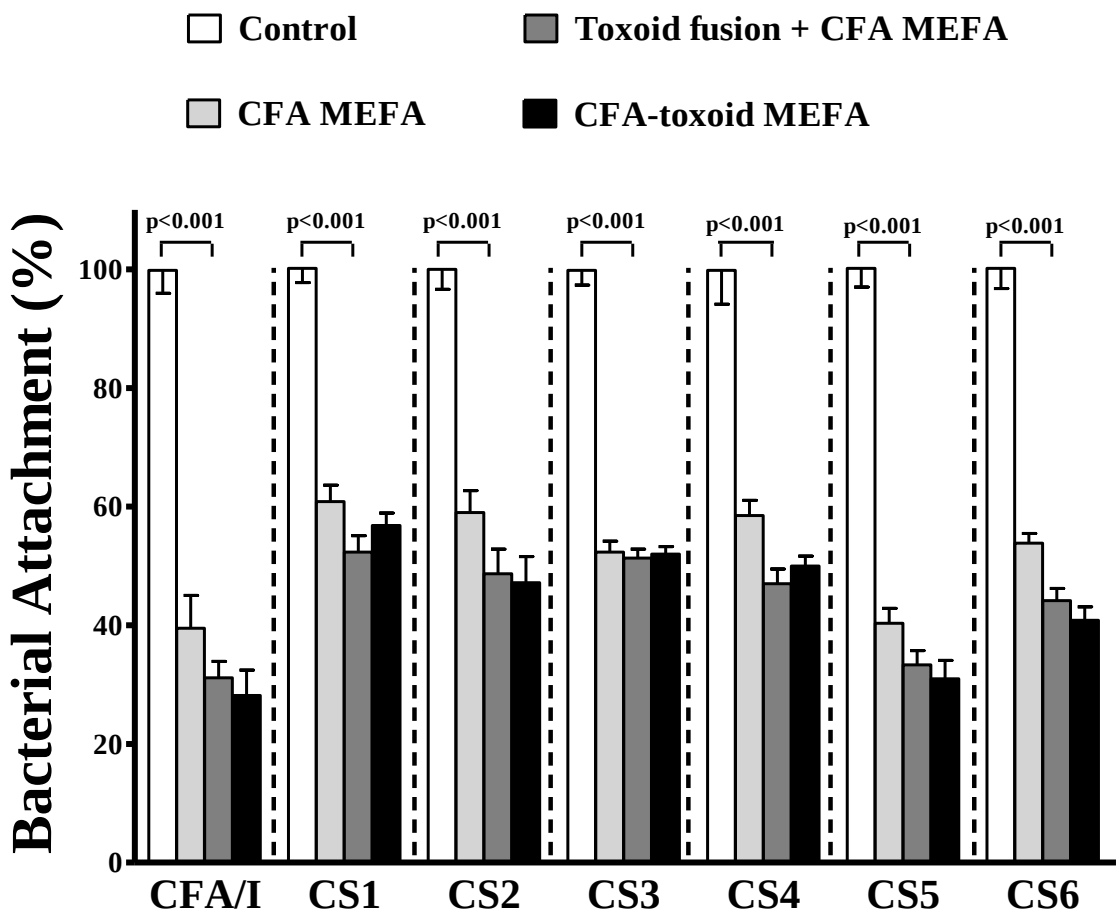

Figure 2.11 Antibody adherence inhibition assay from serum samples of intradermally immunized mice with CFA/I/II/IV MEFA , toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Immunized mouse serum antibodies neutralized ETEC toxins. The serum samples of the immunized mice showed neutralization activity against CT (LT homologue) enterotoxicity in vitro. Intracellular cAMP levels in T-84 cells incubated with CT and serum samples from the mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed neutralizing activity against CT enterotoxicity **(Fig 2.12)**. The cAMP levels within the serum samples of the immunized mice showed no significant difference between groups. In addition, all immunized groups showed a significant neutralization ($p<0.05$) when compared to the control serum.

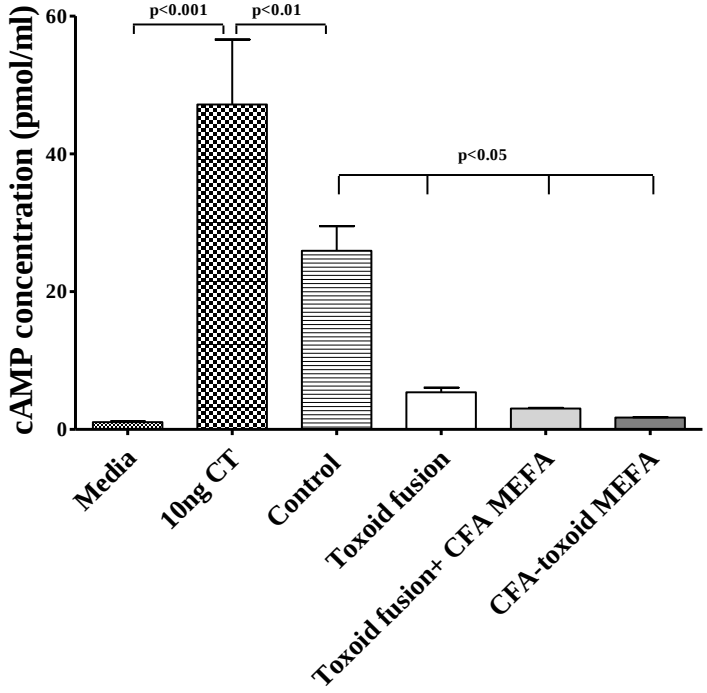

Figure 2.12 *In vitro* mouse serum antibody neutralization activity against CT (LT homologue). Antibody neutralizing activities were measured using cAMP ELISA kit. Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control was incubated with CT (10 ng) and added to T-84 cells. Intracellular cAMP levels (pmol/ml) in T-84 cells were measured using the manufacturer's protocol. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

The serum samples of the immunized mice showed neutralization activity against STa enterotoxicity. Intracellular cGMP levels in T-84 cells incubated with STa and serum samples from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed neutralizing activity against STa enterotoxicity **(Fig 2.13)**.

The cGMP levels within the serum samples of the immunized mice showed no significant difference between groups, but all immunized groups showed a significant neutralization ($p<0.001$) when compared to the control serum.

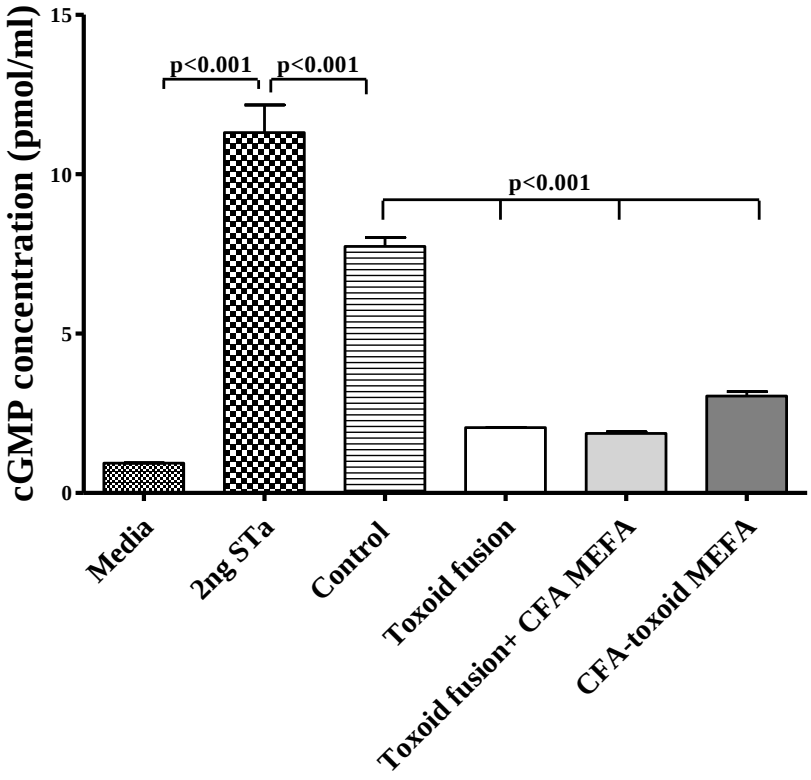

Figure 2.13 *In vitro* mouse serum antibody neutralization activity against STa. Antibody neutralizing activities were measured using cGMP ELISA kit. Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control. was incubated with STa (2 ng) and added to T-84 cells. Intracellular cGMP levels (pmol/ml) in T-84 cells were measured using the manufacturer's protocol. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mouse Intramuscular Immunization Study

For intramuscular injections the same outline was followed as describes for intradermal injections.

IM immunized mice developed anti-adhesin and/or anti-toxin IgG antibodies. ELISA was used to determine serum IgG immune response against the 7 adhesins CFA/1, CS1-6 and against enterotoxins STa and LT. Antibody titrations began at 4-fold (1: 400) dilutions. Mice IM immunized with CFA/I/II/IV MEFA developed anti-adhesin IgG antibody responses for serum Anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5, and anti-CS6 IgG titers were detected at 3.66±0.66, 3.67±0.17, 3.06±0.40, 3.38±0.23, 3.28±0.21, 3.89±0.07 and 3.74±0.10 **(Fig 2.14)**. The titers for mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA were detected at 3.74±0.24, 3.34±0.54, 3.38±0.37, 3.40±0.25, 3.32±0.37, 3.90±0.12 and 3.53±0.36 **(Fig 2.15)**. Mice IM immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had titers at 3.67±0.22, 3.83±0.12, 3.22±0.54, 2.55±0.34, 3.56±0.27, 4.01±0.20 and 3.89±0.15 **(Fig 2.16)**.

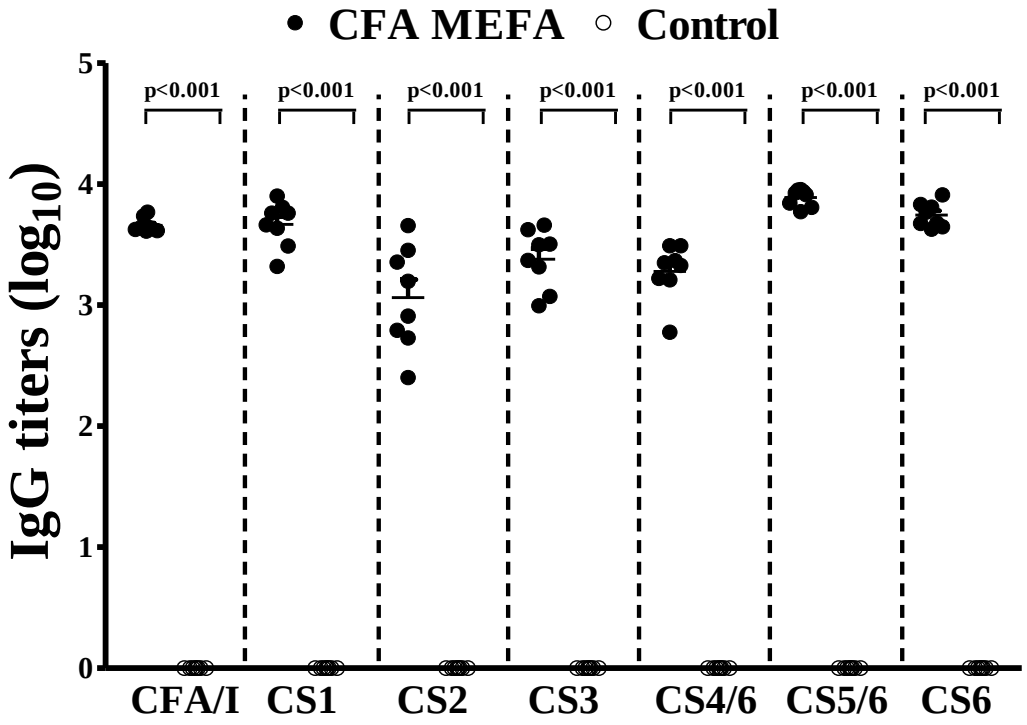

Figure 2.14 Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intramuscularly immunized mice with CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

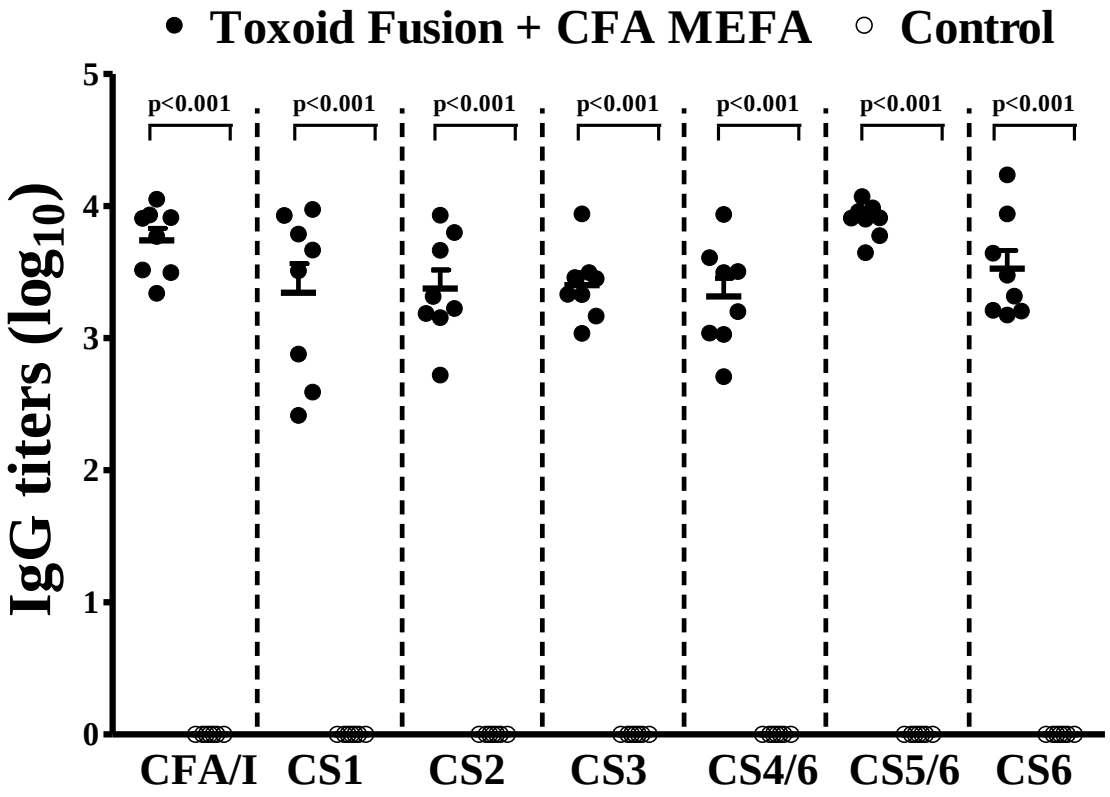

Figure 2.15 Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intramuscularly immunized mice with toxoid fusion 3xSTa_{N12S}-$mnLT_{R192G/L211A}$+ CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

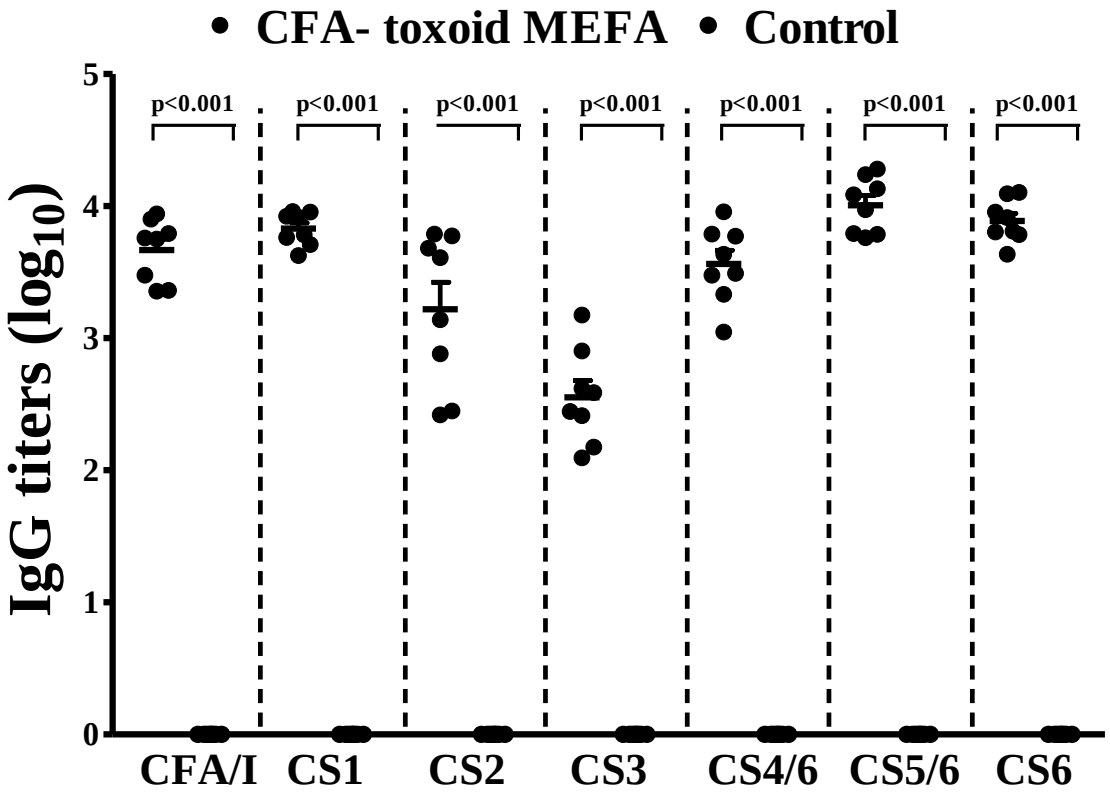

Figure 2.16 Mouse serum anti-CFA/I, -CS1, -CS2, -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intramuscularly immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ developed anti-LT IgG titers at (3.84±0.18), toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA had anti- LT titers detected at (3.81±0.30), and CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had LT titers detected at (3.76±0.21) **(Fig 2.17)**.

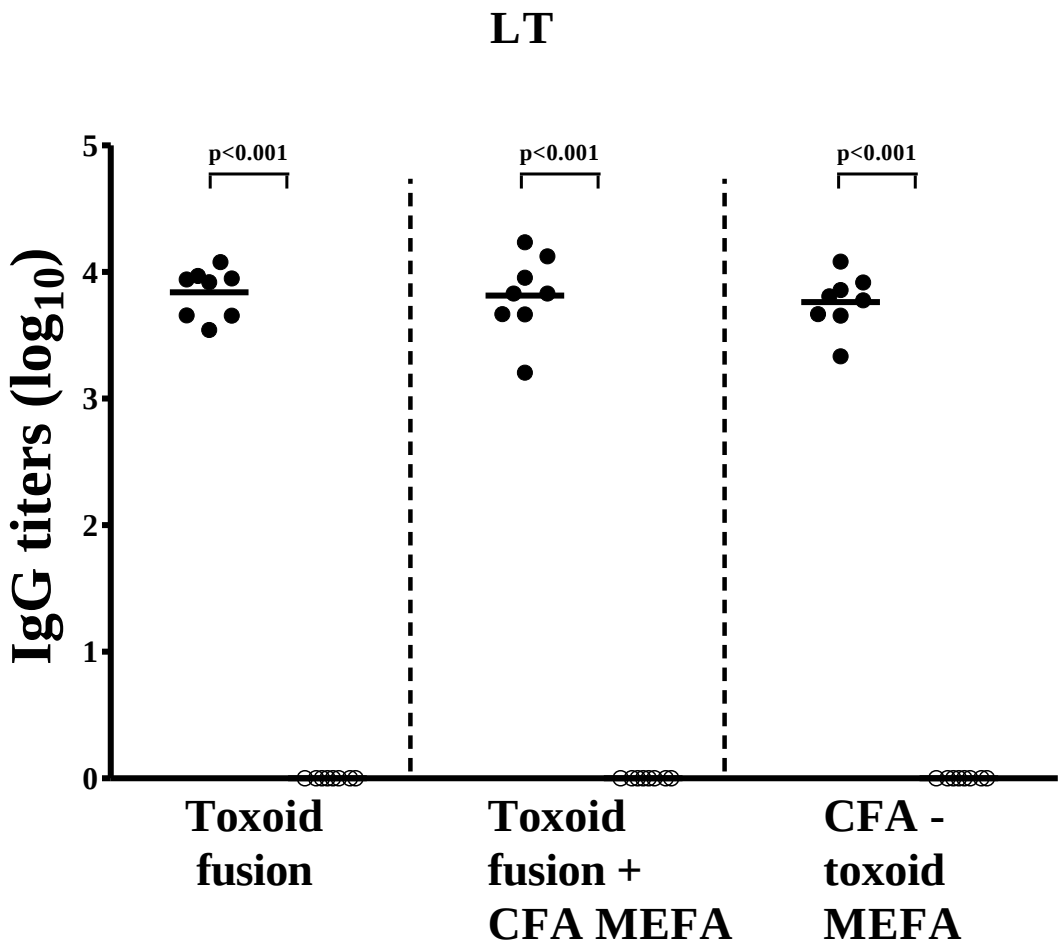

Figure 2.17 Mouse serum anti-LT IgG antibody titers from intramuscularly immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ developed anti-STa IgG titers at (1.75±1.41), toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA induced Anti- STa titers at (0.80±1.04), and mice immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had STa titers detected at (3.38±0.34). Mice immunized with PBS developed no anti-adhesin or antitoxin IgG antibody responses **(Fig 2.18)**.

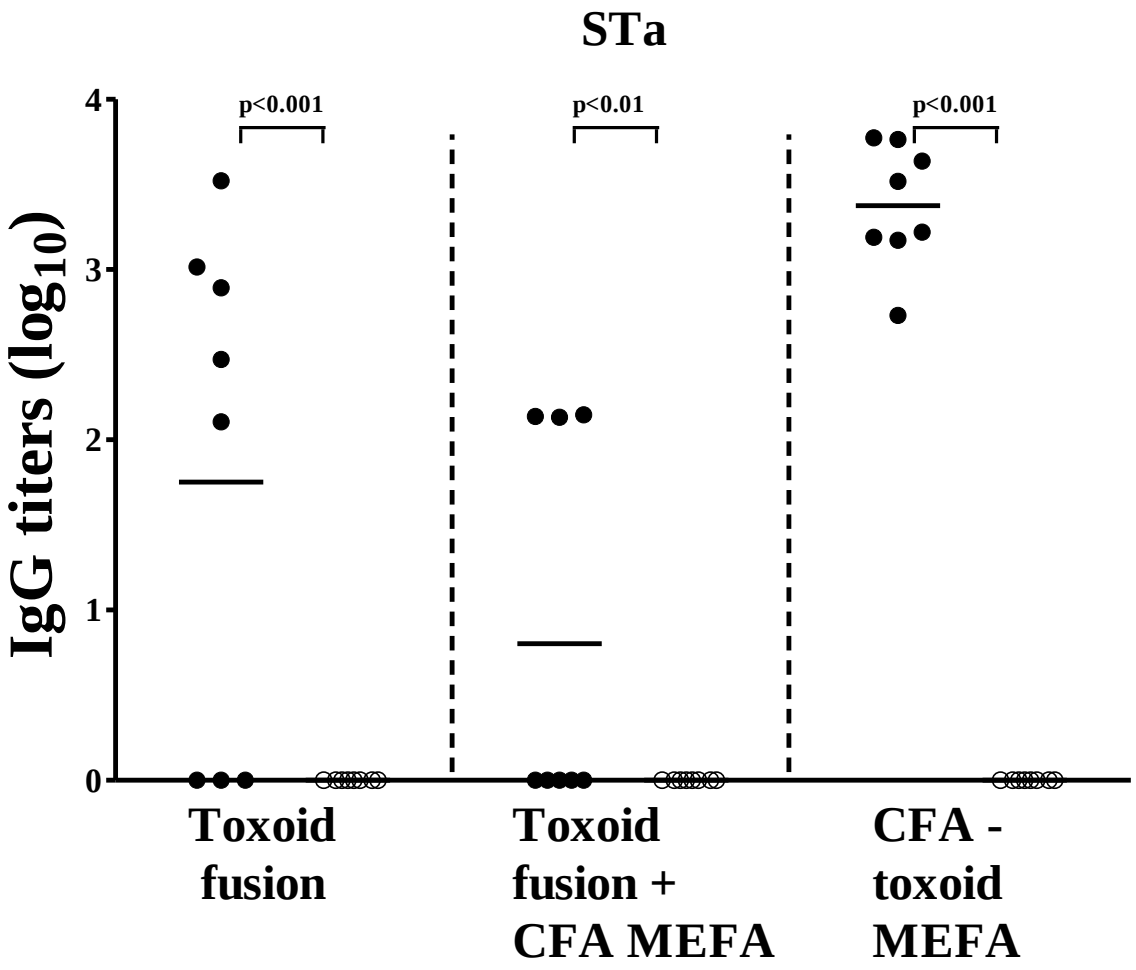

Figure 2.18 Mouse serum anti-STa IgG antibody titers from intramuscularly immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$. (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ immunized mice had high IgG immune response against all 7 adhesions and enterotoxins LT and STa when compared to the control (PBS immunized group).

IM Immunized mouse serum antibodies inhibited bacterial adherence. Mice immunized with CFA/I/II/IV MEFA had an adherence reduction of *E.coli* or ETEC expressing bacteria (CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6 or CS6) to CaCo-2. Bacteria (%) adherent to CaCo-2 cells were 44±8%, 65±10%, 53±7%, 61±5%, 64±6%, 49±7%, and 62±6% **(Fig 2.19)**. Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA had bacteria adherent of 35±8%, 52±9%, 47±9%, 53±5%, 54±8%, 44±5%, and 58±4% to CaCo-2 cells **(Fig 2.20)**. Mice immunized with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ had a bacteria adherent of 33±5%, 58±6%, 47±7%, 56±4%, 51±6%, 44±5%, and 58±4% **(Fig 2.21)**. Immunized mice with CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed significant reduction at adherence to Caco-2 cells when compared to the control group. When CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ antigens were compared using one-way ANOVA, there were no significant differences within groups, indicating that all three antigens are good vaccine candidates **(Fig 2.22)**.

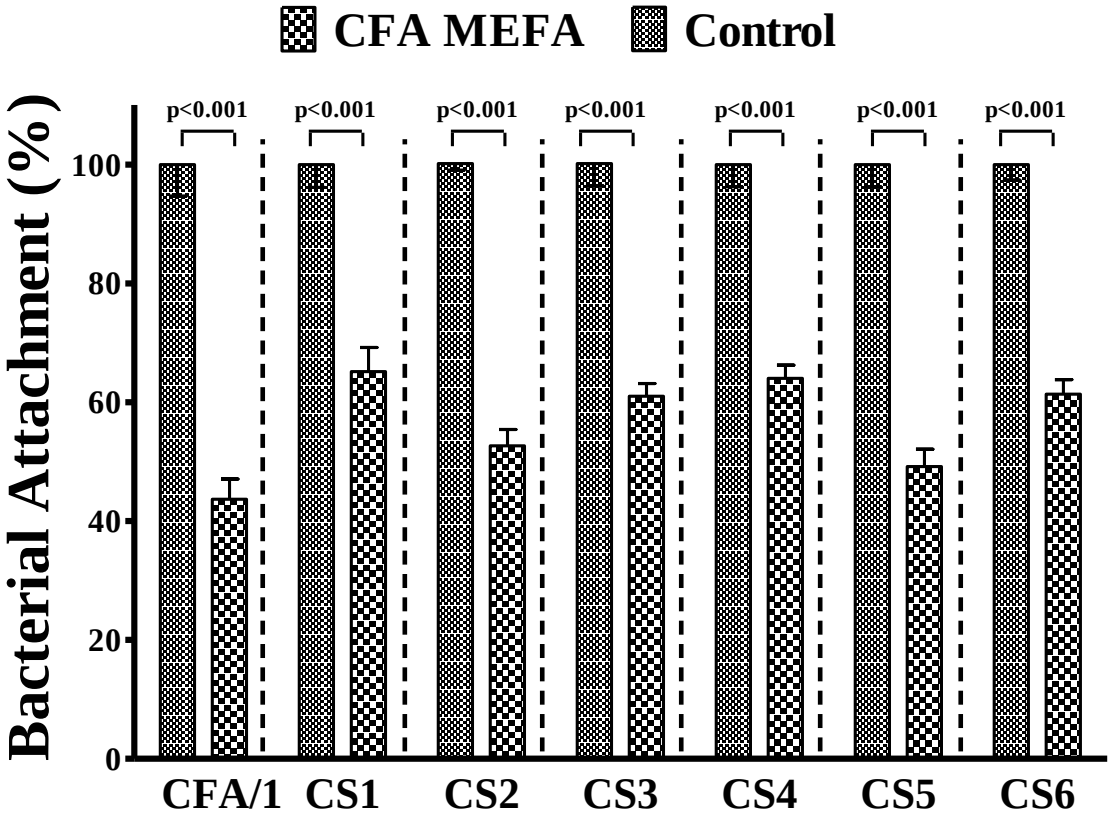

Figure 2.19 Antibody adherence inhibition assay from serum samples of intramuscularly immunized mice with CFA/I/II/IV MEFA and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

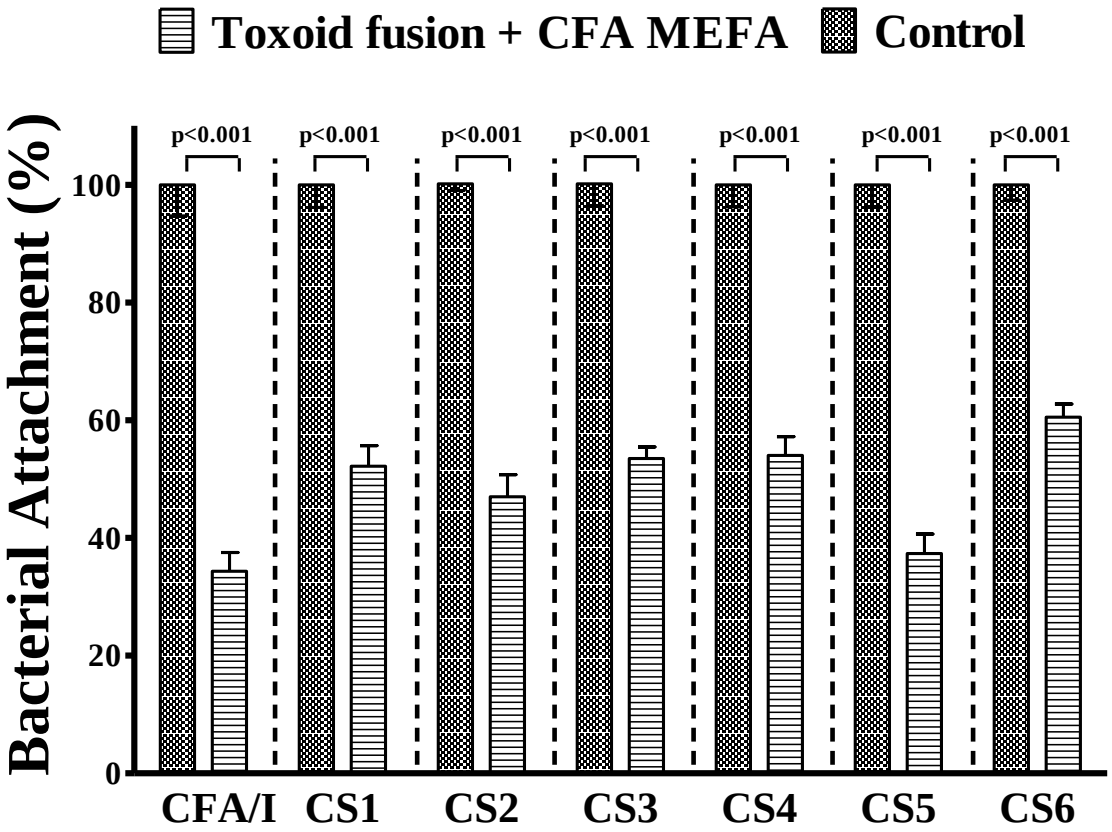

Figure 2.20 Antibody adherence inhibition assay from serum samples of intramuscularly immunized mice with CFA/I/II/IV MEFA + toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

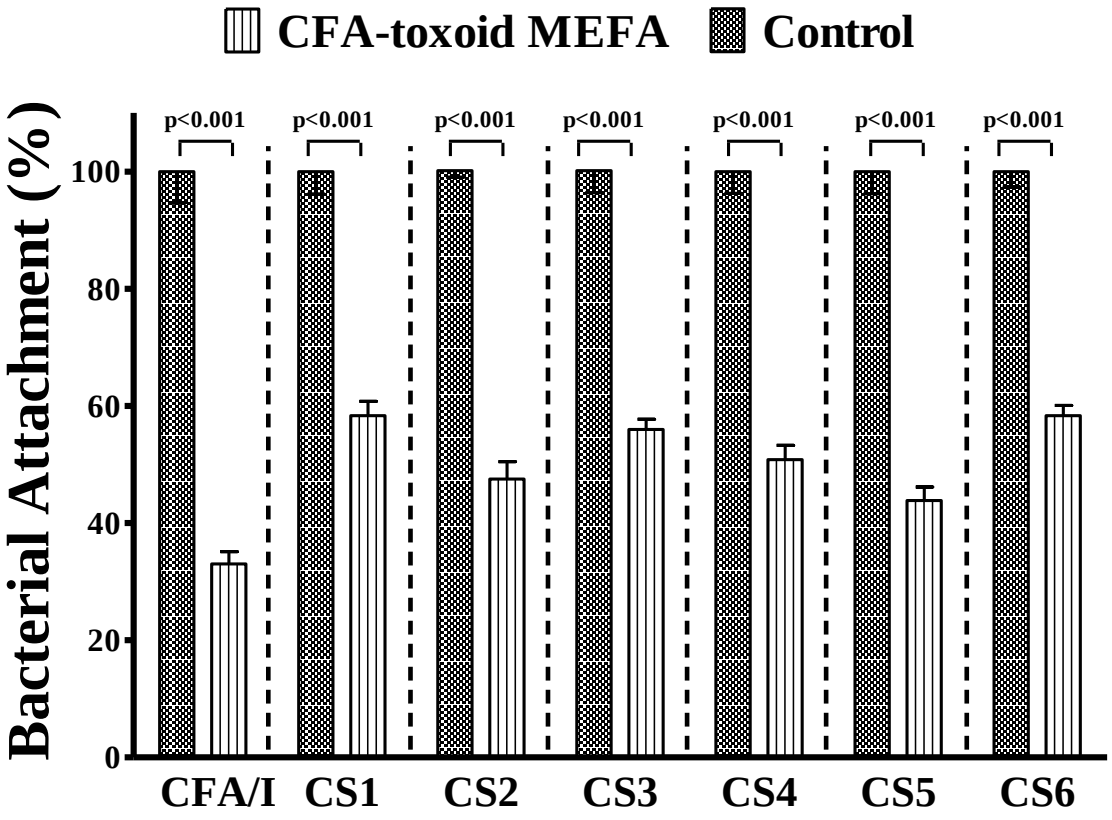

Figure 2.21 Antibody adherence inhibition assay from serum samples of intramuscularly immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

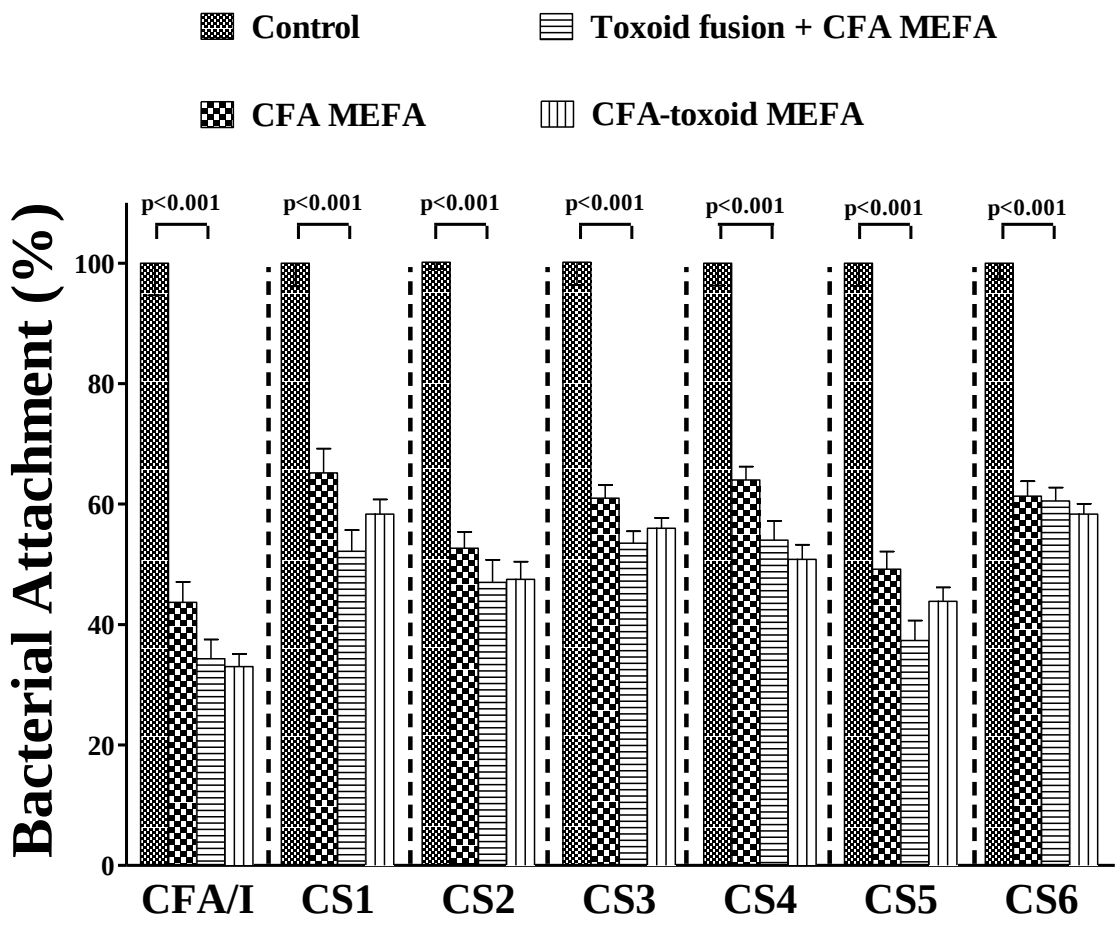

Figure 2.22 Antibody adherence inhibition assay from serum samples of intramuscularly immunized mice with CFA/I/II/IV MEFA , toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$., and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, CS2, CS3, CS4/CS6, CS5/CS6, CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Immunized mouse serum antibodies neutralized ETEC toxins. Intracellular cAMP levels in T-84 cells incubated with CT and serum samples from mice intramuscularly immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed neutralizing activity against CT enterotoxicity the cAMP levels within the serum samples of the immunized mice showed no significant difference between groups. In addition, all immunized groups showed a significant neutralization ($p<0.05$) when compared to the control serum **(Fig 2.23)**.

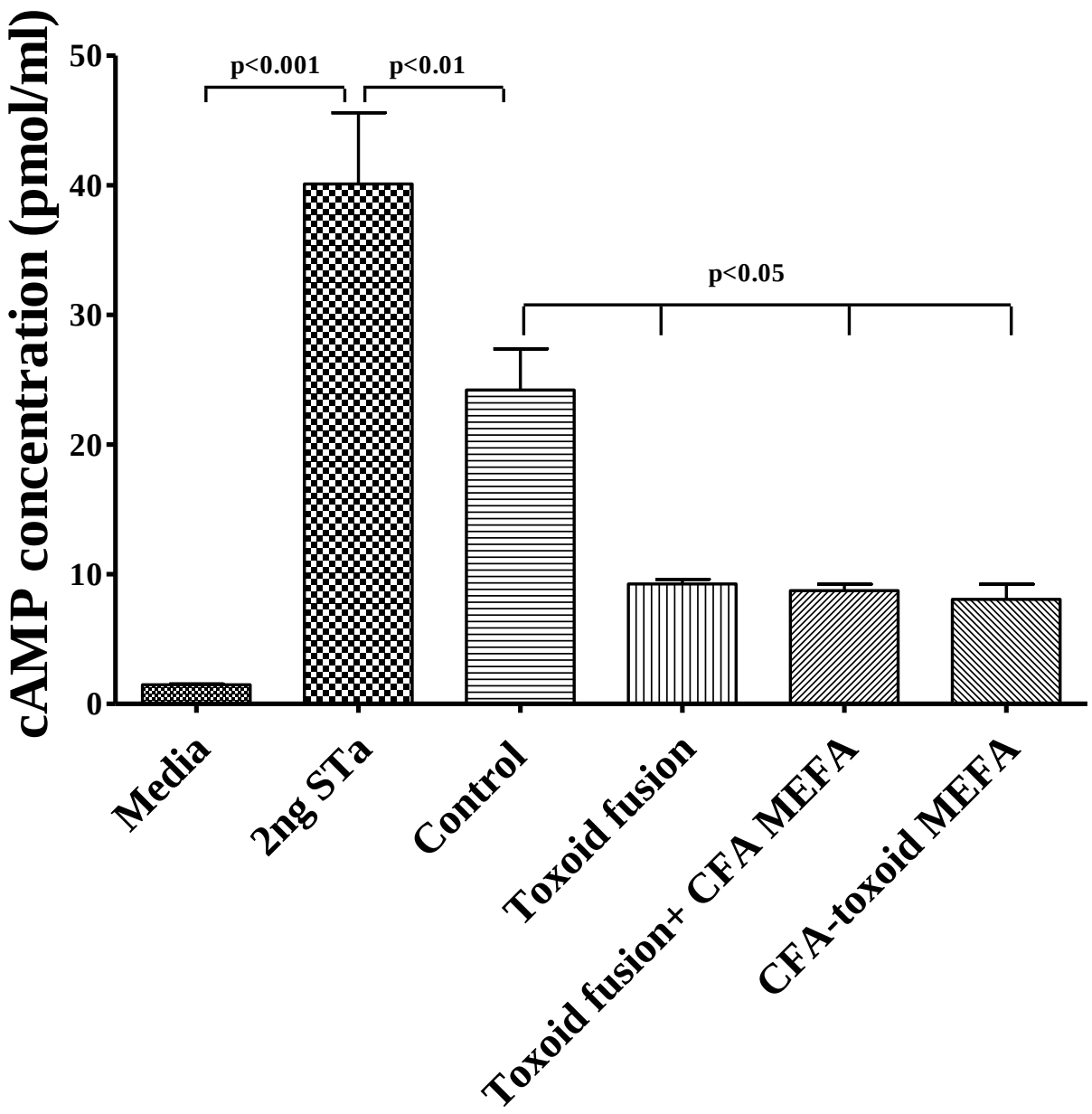

Figure 2.23 *In vitro* mouse serum antibody neutralization activity against CT. (LT homologue). Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control. was incubated with CT (10 ng) and added to T-84 cells. Intracellular cAMP levels (pmol/ml) in T-84 cells were measured using the protocol offered by the manufacturer. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Intracellular cGMP levels in T-84 cells incubated with STa and serum samples from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed neutralizing activity against STa enterotoxicity **(Fig 2.24)**.

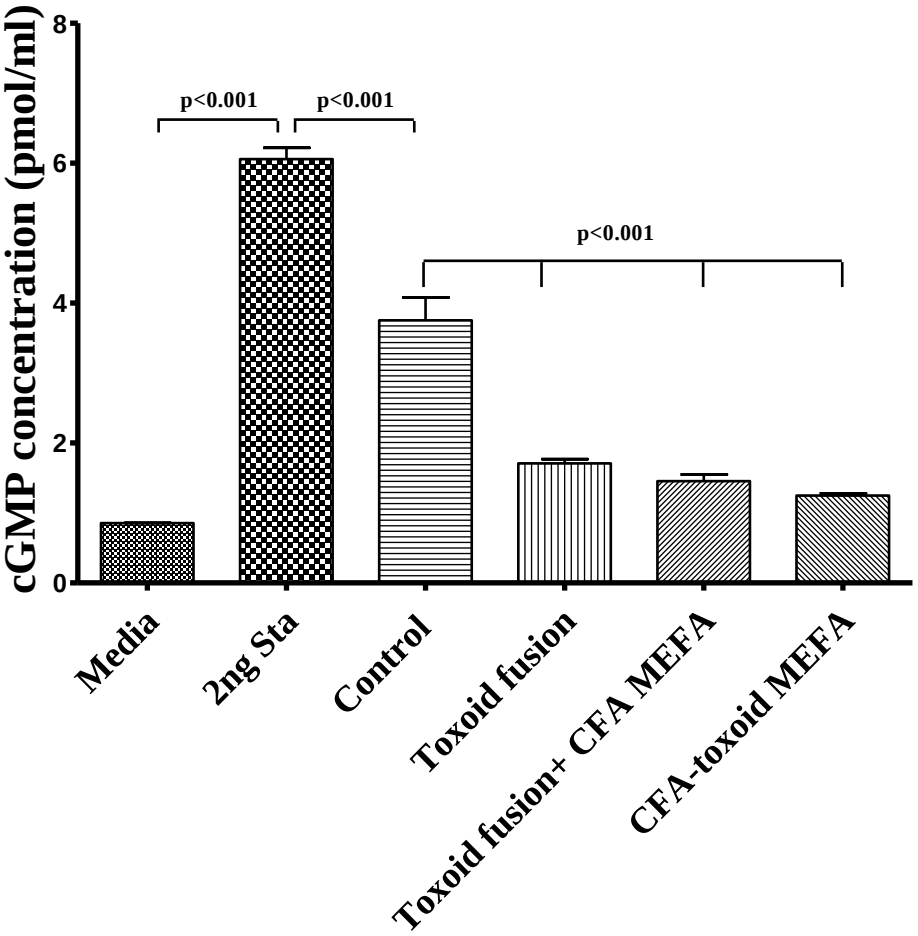

Figure 2.24 *In vitro* mouse serum antibody neutralization activity against STa. Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control. was incubated with STa (2 ng) and added to T-84 cells. Intracellular cGMP levels (pmol/ml) in T-84 cells were measured using the protocol offered by the manufacturer. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

The cGMP levels within the serum samples of the immunized mice showed no significant difference between groups. In addition, all immunized groups showed a significant neutralization ($p<0.001$) when compared to the control serum.

Comparing Mouse Intradermal and Intramuscular Immunization

ID and IM immunized mice developed anti-adhesin and/or anti-toxin IgG antibodies.

Antibody responses of anti-adhesin and anti-toxin IgG titers of mice immunized with CFA/I/II/IV MEFA using ID and IM routes were statistically compared using one-way ANOVA **(Fig 2.25)**. Mice immunized with CFA/I/II/IV MEFA statistically developed greater IgG titers specific to CS4 ($p<0.01$), CS5($p<0.001$), and CS6($p<0.001$) **(Fig 2.26)**. Mice immunized with toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$+ CFA/I/II/IV MEFA demonstrated statistical differences in IgG titers specific to anti-CS4 ($p<0.05$) **(Fig 2.27** and **(Fig 2.28)**. Mice immunized CFA-$3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ had statistically higher IgG titers specific to anti-CS3 ($p<0.001$), anti-CS4 ($p<0.001$), and anti-CS5 ($p<0.001$) **(Fig 2.29 and Fig 2.30)**.

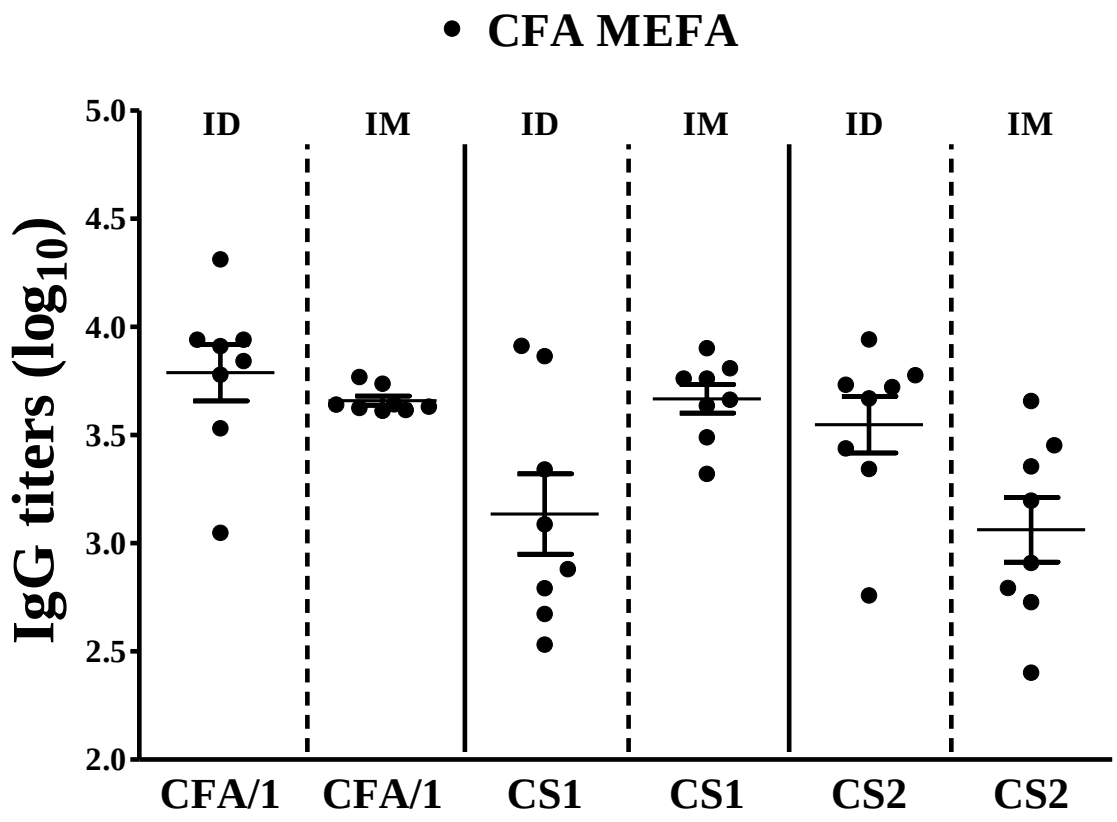

Figure 2.25 Mouse serum anti-CFA/I, -CS1, and anti-CS2 IgG antibody titers from intradermally and intramuscularly immunized mice with CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

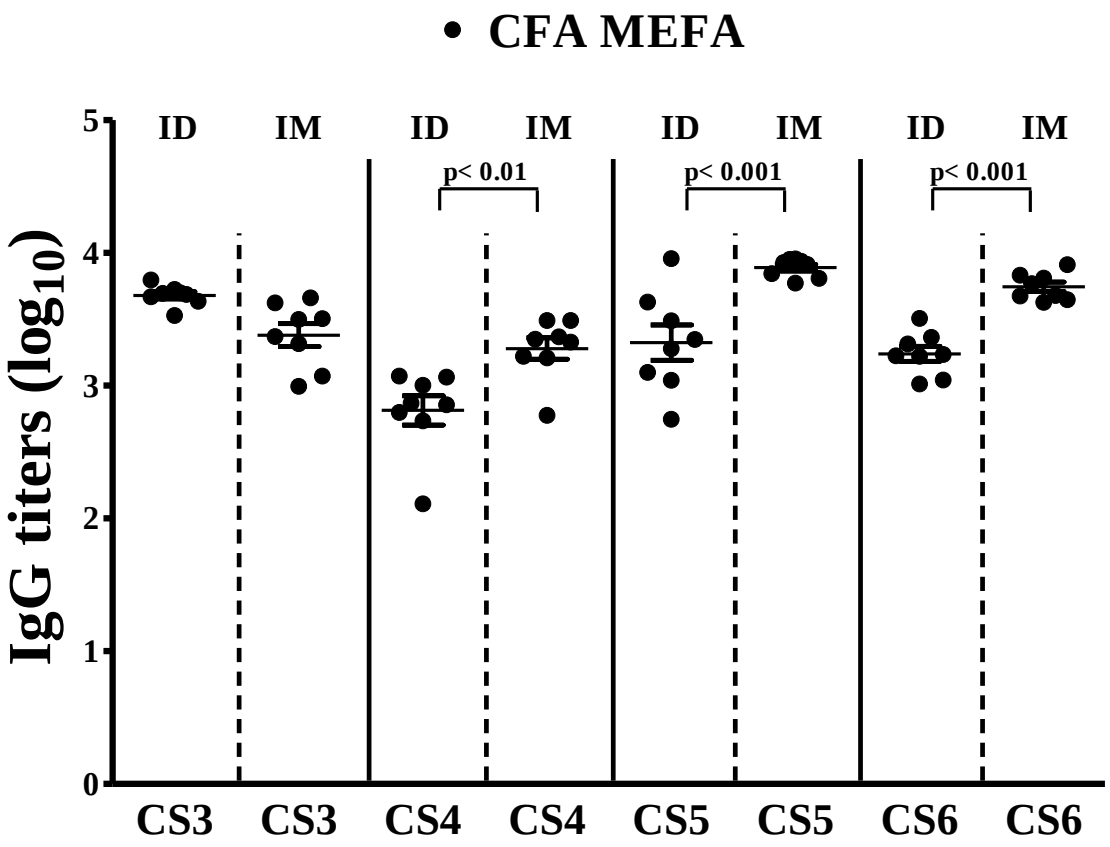

Figure 2.26 Mouse serum anti -CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intradermally and intramuscularly immunized mice with CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

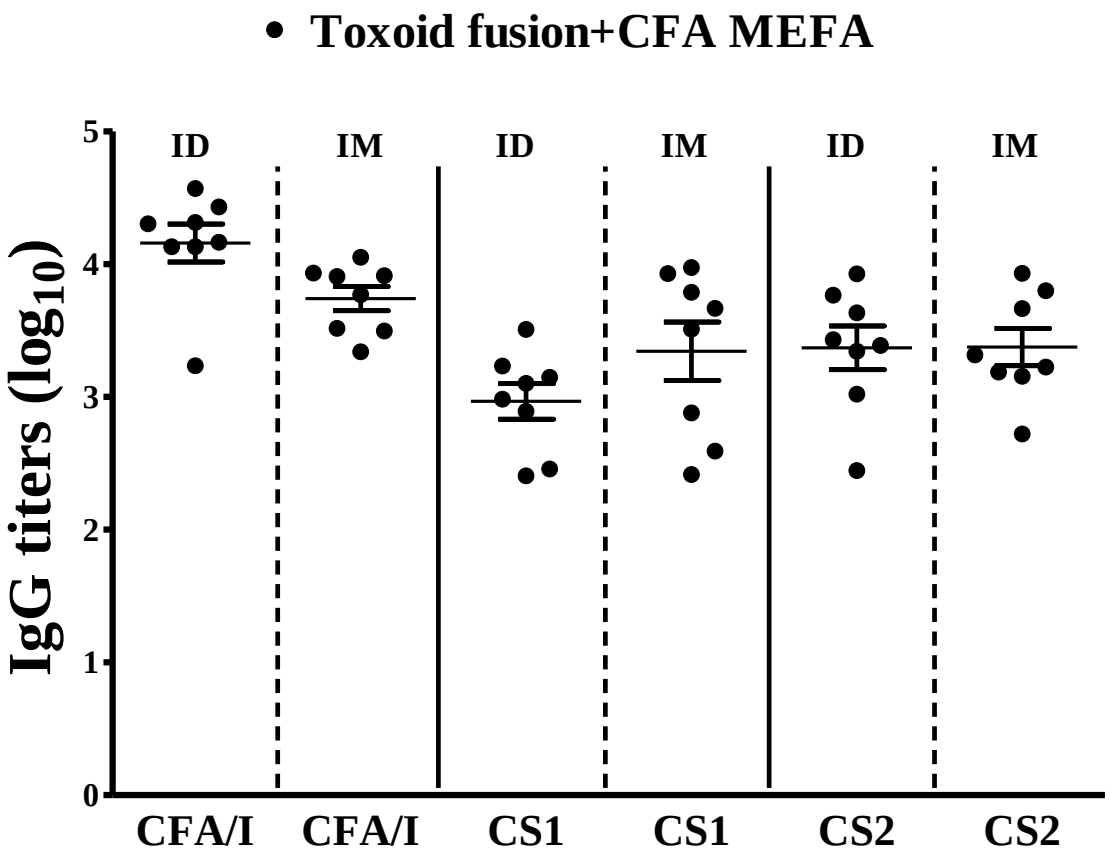

Figure 2.27 Mouse serum anti-CFA/I, -CS1, and anti-CS2 IgG antibody titers from intradermally and intramuscularly immunized mice with toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ + CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

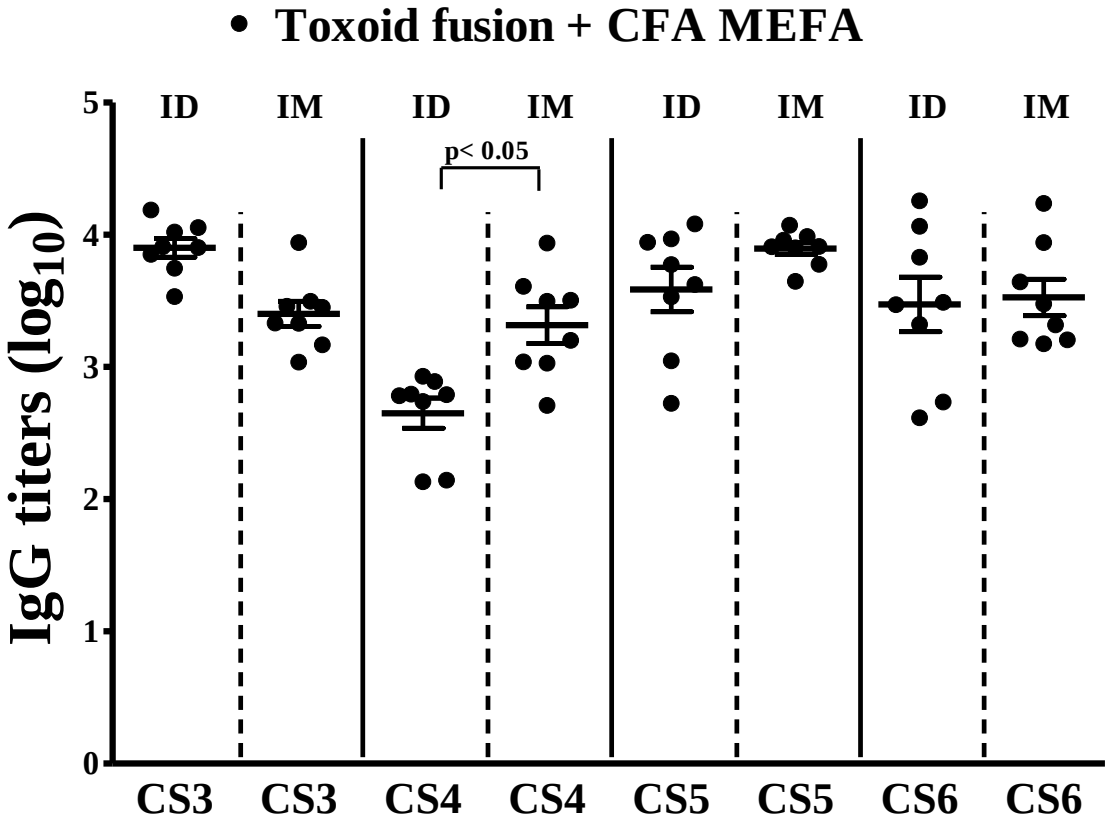

Figure 2.28 Mouse serum anti-CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intradermally and intramuscularly immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

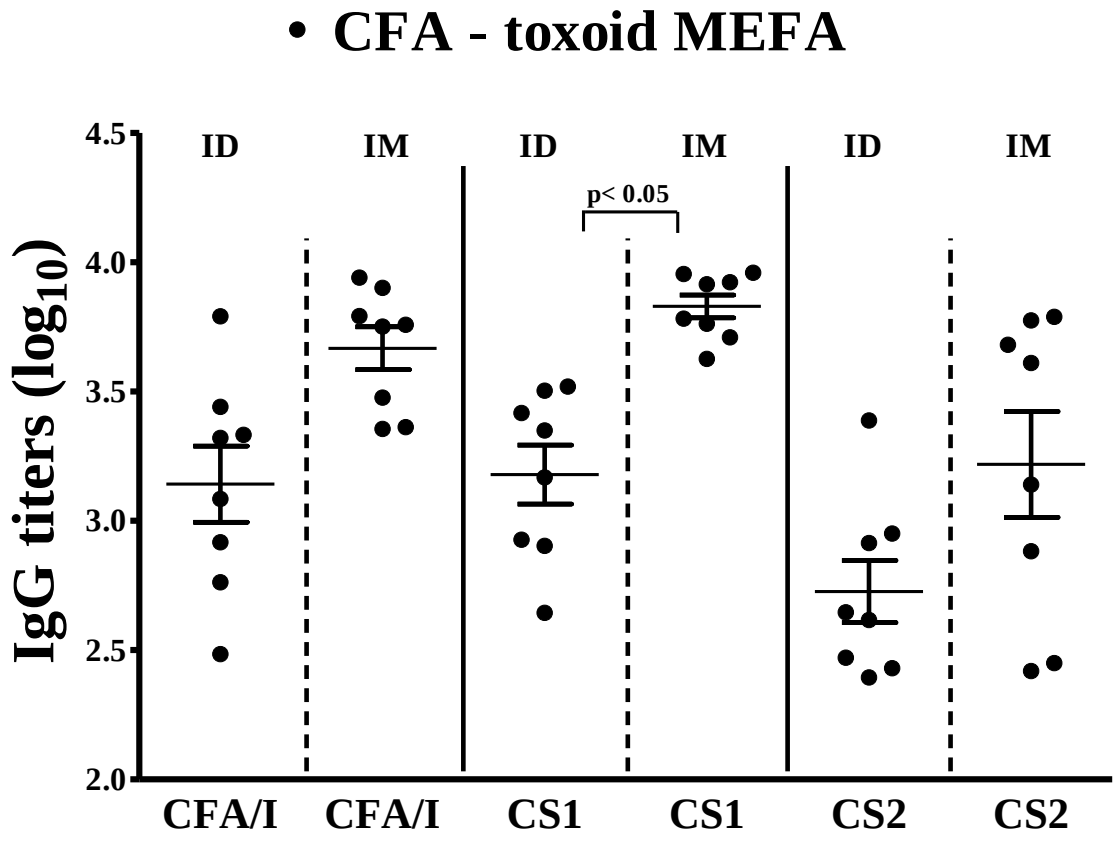

Figure 2.29 Mouse serum anti-CFA/I, -CS1, and anti-CS2 IgG antibody titers from intradermally and intramuscularly immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

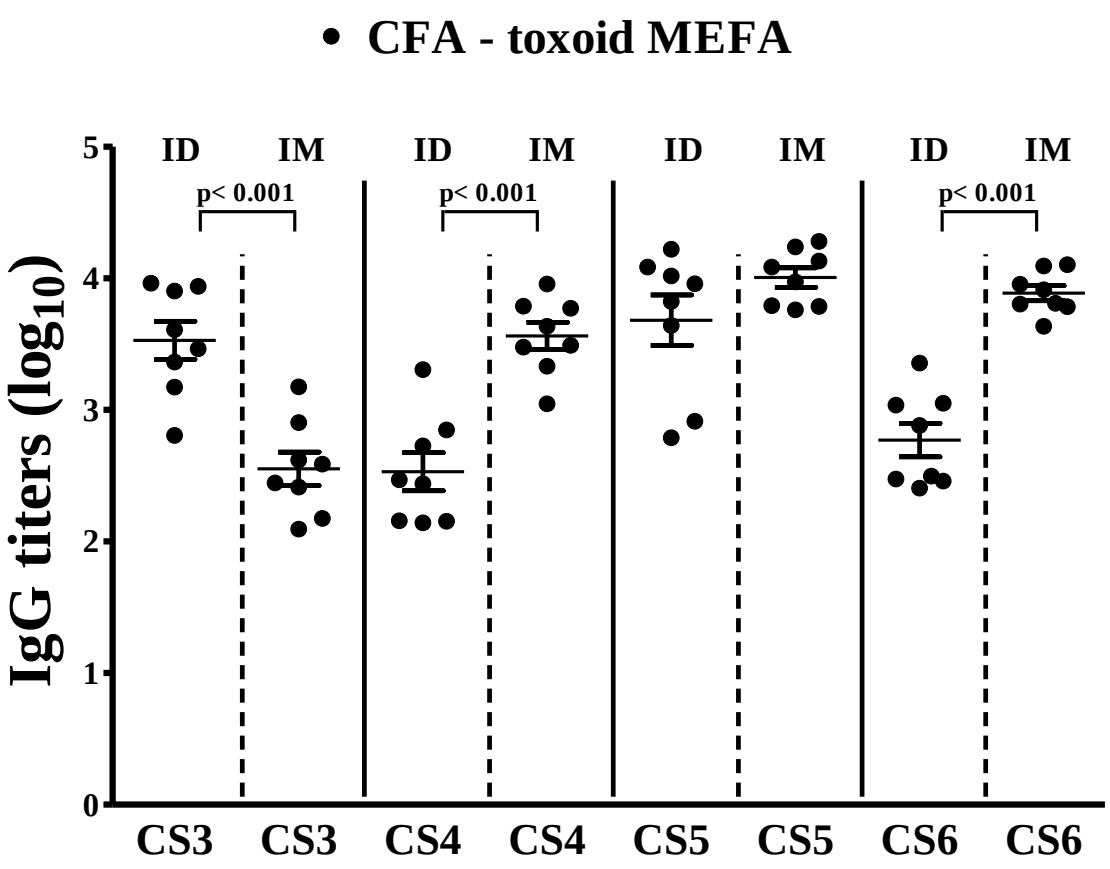

Figure 2.30 Mouse serum anti-CS3, -CS4, -CS5 and anti-CS6 IgG antibody titers from intradermally and intramuscularly immunized mice with CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●) and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ combined with CFA/I/II/IV MEFA, or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ did not statistically show any differences for anti-LT between ID and IM routes **(Fig 2.31)**. The same results were demonstrated for anti-STa **(Fig 2.32)**.

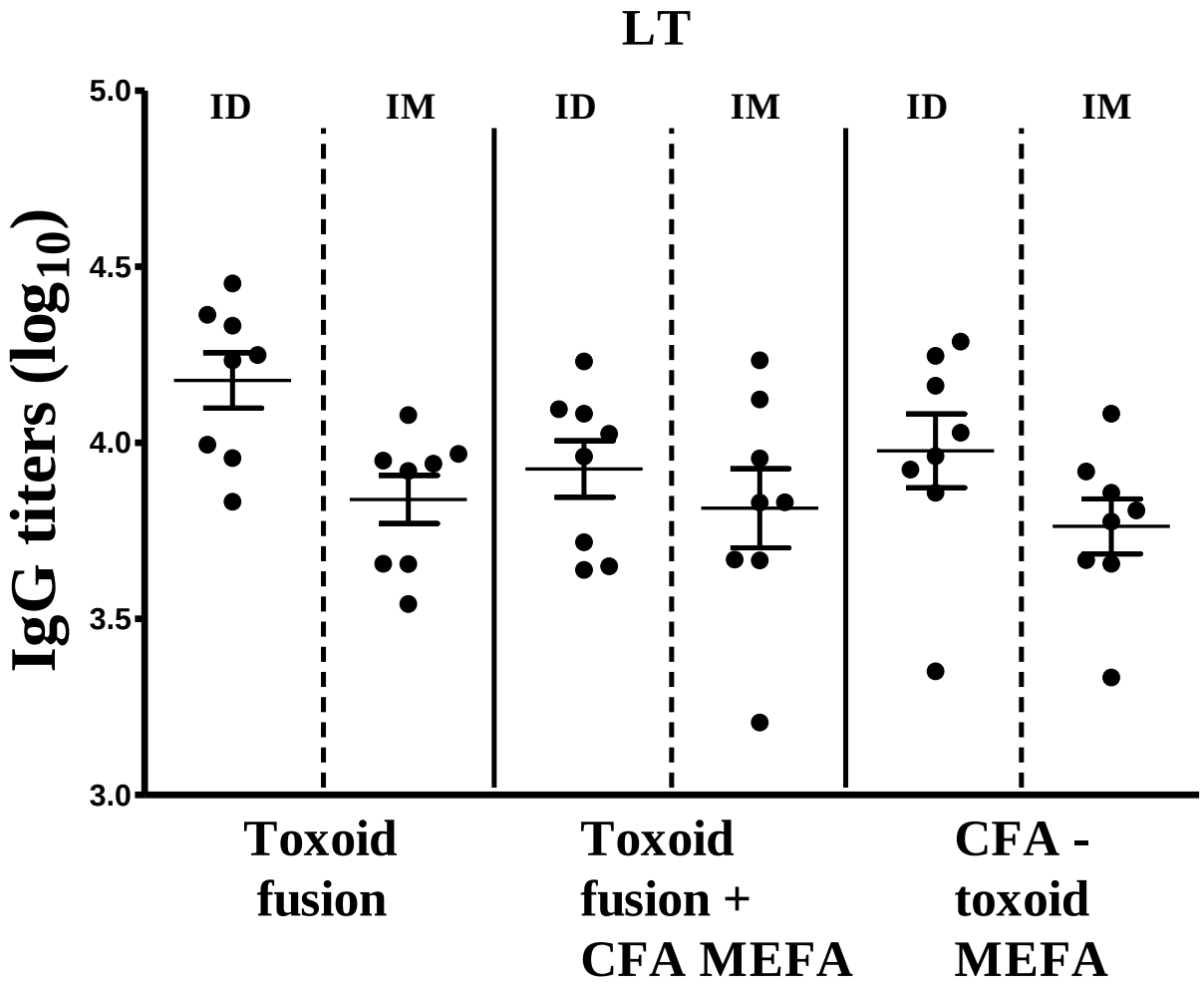

Figure 2.31 Mouse serum anti-LT IgG antibody titers from intradermal and intramuscularly immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

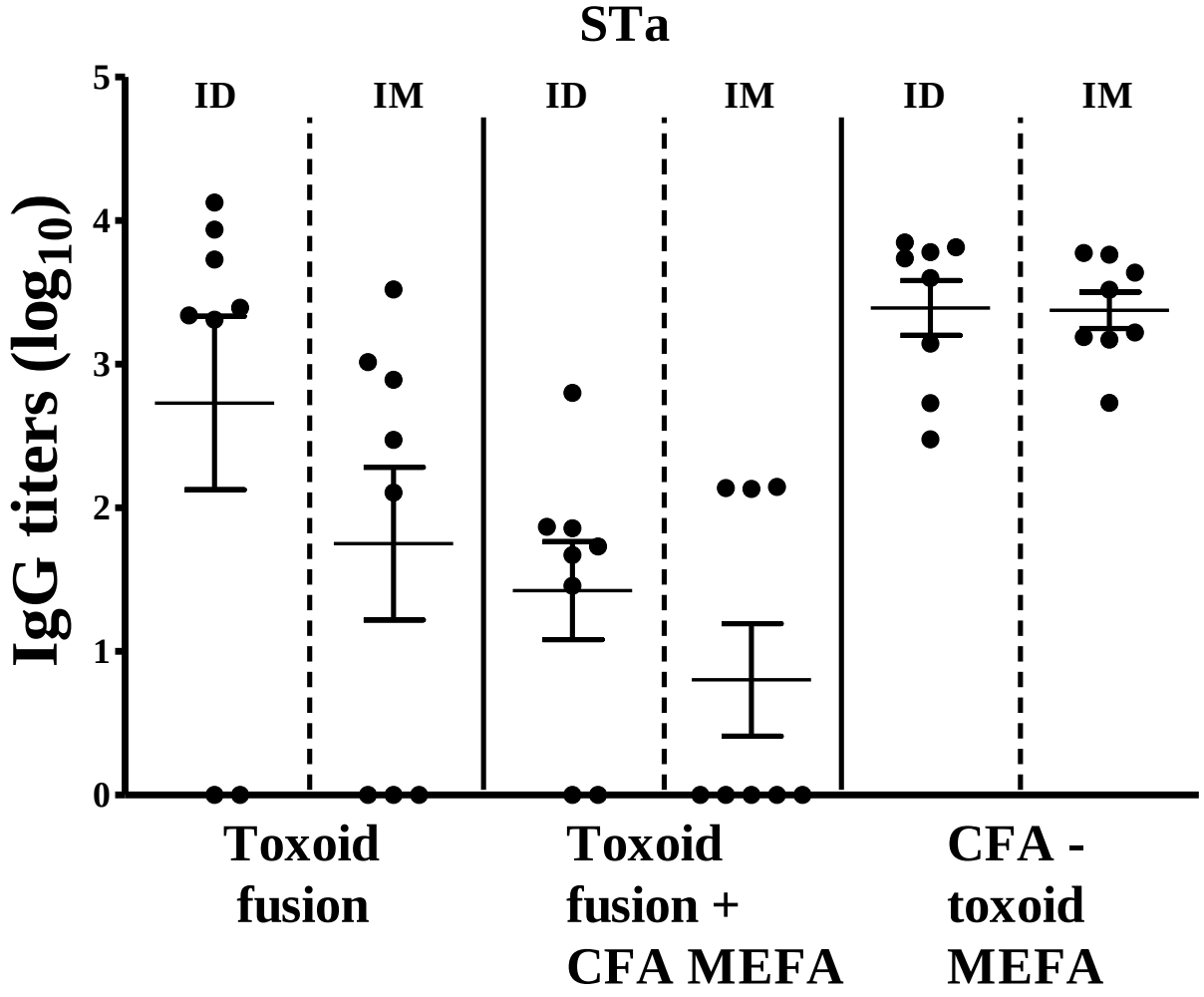

Figure 2.32 Mouse serum anti-STa IgG antibody titers from intradermal and intramuscularly immunized mice with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ (●), and the control mice (○). Bars represent the mean titers of each group. Each dot represents an antigen-specific titer of single mice. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Immunized mouse serum antibodies inhibited bacterial adherence. Mice ID and IM immunized with CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , or CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ showed similar adherence reduction in E. coli or ETEC bacteria expressing CS1, CS2, CS3, CS4/CS6, CS5/CS6 or CS6 to CaCo-2 cells **(Fig 2.33 and Fig 2.34)**. Overall, there were no statistically significant differences in ID and IM routes when bacterial neutralization was compared.

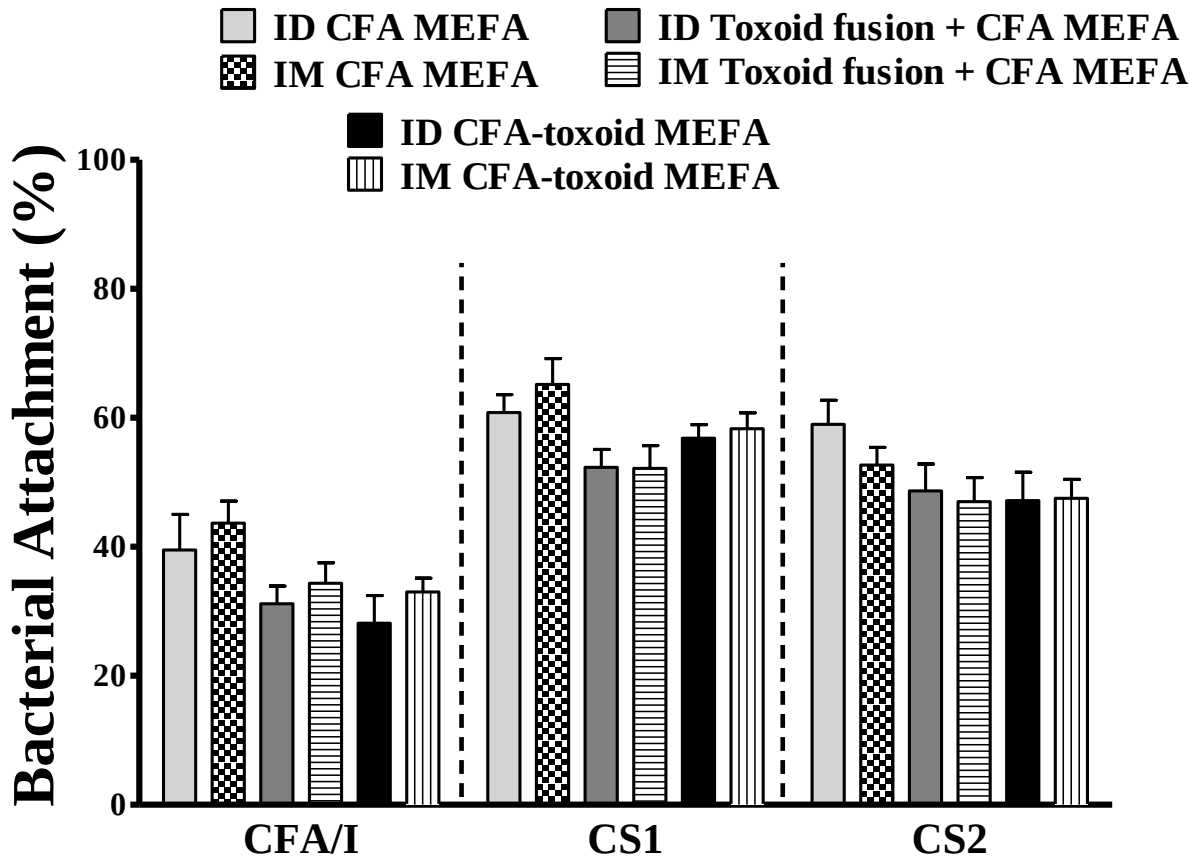

Figure 2.33 Antibody adherence inhibition assay from serum samples of intradermal and intramuscularly immunized mice with CFA/I/II/IV MEFA , toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CFA/I, CS1, and CS2 adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

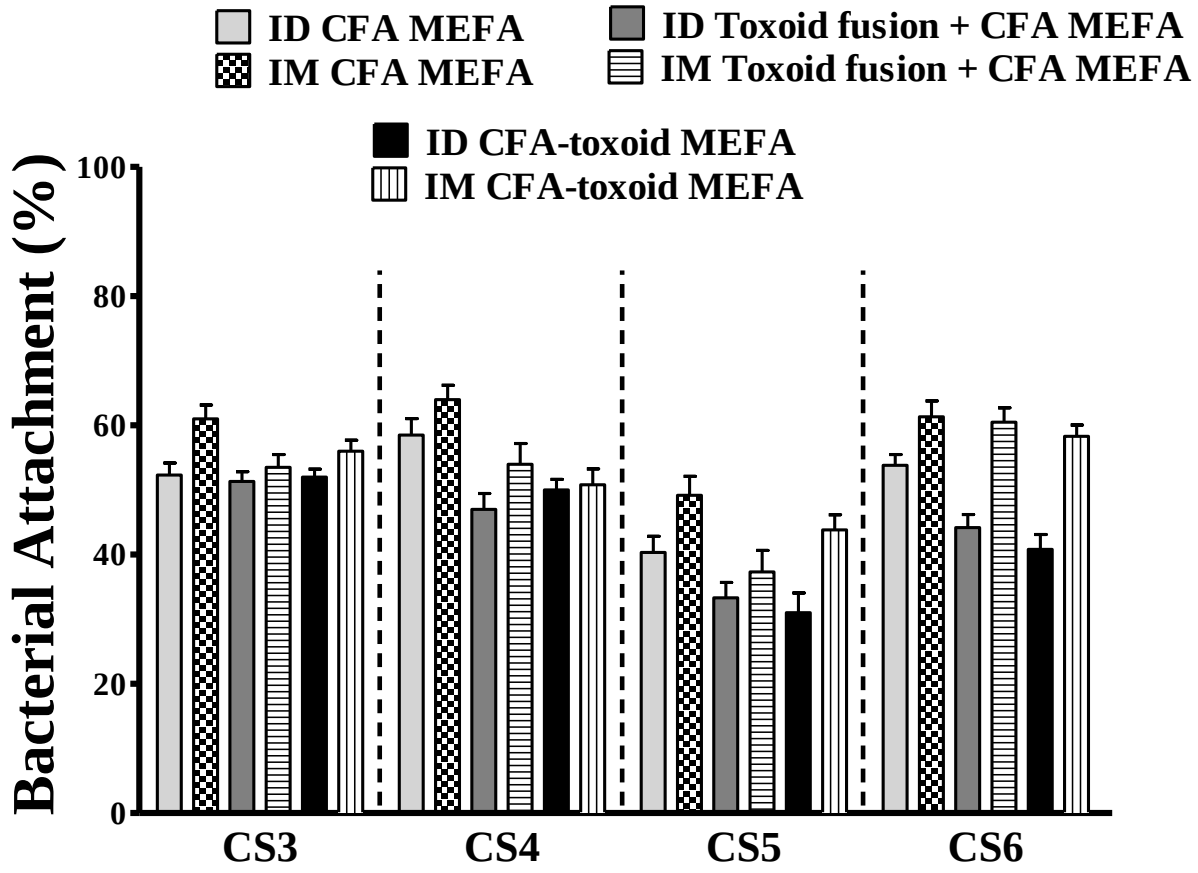

Figure 2.34 Antibody adherence inhibition assay from serum samples of intradermal and intramuscularly immunized mice with CFA/I/II/IV MEFA, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA , CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control serum from immunized mice with PBS. Boxes and bars represent means and standard deviations of bacteria expressing CS3, CS4/CS6, CS5/CS6, and CS6, adherent to Caco-2 cells P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Immunized mouse serum antibodies neutralized ETEC toxins. Intracellular cAMP levels in T-84 cells incubated with CT demonstrates a ($p<0.05$) significant difference in cyclic cAMP levels in routes immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ **(Fig 2.35)**. Mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$+ CFA/I/II/IV MEFA and CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ contained a significance of a ($p<0.01$) **(Fig 2.35)**. The results

suggested that intracellular cAMP had significantly better CT toxin neutralization using intradermal route.

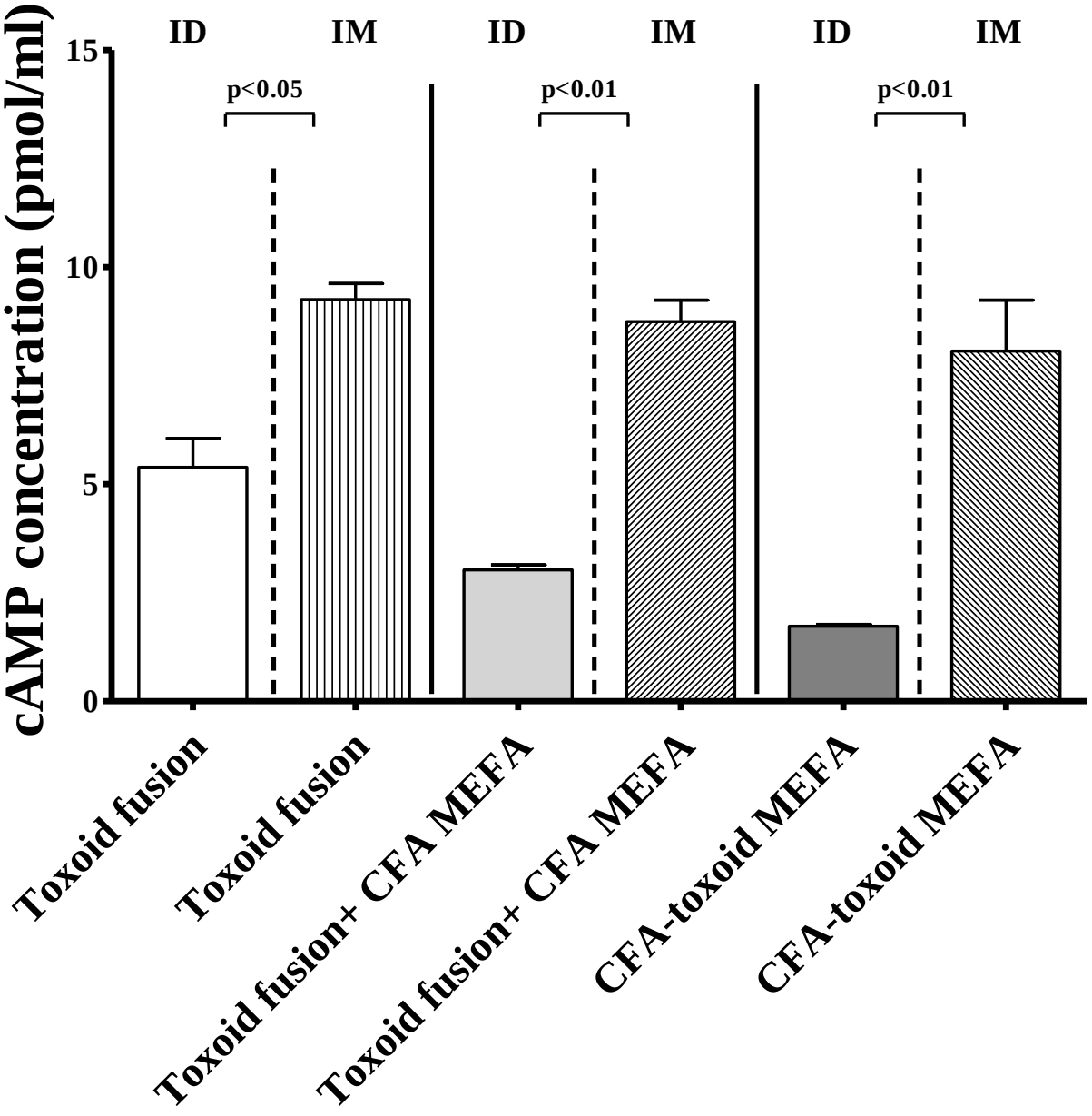

Figure 2.35 *In vitro* mouse serum antibody neutralization activity against CT. (LT homologue). Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control. was incubated with CT (10 ng) and added to T-84 cells. Intracellular cAMP levels (pmol/ml) in T-84 cells were measured by the protocol offered by the manufacturer. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Intracellular cGMP levels in T-84 cells incubated with STa demonstrated no significance in routes that were immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ and toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$+ CFA/I/II/IV MEFA, but there was a significant difference in CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ with ($p<0.01$) **(Fig 2.36)**.

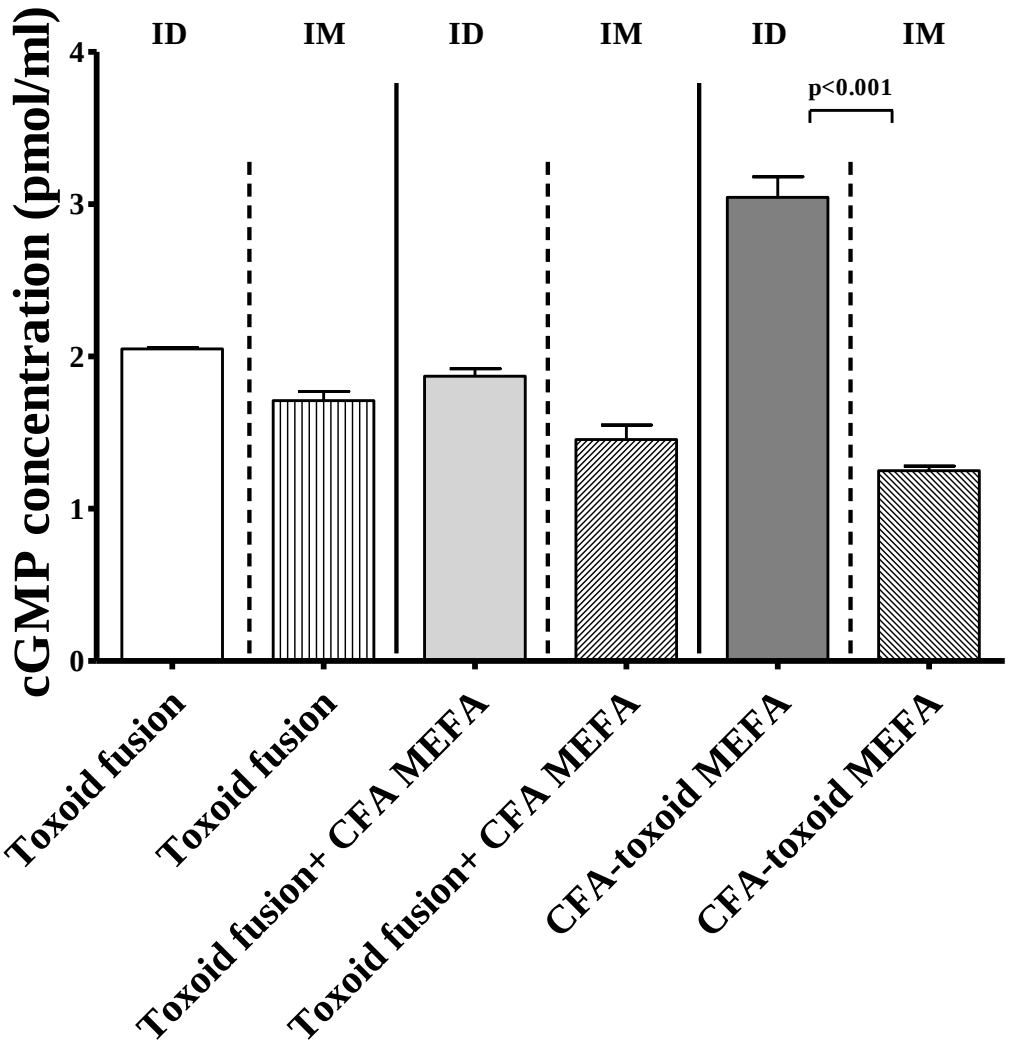

Figure 2.36 *In vitro* mouse serum antibody neutralization activity against STa. Serum from mice immunized with toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, toxoid fusion 3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$ + CFA/I/II/IV MEFA, CFA-3xSTa$_{N12S}$-mnLT$_{R192G/L211A}$, and control. was incubated with STa (2 ng) and added to T-84 cells. Intracellular cGMP levels (pmol/ml) in T-84 cells were measured by the protocol offered by the manufacturer. P-values were calculated using one-way ANOVA with a confidential interval of 95%.

Discussion

In previous studies 6x-His tagged fusion proteins were shown to induce neutralizing antibodies against 7 adhesins as well as LT and ST enterotoxins [64]. However, 6x-His tagged CFA/I/II/IV-3xSTaN12S-dmLT protein was less effective in neutralization of LT and STa enterotoxins [64]. Although, 6x-His tagged proteins are easy to produce, they are less considerable for human vaccines due to little ETEC protection and potential safety concerns. Tag-less toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$, adhesin MEFA (multiepitope fusion antigen) CFA/I/II/IV, and toxoid-adhesin MEFA CFA-$3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ were then constructed and intraperitoneal (IP) or subcutaneous (SC) immunized in mice [64]. The results demonstrated neutralizing activity against antitoxin and/or anti-adhesin antibodies, suggesting that the antigens are potential candidates for the development of a broadly protective ETEC subunit vaccine.

In this study toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$, CFA/I/II/IV MEFA, alone or combined, or toxoid-adhesin MEFA CFA-$3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ were intradermal or intramuscular immunized in mice to determine antigenic characterizations based on route of immunization. Enzyme-linked immunosorbent assay (ELISA) was used to determine if the serum elicited immune response against 7 (CFA/I and CS1-6) adhesins and 2 (STa and LT) enterotoxins. After confirming immune response in serum from immunized mice the bacterial neutralization was tested using the antibody adherence assay. Finally, intercellular cAMP for CT and cGMP for STa were measured using T-84 cells.

For the study intradermal route was used because literature suggested that intradermal (ID) route was rich in antigen presenting cells and that it was an antigen saving route. Intramuscular (IM) route was used because this route is quick absorbing and it prolongs antigen duration. Initially, we thought that ID route was going to function better than IM, because IM route usually

needs larger quantities of antigen based on the muscle used for injection and because ID was supposed to be an antigen sparing route. The results on the other hand, show that there are no significant difference in routes when tested against bacterial neutralization studies. Finally, both routes seem to be effective because they both induced high IgG antibody responses to adhesins and enterotoxins, they significantly neutralized ETEC bacteria expressing CFA/I, CS1-CS6, and they both neutralized cAMP and cGMP intercellular toxins.

Conclusion

Results from ELISA indicated that some adhesins had significant differences in IgG antigenic response based on antigen immunization when comparing ID and IM routes. On the other hand, when comparing ID and IM against anti-STa and anti-LT using ELISA there were no significant difference between both administration routes. In addition, bacterial inhibition assay also demonstrated no significant difference between routes when tested against ETEC expressing bacteria CFA/I, CS1-6. For intercellular cAMP toxin neutralization there were significant differences in the routes. Based on the data ID route had greater levels of toxin neutralization when compared to IM. For cGMP IM induced higher toxin neutralization levels; although, there were no significant difference for toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ and toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$+ CFA/I/II/IV MEFA, the results still demonstrated IM neutralized more toxins. In addition, there was a significant difference in CFA-$3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ with a ($p<0.01$). Finally, both routes had some difference in ELISA, cAMP or cGMP, but there were no significant differences in the bacterial inhibition assay, meaning that both routes demonstrated anti-adhesin characteristics that are the baseline of targeting ETEC.

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