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ANTHRAX TOXIN EFFECTS ON B LYMPHOCYTE FUNCTION

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ANTHRAX TOXIN EFFECTS ON B LYMPHOCYTE FUNCTION

by

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For My Mother

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ANTHRAX TOXIN EFFECTS ON B LYMPHOCYTE FUNCTION

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Bacillus anthracis is a gram-positive spore-forming rod capable of causing cutaneous, gastrointestinal and inhalational anthrax. It has a number of virulence factors of which, the two toxins are of great importance. Lethal toxin is a zinc metaloprotease that cleaves the N terminus of the mitogen-activated protein kinase (MAPK) kinase family 1-7, with the exception of MEK5. This kinase family is responsible for activating the mitogen-activated protein kinase (MAPK) cascade. This cascade includes extracellular signal-regulated protein kinases (ERK), c-Jun NH_2-terminal kinases (JNK) and p38 kinases. The disruption of these signaling pathways has a number of deleterious downstream effects that vary by cell type. Edema factor is a powerful calmodulin-dependent adenylyl cyclase that forms 3',5'-adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). This enzymatic reaction causes an increase in intracellular cyclic AMP (cAMP) in host cells. As cAMP is a prominent second messenger in cellular signaling, edema toxin has a wide array of effects on numerous cell types and functions.

To determine the effects of the anthrax toxins on the adaptive immune response, B lymphocytes were exposed to LeTx or EdTx *in vitro*. LeTx and EdTx both inhibit B cell activation in different manners. LeTx inhibited B cell proliferation but not migration, while EdTx inhibited B cell migration but not proliferation. LeTx and EdTx altered expression patterns of B cell activation markers. EdTx inhibited MIP-1α and MIP-1β while enhancing IL-6 production. Previously unseen in any cell type EdTx was demonstrated to be cytotoxic to naïve B cells.

The research presented in this report illustrates the inhibitory effects of LeTx and EdTx on B lymphocytes, providing valuable insight into the immunoevasion tactics of *B. anthracis*.

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Chapter 1 Introduction

1.1 History

Bacillus anthracis is a gram-positive, spore-forming, non-motile bacillus [1]. Typically a zoonotic disease, anthrax is associated with herbivores such as goats, sheep, and cattle. The bacteria and its resultant disease, anthrax, have a long association with man, dating from biblical plagues through Robert Koch and the very beginnings of microbiology to present day. It is postulated that the fifth and sixth plagues mentioned in Exodus were anthrax [2]. The poet Virgil is believed to have described anthrax in the third book of Georgics [3]. *Bacillus anthracis* as the bacterial cause of anthrax was first established by Robert Koch in 1876 [2-4]. The first identified cases of human inhalational infection with *Bacillus anthracis* occurred in the wool sorting industry in 19th century England. This disease, known as "Wool sorters disease", was first linked to the bacteria that both Koch and Pasteur had identified as the cause of anthrax by Dr. J. H. Bell in 1879 [4]. This was as a result of processing contaminated goat hair and alpaca wool imported from India and is believed to be the origin of the bacteria in England. The first effective live vaccine was developed against anthrax by Louis Pasteur in 1881. The cutaneous form of the disease is the most common and is associated with those working closely with infected animals or animal products.

The development of *B. anthracis* as a bioweapon dates back to World War I when German agents in the U.S. injected horses bound for Europe with the bacteria. Numerous countries had begun such biowarfare programs by World War II, including the Japanese in Manchuria, and even the British, who tested methods for dispersal of spores on Gruinard Island off Scotland. The resulting quarantine of the island for nearly 50 years is a testament to the persistence of the spores and their usefulness for denying territory to adversaries. The USA had an active bioweapons program from WWII until 1969 when President Richard Nixon ended it. The drafting of international treaties banning the use of biological weapons, such as the International Biological Weapons Convention, has been ineffective in preventing rogue states from continuing these programs as evidenced by the admission of such programs by Iraq in 1995, while it is believed many more operate such programs [5, 6]. The largest known release of *B. anthracis* occurred in The Soviet Union in 1979 [7-9]. The accidental release at a bioweapons facility in Sverdlosk resulted in 96 casualties. Most recently, in 2001 anthrax was released in an episode of domestic terrorism *via* the US Postal Service resulting in 22 cases of anthrax, 11 inhalational and 11 cutaneous. Of the 11 inhalational cases five were fatal even with medical treatment [10]. This high mortality despite antibiotic therapy and the efforts of the medical community spurred a renewed interest in anthrax research.

1.2 The Bacteria

A Gram-positive bacillus, *Bacillus anthracis* is capable of surviving in unfavorable environments by the formation of a spore. In favorable conditions the cells grow in long chains and form distinctive colonies on agar surfaces. These colonies are opaque with a ground glass appearance with rough outer edges and are nonhemolytic on sheep blood agar. In unfavorable conditions the cells form a spore that is very hardy and capable of surviving for many years in the environment. It is positively identified by lysis by gamma-phage [11].

1.3 Epidemiology

Anthrax, the disease caused by *B. anthracis*, occurs on every continent but Antarctica with the disease considered endemic in South America, Asia and Africa. Occasional outbreaks occur in North America and Europe and rarely in Australia. These outbreaks usually occur in summer months or during dry periods with high temperatures that follow periods of heavy rain. The most recent outbreak in Australia occurred in 2007 in New South Whales [12]. The previous outbreak in the region dates back to 1939, which may indicate possible dormancy of spores for greater than 60 years. A massive outbreak occurred in a wildlife reserve in Zimbabwe in 2004 [13]. Alberta Canada experienced an outbreak in 1999 that resulted in the death of 29 cattle and one horse [14].

In the United States significant outbreaks in cattle occurred in North and South Dakota, Minnesota and Texas in the summer of 2005.

Human infections occur sporadically, usually associated with contact with infected animal products. An estimated 100,000 cases of human anthrax occur each year worldwide. An outbreak in Thailand that included both oropharyngeal and cutaneous anthrax occurred in 1984 when undercooked meat from cattle and water buffaloes were brought in from Burma [15]. Over 6,000 cases of cutaneous anthrax occurred in Zimbabwe in 1979-1980 [16]. Twenty five cases were reported in Paraguay in 1987 [17]. Three fatal cases were reported in France in 1997 [18]. The number of cases in the United States has decreased to an average of one case per year since 1980 due to the use of the veterinary vaccine, which concomitantly decreased the number of animal cases.

1.4 Human Disease

Anthrax occurs in four forms dependent on the route of infection. These forms include cutaneous, gastrointestinal, oropharyngeal and inhalational. Cutaneous anthrax is the most common form of the disease accounting for 95% of all cases [19]. A vesicle containing serosanguineous fluid forms at the site of infection. The vesicle then ruptures forming a painless necrotic ulcer. This ulcer becomes a black eschar which suppurates after 2-3 weeks leaving an eschar. This black eschar is believed to be the reason for the

name anthrax, which is the Greek word for coal. Untreated, cutaneous anthrax can result in 10 to 20% mortality, however, with treatment mortality falls to less than 1% [19].

Oropharyngeal and gastrointestinal anthrax result from ingestion of contaminated meat. The incubation period for gastrointestinal anthrax is 2-5 days. Ulcerative lesions form from the oral cavity to the cecum resulting in a high mortality of up to 50% [20]. The oropharyngeal and gastrointestinal forms of anthrax form are very rare.

Inhalational anthrax occurs as a result of inhaling aerosolized *B. anthracis* spores. Historically, this has occurred when animal hides or sheep's wool, contaminated with anthrax spores, were processed, hence, the name "wool sorters disease." However, the accidental release of anthrax spores from a biological weapons factory in Sverdlosk in 1979, as well as the intentional release of anthrax spores in the mail in 2001, has garnered much attention for this form of the disease. The 2001 release resulted in a mortality rate of 45% despite antibiotic treatment. The incubation period for this form of the disease is usually 1-6 days and results in early non-specific "flu-like" symptoms including fever, malaise, fatigue, and myalgia [21-23]. These early sequelae are followed by dispnea, cyanosis chest pain and stridor. X-rays reveal widening of the mediastinum and pleural effusion. Delay of treatment until the onset of symptoms greatly reduces the prognosis.

1.5 Treatment and Prophylaxis

Most environmental and clinical isolates of *B. anthracis* are susceptible to numerous antibiotics including the penicillins, tetracyclines, and the fluoroquinolones;

however, spontaneous resistance to multiple antibiotics in *B. anthracis* was easily demonstrated in the literature [24]. The choice of antibiotic for clinical use is dependent on the suspected route of infection. Cutaneous anthrax may be treated with amoxicillin, if no aerosol is suspected. Inhalational, gastrointestinal and oropharyngeal should be treated with multiple intravenous antibiotics. Ciprofloxicin is the drug of choice for inhalational anthrax and is the drug kept in the national stockpile; however, numerous other antibiotics have been shown to be effective in animal models. Prophylactic treatment with antibiotics before onset of symptoms greatly enhances the prognosis and may be combined with administration of the anthrax vaccine. Currently, there is only one anthrax vaccine licensed for use in the United States, Anthrax Vaccine Adsorbed (Biothrax) produced by Emergent Biosolutions (formerly BioPort) in Gaithersburg, MD. The vaccine consists of sterile filtrate of an unencapsulated strain of *B. anthracis* adsorbed to aluminum hydroxide. The predominant immunogen in the vaccine is protective antigen (PA); however, some lots contain small amounts of lethal factor (LF) and edema factor (EF) [4]. The vaccine is recommended for industrial workers that may come in contact with contaminated animal products, and laboratory workers conducting research on the Ames strain. Military personnel are vaccinated to protect them against the threat of anthrax use as a bioweapon.

1.6 Virulence Factors

A number of virulence factors have been identified in *B. anthracis* infection. The most important virulence factors are lethal toxin (LeTx) and edema toxin (EdTx) encoded on the pXO1 plasmid and a poly-D-glutamic acid capsule synthesis gene encoded on pXO2. The production of the protein toxins is regulated by *atxA* located on pXO1, while induction of the capsule synthesis gene is regulated by *acpA* on pX02. The expression of these genes is regulated by temperature and bicarbonate, and an additional level of control is imposed by the gene *abrB* which limits induction of the toxin genes while the bacteria are in the logarithmic phase of growth [25].

1.6.1 Capsule

The capsule is antiphagocytic and poorly immunogenic and is required for dissemination from the lungs to other areas of the body. The capsule is needed for virulence in humans and strains of the bacteria that have been cured of the pX02 plasmid are considered to be attenuated [26, 27]. In fact, the former Soviet Union uses an attenuated unencapsulated strain as a live vaccine in humans. Likewise, pX02-deficient strains are not considered to be select agents by CDC.

1.62 Lethal Toxin

The presence of anthrax toxin was first demonstrated in 1954 [28]. The exotoxins, lethal toxin in particular, have been studied extensively. Both toxins are AB toxins, made up of two components, an active-component and a receptor binding component. In both toxins, the receptor-binding component (PA) attaches the complex to the host cell receptors Tumor Endothelial Marker 8 (TEM8) or Capillary Morphogenesis Gene-2 (CMG2) [29, 30]. These two host cell receptors seem to be ubiquitously present on different immune cell types. After binding to the cell membrane, they are then cleaved by furin, a host cell protease[31]. Once modified, the PA molecules form a heptamer which forms a pore aiding in delivery of the active components into the cell [32]. Each heptamer has three binding sites for either active component. Heptamerization results in endocytosis of the PA heptamer, and the acidic pH of the endosomes enables EF or LF to pass through the pore into the host cell cytosol [33].

Lethal toxin's active component is lethal factor, a zinc metaloprotease which cleaves the N terminus of the mitogen-activated protein kinase kinase (MAPKK) family of proteins (1-7) with the exception of MEK5 [34-37]. These MAPKK proteins are responsible for activating the mitogen-activated protein kinase (MAPK) cascade. This cascade includes extracellular signal-regulated protein kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 kinases. The disruption of these signaling pathways has a number of deleterious downstream effects that vary by cell type.

1.6.3 Edema Toxin

Edema toxin, which utilizes the same PA tissue receptor-binding component as lethal toxin, delivers a different active component, edema factor. Edema factor is a powerful calmodulin-dependent adenylyl cyclase which forms 3',5'-adenosine monophosphate, or cyclic AMP (cAMP) from adenosine triphosphate (ATP). This results in an increase in intracellular cAMP in host cells [38]. Since cAMP is a prominent second messenger in cellular signaling, edema toxin has a wide array of effects on numerous cell types and functions.

1.7 Toxin Effect on Immune cells

1.7.1 Innate Immune Cells

B. anthracis toxins aid in immune evasion by interfering with the immune response. Anthrax toxins have been shown to affect a wide range of cell types including those of both the innate and adaptive immune defenses. Innate defense cell types affected by anthrax toxins include macrophages, monocytes, polymorphonuclear cells and dendritic cells.

The macrophage was implicated as a key player early on in the study of the disease. The toxin effects on macrophages have been studied extensively. Lethal toxin

has been shown to induce apoptosis in murine macrophages in a strain-dependent manner [39, 40]. This strain-dependent effect has been shown to be associated with polymorphism in the NALP1b locus, implicating another mechanism other than MAPKK cleavage which occurs in both LeTx-sensitive and resistant strains of mice [41]. Both LeTx and EdTx have been shown to inhibit pro-inflammatory cytokine production [34, 40, 42-44]. Additionally, LeTx has been shown to inhibit NF-κB and interferon regulatory factor (IRF)-3, both vital to pro-inflammatory cytokine expression [40, 45, 46]. Both lethal toxin and edema toxin have been shown to impair macrophage chemotaxis [47, 48].

Polymorphonuclear (PMNs) cells have also been shown to be impaired by both EdTx and LeTx. In these cells, both toxins inhibit the oxidative burst response [49]. EdTx has been shown to impair phagocytosis by PMNs, while LeTx has been shown to impair PMN actin-based motility [50, 51].

Dendritic cells (DC), largely ignored until recently, have been shown to have a similar role as the macrophage in disease progression. LeTx has been shown to have effects on DCs similar to those seen in macrophages [52, 53]. They display the same strain-specific LeTx-induced apoptosis in the DC as seen in macrophages [52, 53]. However, LT has been shown to kill resistant DC strains in a caspase-1 independent manner. LeTx impairs pro-inflammatory cytokines, TNF-α and IL-6, in LPS-stimulated murine DCs [54]. Similar results have been observed in human DCs [55, 56]. Additionally, LPS-induced activation markers CD40, CD86 and CD80 are down

regulated by LeTx [54]. EdTx has also been shown to impair DC production of IL-12p70 and TNF-α secretion in infected DC cultures while LeTx inhibits IL-10 and TNF-α [57].

1.7.2 Adaptive Immune Response

While the effects of the toxins on innate immunity are of considerable importance during acute infection, the effect of both toxins on the adaptive immune response has been shown to be considerable in T lymphocytes. It has been demonstrated that neither EdTx or LeTx affect T cell viability in Jurkat cells, an immortalized T cell line [58]. However, the same study showed impaired activation and proliferation of both LeTx- and EdTx-treated T-cells. This impairment included the inhibition of CD25 and CD69 [58]. Fang *et al.* showed that MAPK kinase dependent IL-2 production was inhibited in LT treated human $CD4^+$ T-cells *in vitro* [59]. Similar results were discovered in a more comprehensive study utilizing primary murine T-cells [60]. Additionally, it was shown that $CD4^+$ T-cells from mice injected with LeTx and to a lesser extent EdTx had decreased cytokine production after stimulation of T-cell receptor (TCR) by anti-CD3 and anti-CD28 [60]. T-cells of LeTx-injected mice had decreased phosphorylation of kinases in the MAPK pathway involved in T-cell activation including ERK1/2, p38 and ATF-2 [60]. Additionally, AKT of the serine threonine pathway was inhibited, as well as GSK-3 [60]. EdTx had little or no effect on these signaling pathways [60]. Paccani *et al.*

showed that CXCR4-mediated T-cell chemotaxis was impaired by both LeTx and EdTx [47].

The anthrax toxin effect on B lymphocytes remains one of the least examined potential mechanisms of immune evasion that *B. anthracis* harbors. Fang *et al.* demonstrated that LeTx was not lethal for B lymphocytes but did cleave MEK-1 and 2 in PAM- and LPS-stimulated B lymphocytes [61]. Phosphorylation of ERK, JNK and p38 in PAM-stimulated B lymphocytes was impaired by LeTx [61]. IgM production and proliferation of stimulated B lymphocytes was also significantly impaired in LeTx-treated B lymphocytes [61]. This study demonstrated that B lymphocytes were indeed targets of LeTx, but a number of B cell functions were left unexamined, while the effects of EdTx on B lymphocytes were not examined at all.

A number of B cell functions are important for the adaptive immune response to be effective. B cell migration is vital for B cell - T cell interactions. B cell activation is an important step in the adaptive immune response. A number of different cytokines and chemokines are produced by B cells contributing to both the innate and adaptive immune response. Clonal expansion is another B cell function vital to an effective B cell response to antigen. Determining the effects that these toxins have on B cells is important in our understanding of the overall mechanism by which *B. anthracis* evades the immune system. The subject of this dissertation was focused on testing the hypothesis that <u>B cell function is directly impaired by anthrax toxins.</u>

Chapter 2: Materials and Methods

2.1 Isolation of B lymphocytes

Spleens were collected from 8-12 week old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) after humane euthanization. Spleens were pressed through a 70 μm cell strainer (BD Falcon, Bedford, MA) to achieve a single cell suspension. Red blood cells were lysed using Red blood cell lysing buffer (Sigma, St. Louis, MO). The remaining lymphocytes were processed to negatively select untouched resting B lymphocytes using a B cell isolation kit (Miltenyi Biotec, Auburn, CA), and the autoMACS separator magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Purified resting B lymphocytes were suspended in complete RPMI 1640 (GIBCO, Carlsbad, CA) containing 10% Fetal bovine serum (HyClone, Logan, UT), 100 μg/ml penicillin and streptomycin (Sigma Aldrich, St. Louis, MO), 1 mM sodium pyruvate (Sigma, Aldrich, St. Louis, MO) and 50 μM 2-ME (Sigma, St. Louis, MO).

2.2 In vitro exposure to toxins

Direct effects of the anthrax toxins on murine B lymphocytes were determined by incubating purified resting B lymphocytes with 2.5 μg/ml of PA with 1.0 μg/ml EF

and/or 1.0 μg/ml LF or PA, LF or EF alone. B cell viability was measured using propidium iodide (PI) staining (Sigma Aldrich, St. Louis, MO). Cells were seeded into 48 well plates at a concentration of $1x10^6$/ml and exposed to toxins for 1, 4, 8, 12, and 24 h. Each 100 μl aliquot of cells was washed twice with sterile PBS and resuspended in 100 μl of staining buffer (PBS with 1% FBS). PI was added to reach a final concentration of 1 μg/ml and incubated for 10 min. at room temperature. Staining buffer was added to reach a final volume of 400 μl, and samples were analyzed by flow cytometry on the FACSCanto (Beckton Dickinson, Franklin Lakes, NJ) for PI staining.

2.3 cyclic AMP assay

Intracellular cyclic AMP was determined by Enzyme Immunoassay. The murine splenic resting B lymphocytes were plated at a concentration of $5x10^5$ cells/200μl RPMI in 96-well tissue culture plates (Costar, Corning, NY). After the experimental treatments were set up, assay plates were incubated at 37°C with 5% CO_2 for 1, 4, 12, and 24 hours. Plates were centrifuged at 600 xg for 10 minutes and supernatants were removed. Cells were lysed with 200μl 0.1N HCl for 10 minutes. Plates were centrifuged again and supernatants were harvested. Subsequently, cAMP was measured by a Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions.

2.4 Cell proliferation assays

The effect of anthrax toxins on B lymphocyte proliferation was determined using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR). Resting B lymphocytes were washed twice with staining buffer and resuspended at a concentration of 2 x 10^7 cells/ml. An equal volume of 2.5μM CFSE was added to the cell suspension and mixed thoroughly. Cells were incubated at room temperature for 10 min in the dark. Cells were then washed and resuspended at 2x10^7 cells/ml in complete RPMI 1640 and incubated at 37°C with 5% CO_2 for 30 min. Cells were then washed and resuspended at 4x10^5 cells/ml in complete RPMI 1640. Cells were then plated 1 ml per well in 24-well tissue culture plates (Corning, Corning, NY). Cells were then exposed to the anthrax toxins or components for 4 hours and reacted with 5 μg/ml anti-CD40 antibody (eBioscience, San Diego, CA) and 5 ng/ml IL-4 (eBioscience, San Diego, CA) and allowed to incubate for 72, 96, or 120 hours. Cells were stained with PI and analyzed using the FACSCanto (Beckton Dickinson, Franklin Lakes, NJ), gating on forward and side scatter and PI negative for live cells. The results were analyzed using FCS Express V3 cell proliferation analysis software (DeNovo Software, Los Angeles, CA).

2.5 Cytokine and Chemokine assays

Murine resting B lymphocytes were treated with toxins or media alone for 4 hours and stimulated with 5 μg/ml anti-IgM, 5 μg/ml anti-CD40 or 5 ng/ml IL-4 for 48 h. Supernatants were collected and Eotaxin, G-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), TNFα, IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, , MIG, MIP-1α, MIP-1β, MIP-2, RANTES, and VEGF were measured using a 32-plex cytokine assay (Millipore, Billerica, MA) according to manufacturer's instructions.

2.6 Migration Assay

Resting B lymphocytes were treated with the anthrax toxins as described and seeded at $5x10^5$ cells per 100 μl in the upper well of a 24 well, 5 μm transwell filter chamber (Corning, Corning, NY. A volume of 600 μl of medium alone or with 1 μg/ml BCA-1 (R&D Systems, Minneapolis, MN) or MIP-3β (R&D Systems, Minneapolis, MN) was added to the lower chamber, and the cells were incubated at 37°C for 3 hours. Subsequently, cells in the lower chamber were counted using a hemocytometer, and the percent migration was calculated as (migrated cells*100/input cells). Specific migration was calculated as percent chemokine migration minus percent control migration.

2.7 Surface staining

Resting B lymphocytes were treated and stimulated as described. For surface staining, cells were washed twice with staining buffer and resuspended at $2x10^7$ cells/ml in staining buffer and incubated with 0.5 μg of anti-CD16/32 per million cells for 10 minutes at 4°C. Cells were then stained with fluorochrome-labeled antibodies for 20 minutes in the dark at 4°C. Antibodies used included, CD45R, CD69, CD86, MHCII, CD23, CCR7 (eBioscience, San Diego, CA), and CXCR5 (BD Bioscience, San Jose, CA). Fluorochromes used were PE, FITC, PE Cy5, APC, and PE Cy7. Cells were washed three times with staining buffer and resuspended in 500 μl of staining buffer with PI or 7-AAD added, and the cells were analyzed on the FACSCanto (Beckton Dickinson, Great Lakes, NJ). Viable cells were distinguished using forward and side scatter and propidium iodide or 7AAD.

Chapter 3: Direct Inhibition of B lymphocyte function by anthrax toxins.

3.1 Anthrax EdTx but not LeTx is lethal to murine B lymphocytes in vitro

To determine the effects of anthrax toxins on B lymphocyte viability, B lymphocytes were isolated from mouse spleens using antibody-conjugated magnetic beads to negatively select for B lymphocytes. B lymphocytes were treated with LeTx, EdTx, LeTx plus EdTx, PA alone, LF alone, EF alone or media only for 4, 8, and 24 h. Toxin-mediated cell death was measured by Annexin V and Propidium Iodide staining with Annexin V^-/PI^- being healthy cells, Annexin V^+/PI^- being apoptotic and Annexin V^+/PI^+ being necrotic. We saw no significant change in the percentage of healthy cells between control and LeTx-treated cells. There was a noticeable decrease in the percentage of healthy cells in the EdTx group as early as 8 h with the difference more dramatic by 24 h (**Figure 3.1**). The edema toxin-mediated cell death was observed to be dose and time dependent with a significant increase in naïve B cell killing at an EdTx concentration as low as 10 ng/ml ($P<0.05$) **(Figure 3.2)**.

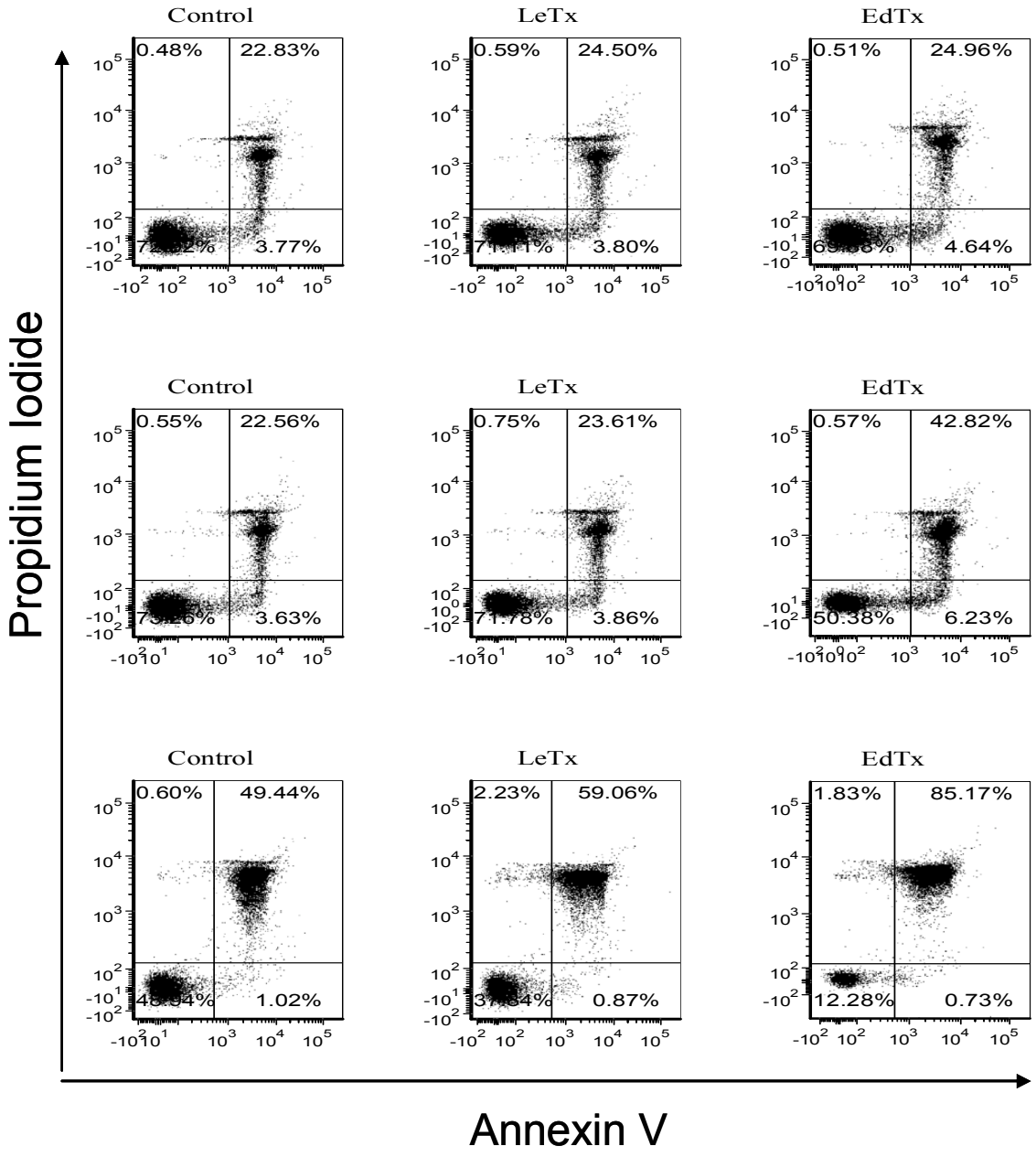

Figure 3.1 Anthrax toxin effects on B lymphocyte Viability. Naïve B lymphocytes were treated with 2.5 µg/ml of PA and 1.0 µg/ml LF or EF and incubated at 37°C and 5% CO_2 for 4, 8, and 24h. Viability was assessed using Annexin V and PI staining with at least 30,000 events recorded per group. The data are from one experiment and are representative of at least three independent experiments.

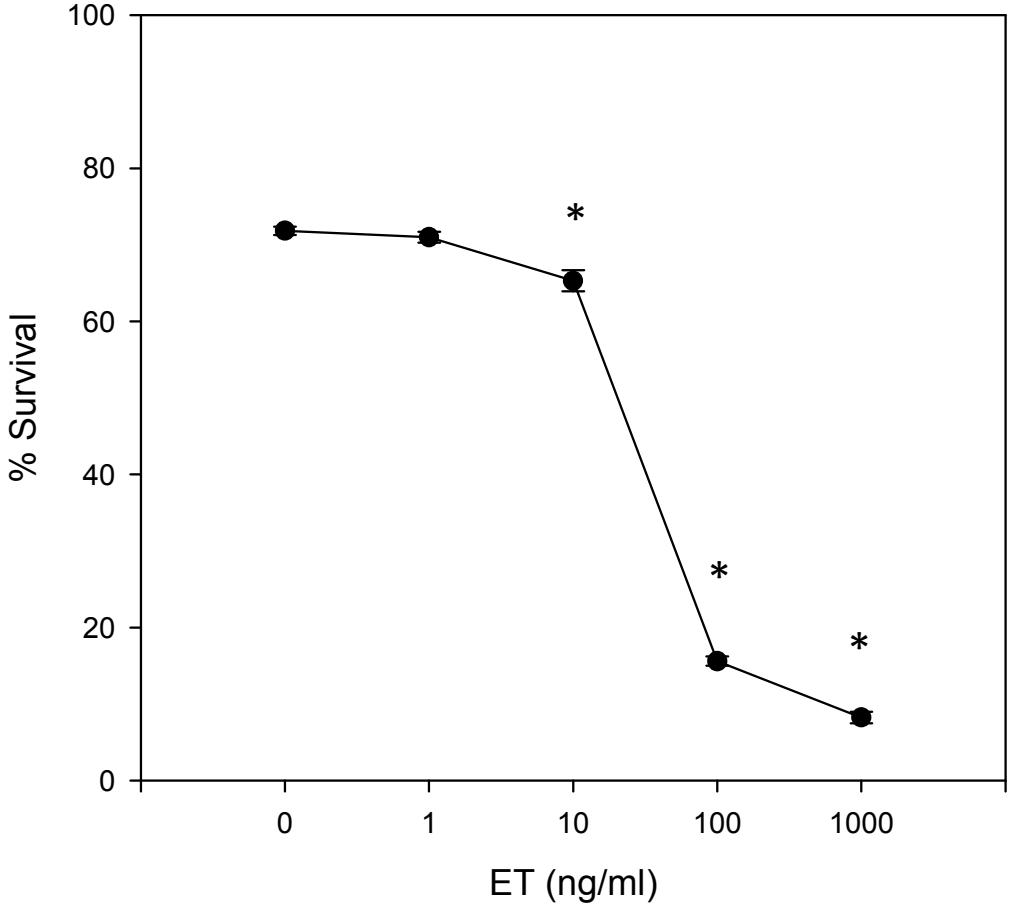

Figure 3.2 Toxicity of EdTx on naïve Murine B lymphocytes. Naïve Balb/c B lymphocytes were incubated with various concentrations of EdTx consisting of a ratio of PA to EF of 2.5:1. After 24 h incubation at 37°C and 5% CO_2, Survival was measured by PI staining of cells and read on a flow cytometer. The line represents the mean of triplicate values ± standard error. The data are from one experiment and are representative of three independent experiments. Asterisks denote a statistically significant difference between untreated and edema toxin-treated cells ($p<0.05$ by the Dunnett's test).

3.2 cAMP levels in EdTx treated B lymphocytes

To determine if EdTx, a potent adenylyl cyclase, was able to enter B lymphocytes, we exposed murine splenic B lymphocytes to EdTx for 1, 4, 8, 12, and 24 h. EdTx treatment resulted in significant increases in cAMP over controls at all time points, peaking at 12 hours after exposure **(Figure 3.3).**

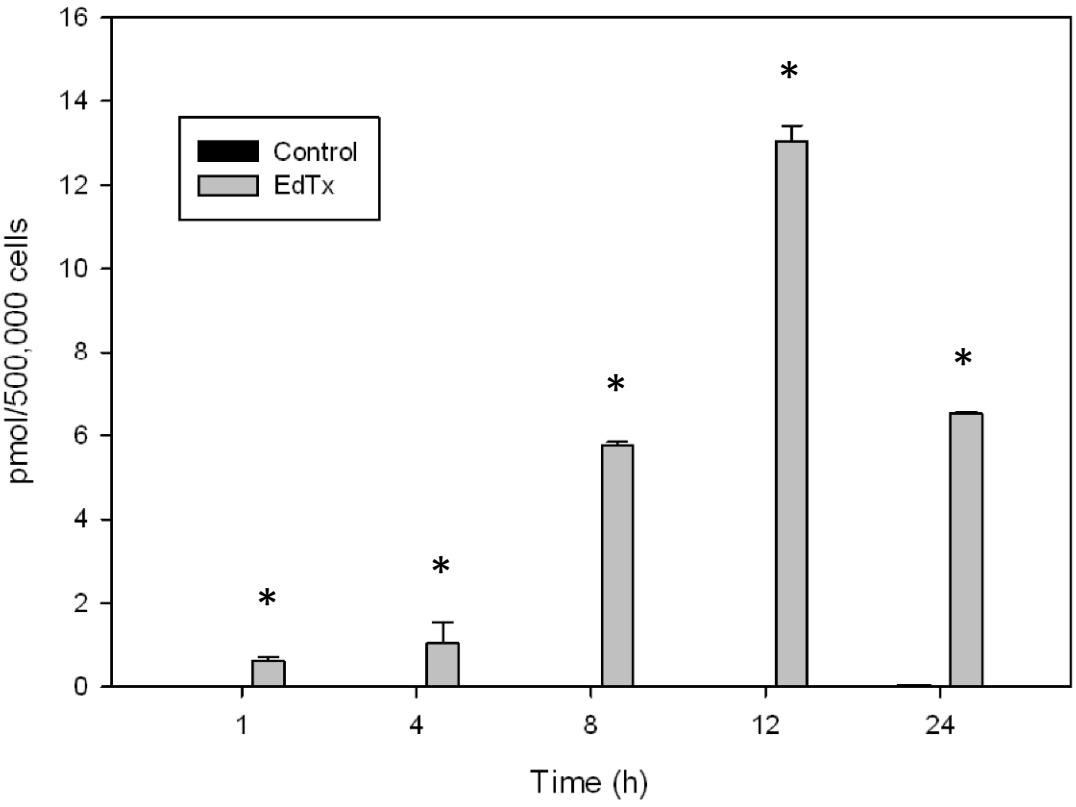

Figure 3.3 Cyclic AMP production in Edema Toxin treated Murine Naïve B lymphocytes. Murine naïve B lymphocytes were treated with media, 1µg/ml edema factor and 2.5µg/ml of protective antigen or protective antigen alone for 1, 4, 12 and 24 hours. After indicated incubation times, intracellular cyclic AMP was measured using an enzyme immunoassay. The line represents the mean of triplicate values ± standard error. The data are from one experiment and are representative of three independent experiments. Asterisks denote a statistically significant difference between untreated and edema toxin treated cells ($p<0.05$ by the Student's t-test).

3.3 Activation markers are altered on EdTx and LeTx- treated naïve B lymphocytes.

To determine the effects of EdTx and LeTx on naïve B cell activation markers, murine naïve B lymphocytes were incubated for 24 h with 2.5 µg/ml of PA and 1.0 µg/ml of EF, LF or both. Surface expression of activation markers was measured by staining cells with fluorochrome-labeled antibodies to CD86, CD69, CD23 and MHCII, and the cells were analyzed on a flow cytometer. Cells were gated on forward- and side-scatter to eliminate dead cells and cell debris. Edema toxin treatment alone resulted in higher CD86 expression in live gated cells while LT had no effect **(Figure 3.4)**. Neither EdTx nor LeTx had any perceptible effect on CD69 surface expression **(Figure 3.5)**. Both EdTx and LeTx decreased CD23 surface marker expression on naïve B cells **(Figure 3.6)**. EdTx increased MHCII surface marker expression, while LeTx had no effect **(Figure 3.7)**.

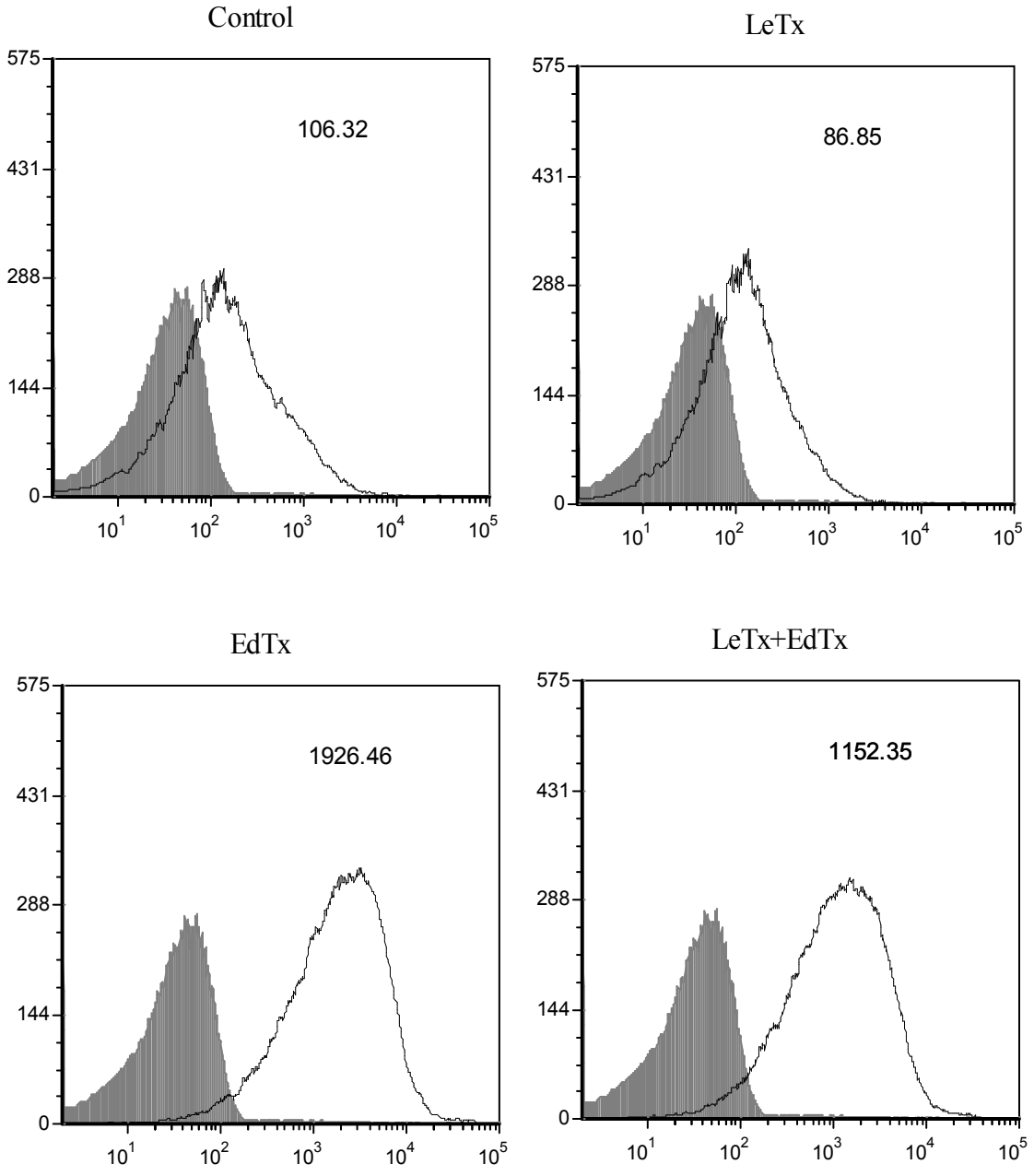

Figure 3.4 CD86 expression on toxin-treated naïve B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF, and LF during incubation at 37°C with 5% CO_2 for 24h. CD86 expression was measured using flow cytometry (solid black line). Shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

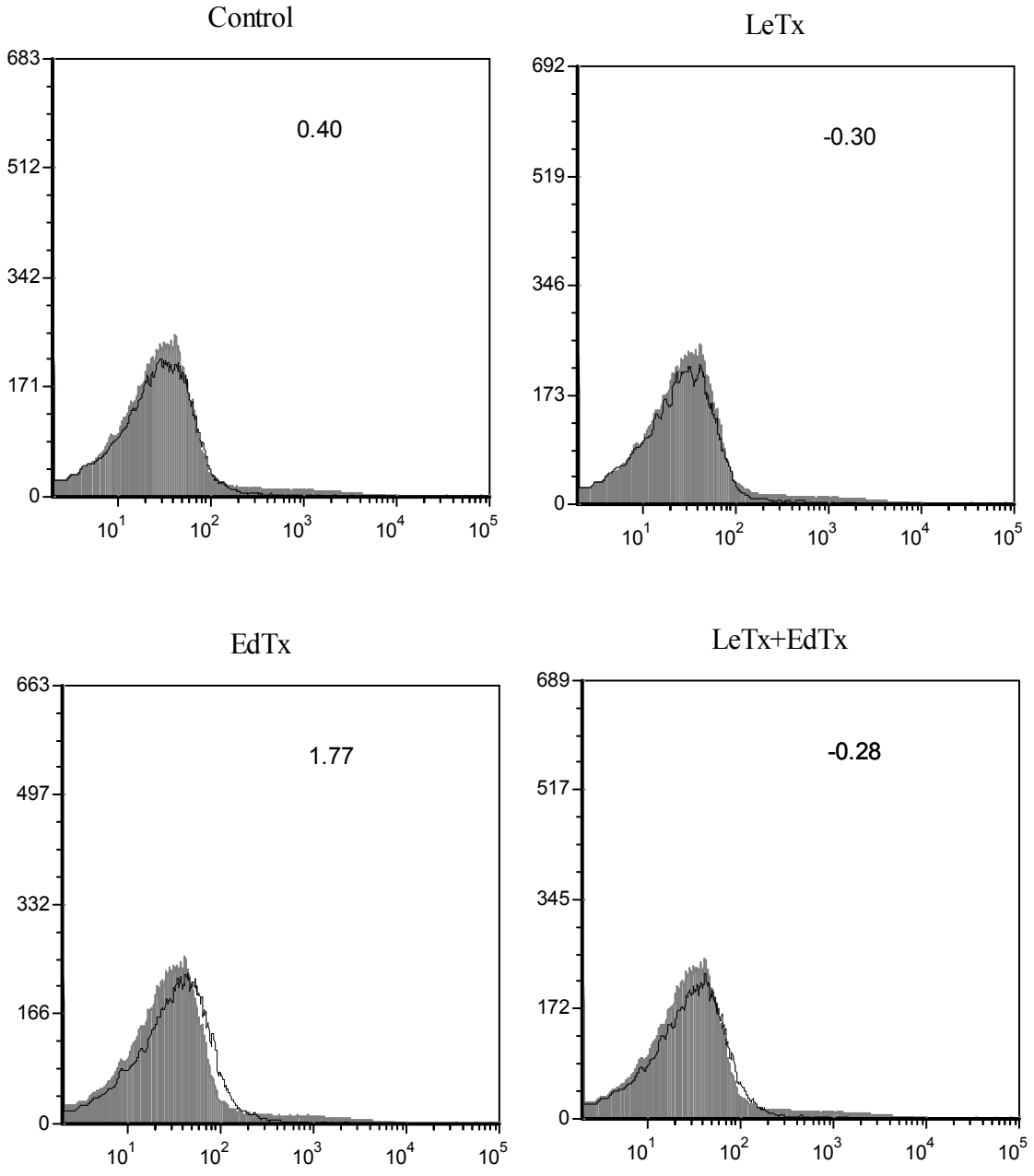

Figure 3.5 CD69 expression on naïve B lymphocytes exposed to anthrax toxin proteins. Naïve B lymphocytes were isolated from female BALB/c mice and exposed to 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 24h. CD69 expression was measured using flow cytometry (solid black line). Shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

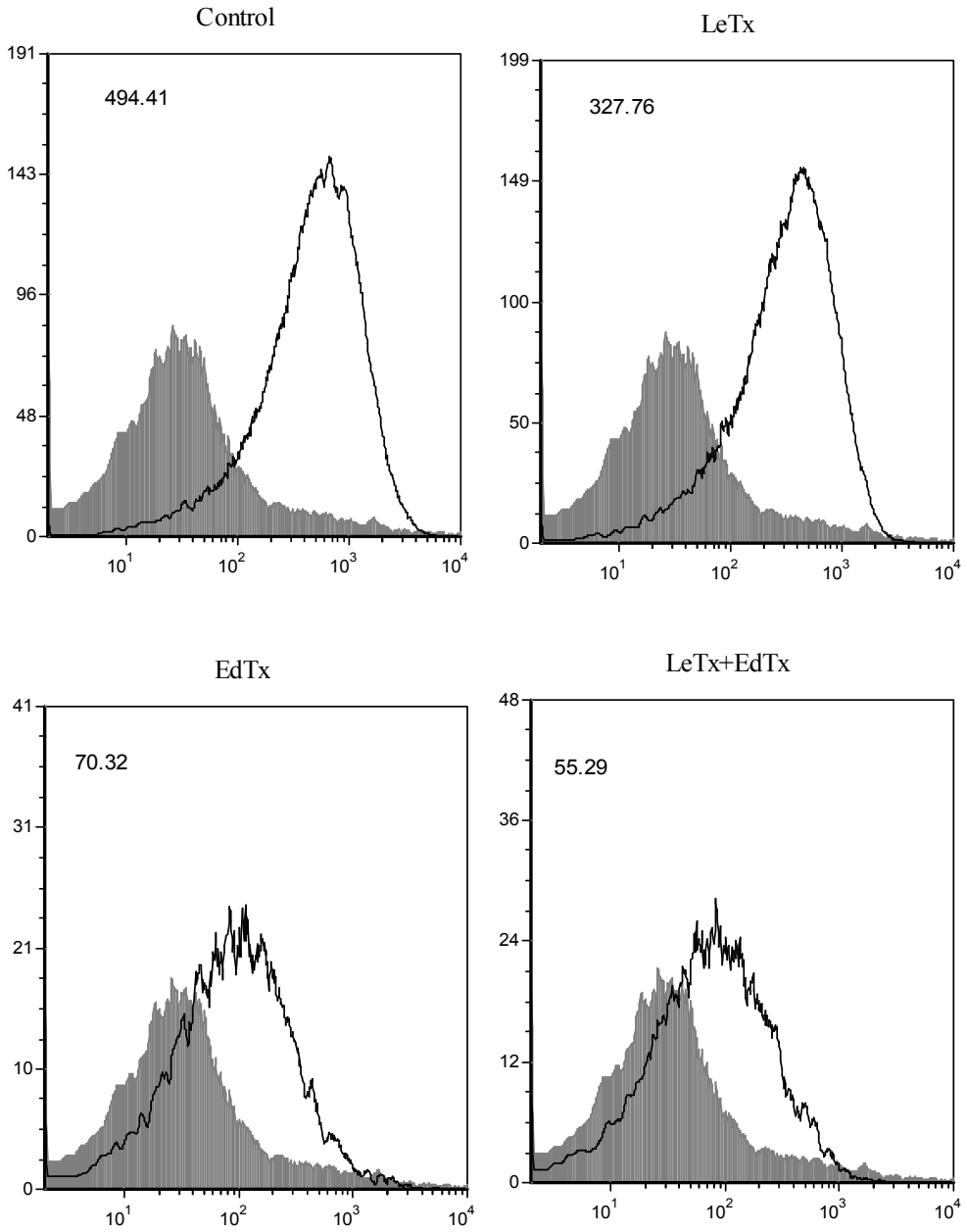

Figure 3.6 CD23 expression on naïve B lymphocytes exposed to **anthrax toxin proteins.** Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5%CO_2 for 24h. CD23 expression was measured using flow cytometry (solid black line). Shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

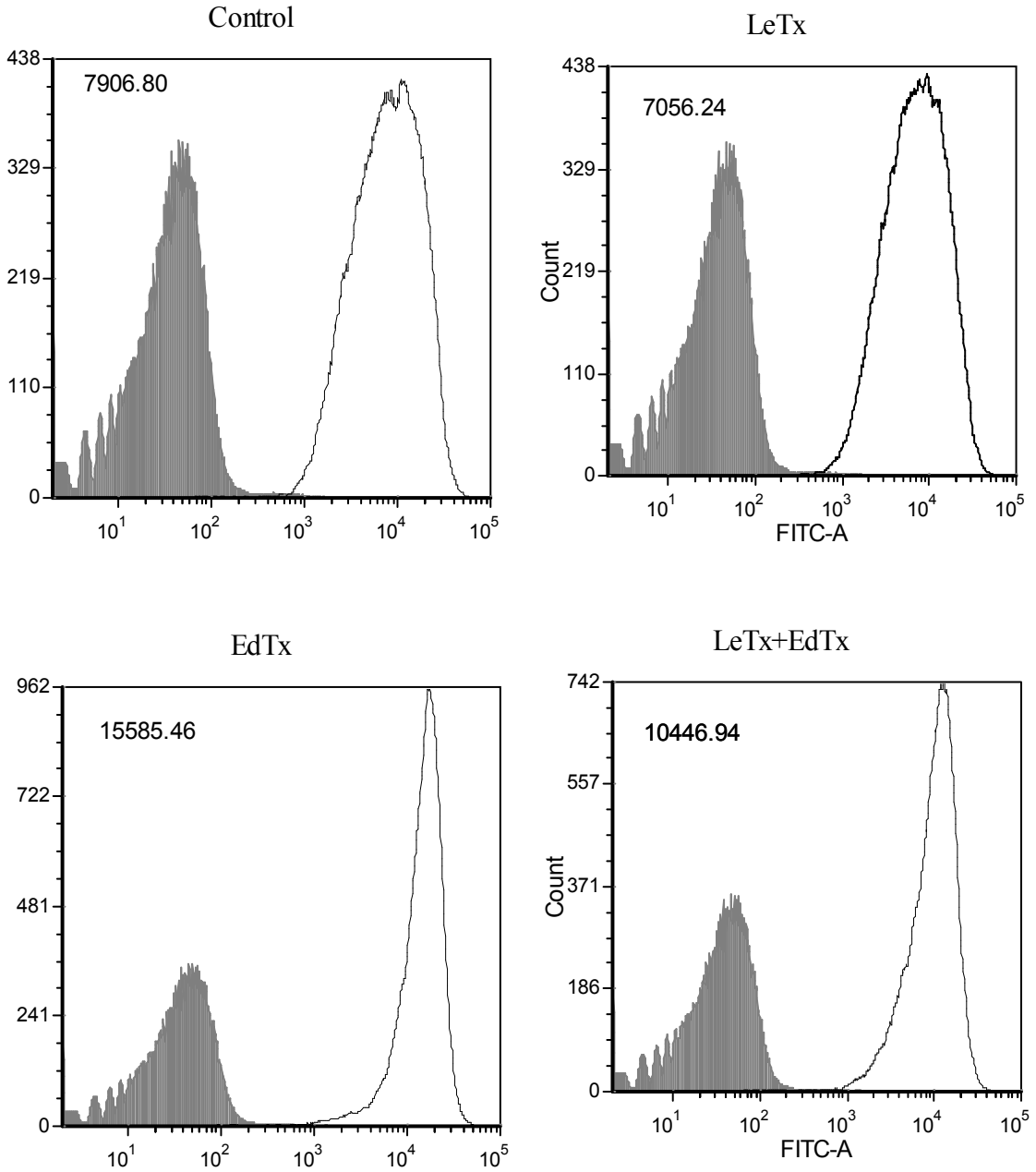

Figure 3.7 MHCII expression on naïve B lymphocytes exposed to anthrax toxin proteins. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 24h. CD86 expression was measured using flow cytometry (solid black line). Shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

3.4 Activation markers are altered on anti-CD40/IL-4 stimulated, toxin-treated B lymphocytes.

To determine the effects of EdTx and LeTx on T cell-mediated B lymphocyte activation, naïve B cells were exposed to 2.5 µg/ml of PA and 1.0 µg/ml of EF, LF or both, and incubated 4 h. Cells were then stimulated with 5 µg/ml of anti-CD40 antibody and 5 ng/ml of IL-4. Surface expression of activation markers was measured by staining cells with fluorochrome-labeled antibodies to CD86, CD69, CD23, and MHCII, and then the cells were analyzed on a flow cytometer. Cells were gated on forward- and side-scatter to eliminate dead cells and cell debris. Edema toxin treatment resulted in an increase in CD86 surface expression, while LeTx had no effect **(Figure 3.8)**. CD69 expression was not affected by EdTx but its production was impaired by LeTx **(Figure 3.9)**. Neither EdTx nor LeTx had any perceptible effect on CD23 surface expression **(Figure 3.10)**. Both EdTx and LeTx treatment resulted in a decrease in MHCII expression in stimulated B cells **(Figure 3.11)**.

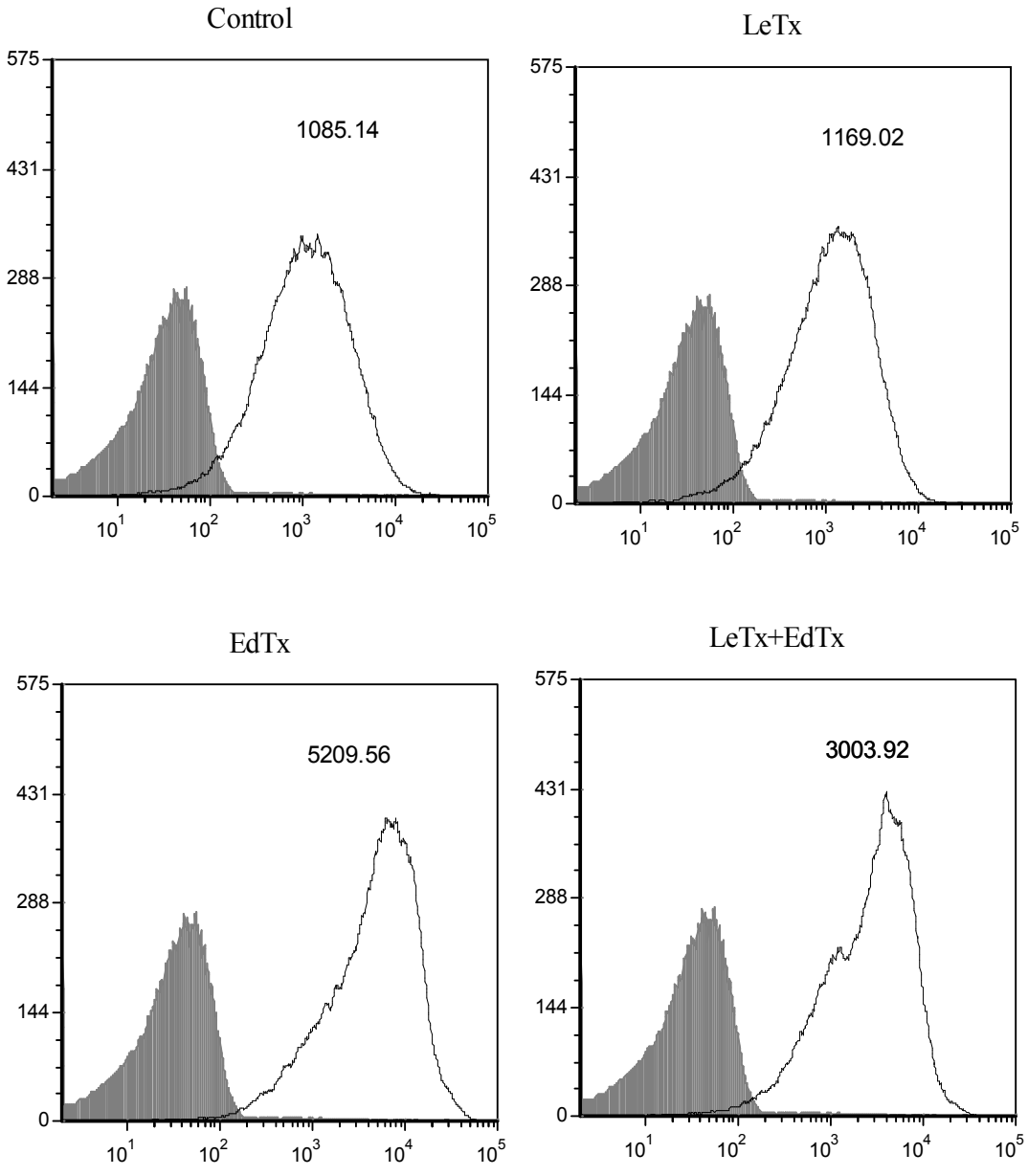

Figure 3.8 CD86 expression on anti-CD40/IL-4-stimulated murine B lymphocytes exposed to anthrax toxin proteins. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4h. Cells were then stimulated with 5μg/ml anti-CD40 and 5ng/ml IL4. After 24h, cells were stained with fluorochrome-labeled anti-CD86. Then, CD86 expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

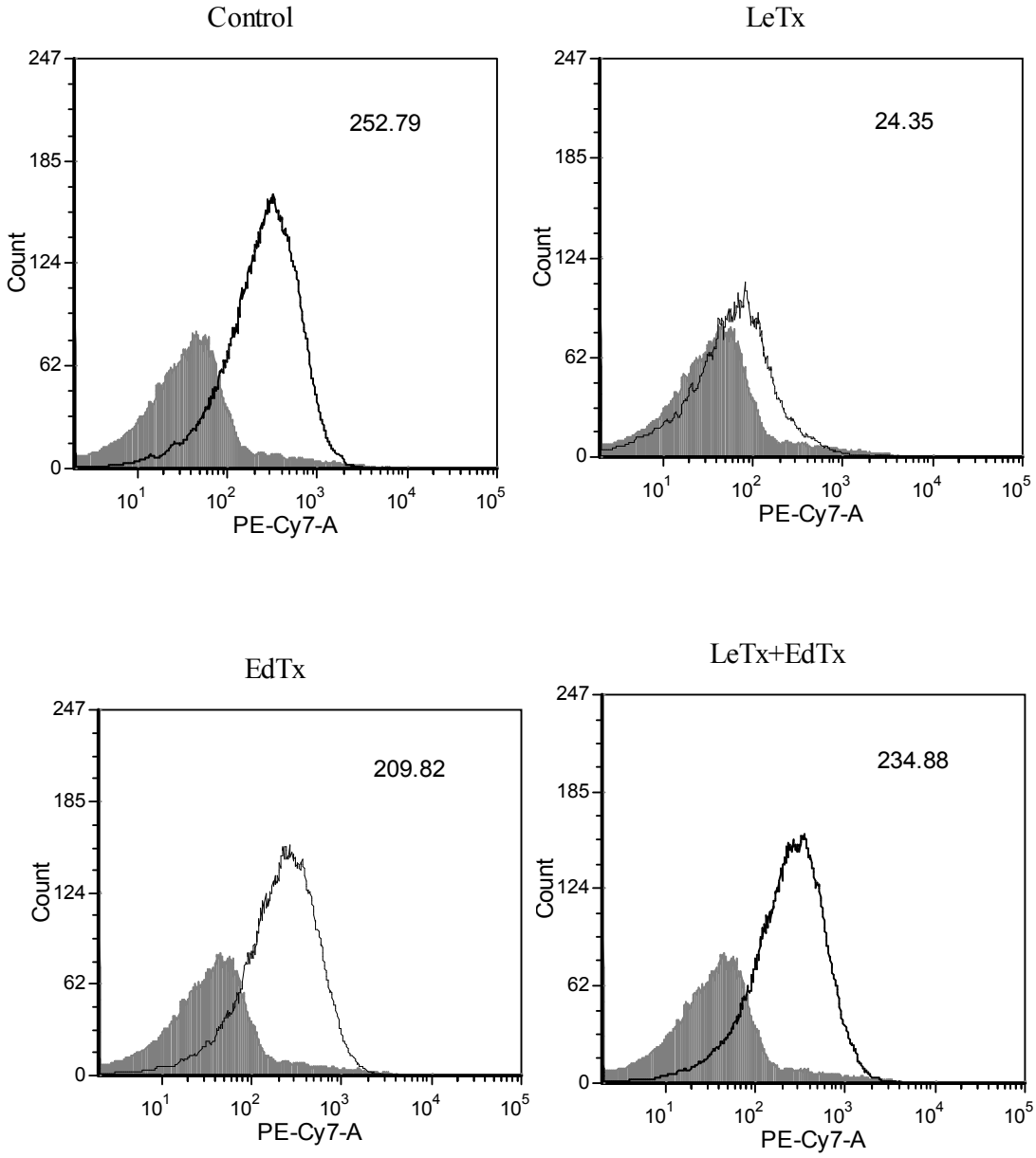

Figure 3.9 CD69 expression on anthrax toxin-treated anti-CD40/IL-4-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5 μg/ml anti-CD40 and 5ng/ml IL4. After 24 h, cells were stained with fluorochrome labeled anti-CD69 and CD69 expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

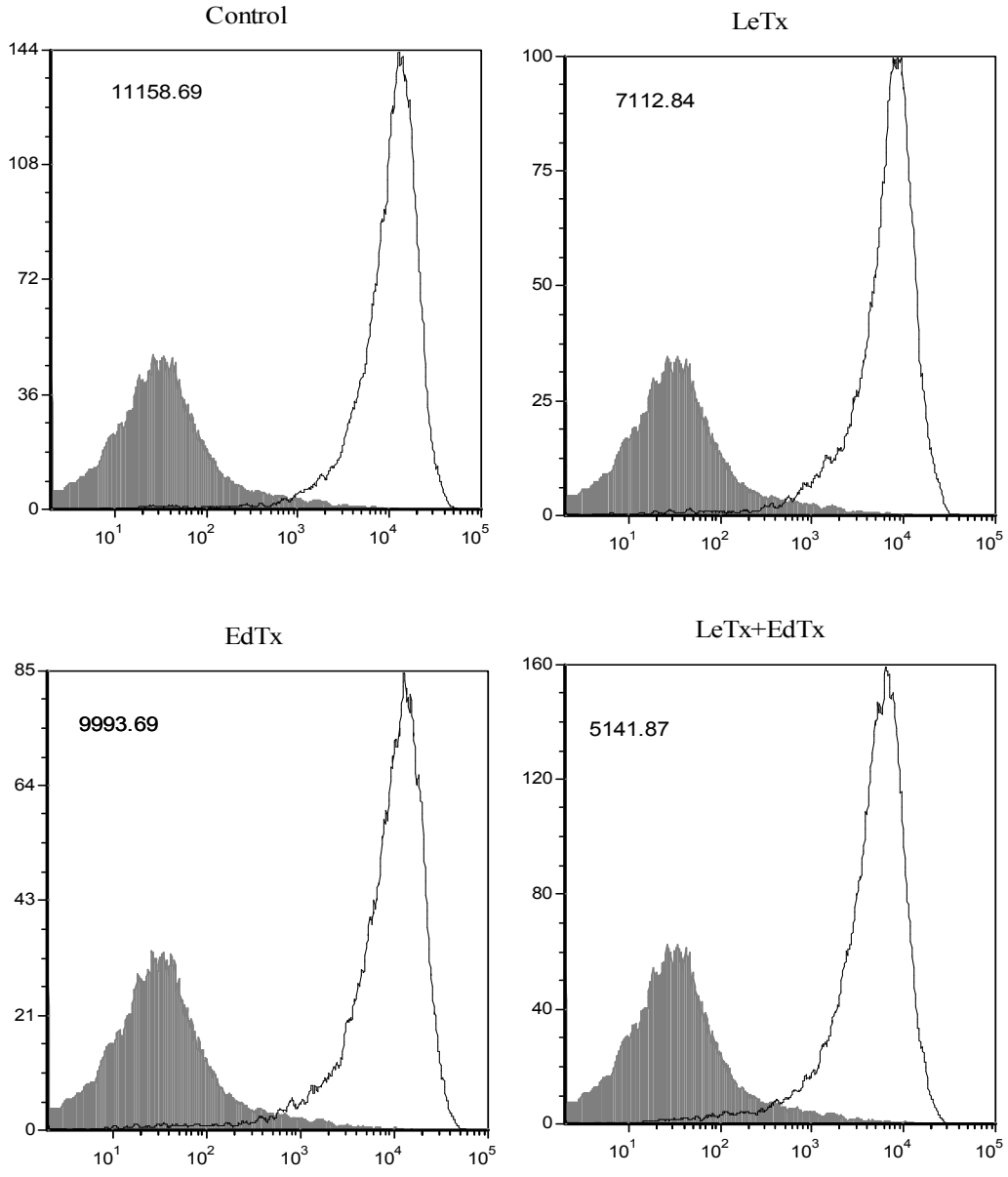

Figure 3.10 CD23 expression on anthrax toxin-treated anti-CD40/IL-4-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4h. Cells were then stimulated with 5 µg/ml anti-CD40 and 5 ng/ml IL4. After 24 h, cells were stained with fluorochrome labeled anti-CD23 and CD23 expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

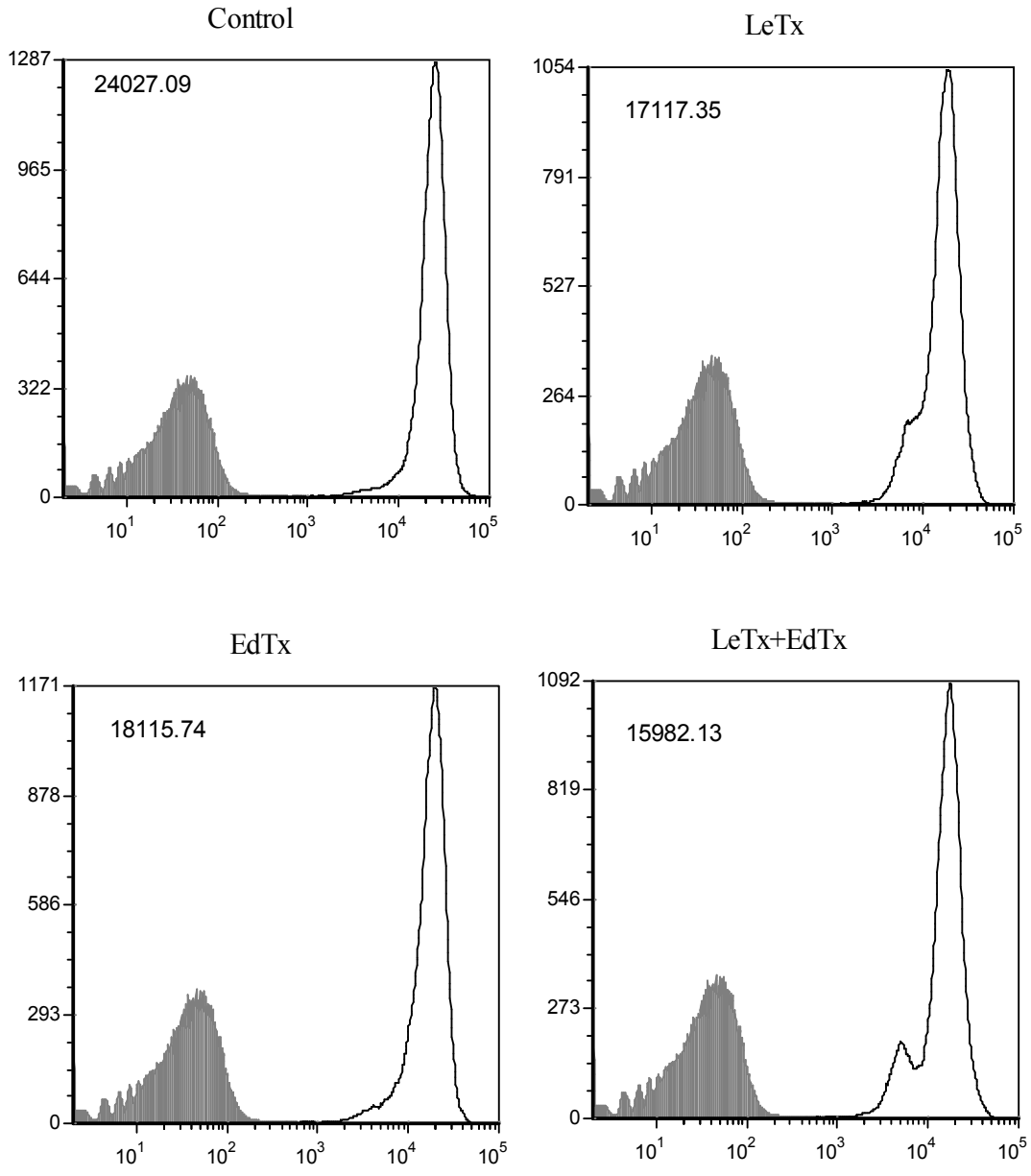

Figure 3.11 MHCII expression on anthrax toxin-treated anti-CD40/IL-4-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4h. Cells were then stimulated with 5μg/ml anti-CD40 and 5ng/ml IL4. After 24 h, cells were stained with fluorochrome labeled anti-MHCII and MHCII expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

3.5 Activation markers on anti-IgM stimulated, toxin-treated B lymphocytes are altered.

To determine the effects of EdTx and LeTx on B cell receptor (BCR)-mediated B lymphocyte activation, naïve B cells were exposed to 2.5 μg/ml of PA and 1.0 μg/ml of EF, LF, or both and incubated for 4 h. Cells were then stimulated with 5 μg/ml of anti-IgM. Surface expression of activation markers was measured by staining cells with fluorochrome-labeled antibodies to CD86, CD69, CD23 and MHCII and cells were analyzed on a flow cytometer. Cells were gated on forward- and side-scatter to eliminate dead cells and cell debris. EdTx caused an increase in CD86 surface expression while LeTx impaired CD86 surface expression **(Figure 3.12)**. CD69 expression was not affected by either EdTx or LeTx **(Figure 3.13)**. EdTx caused a decrease in CD23 expression, while LeTx had no effect **(Figure 3.14)**. Both EdTx and LeTx resulted in a decrease in MHCII expression in BCR-stimulated B cells **(Figure 3.15)**.

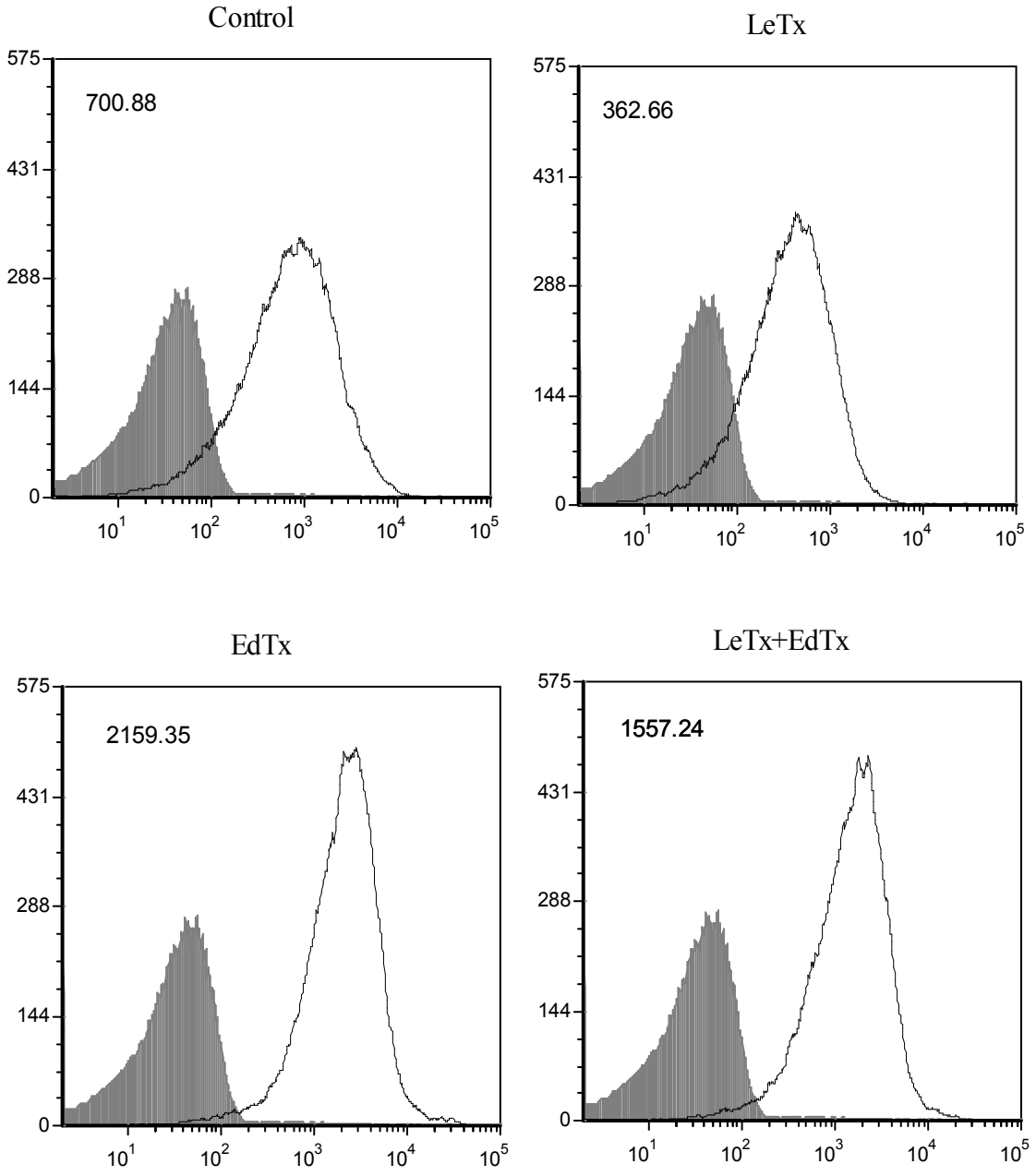

Figure 3.12 CD86 expression on toxin-treated anti-IgM-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice, and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5 µg/ml anti-IgM. After 24h, cells were stained with fluorochrome labeled anti-CD86 and CD86 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

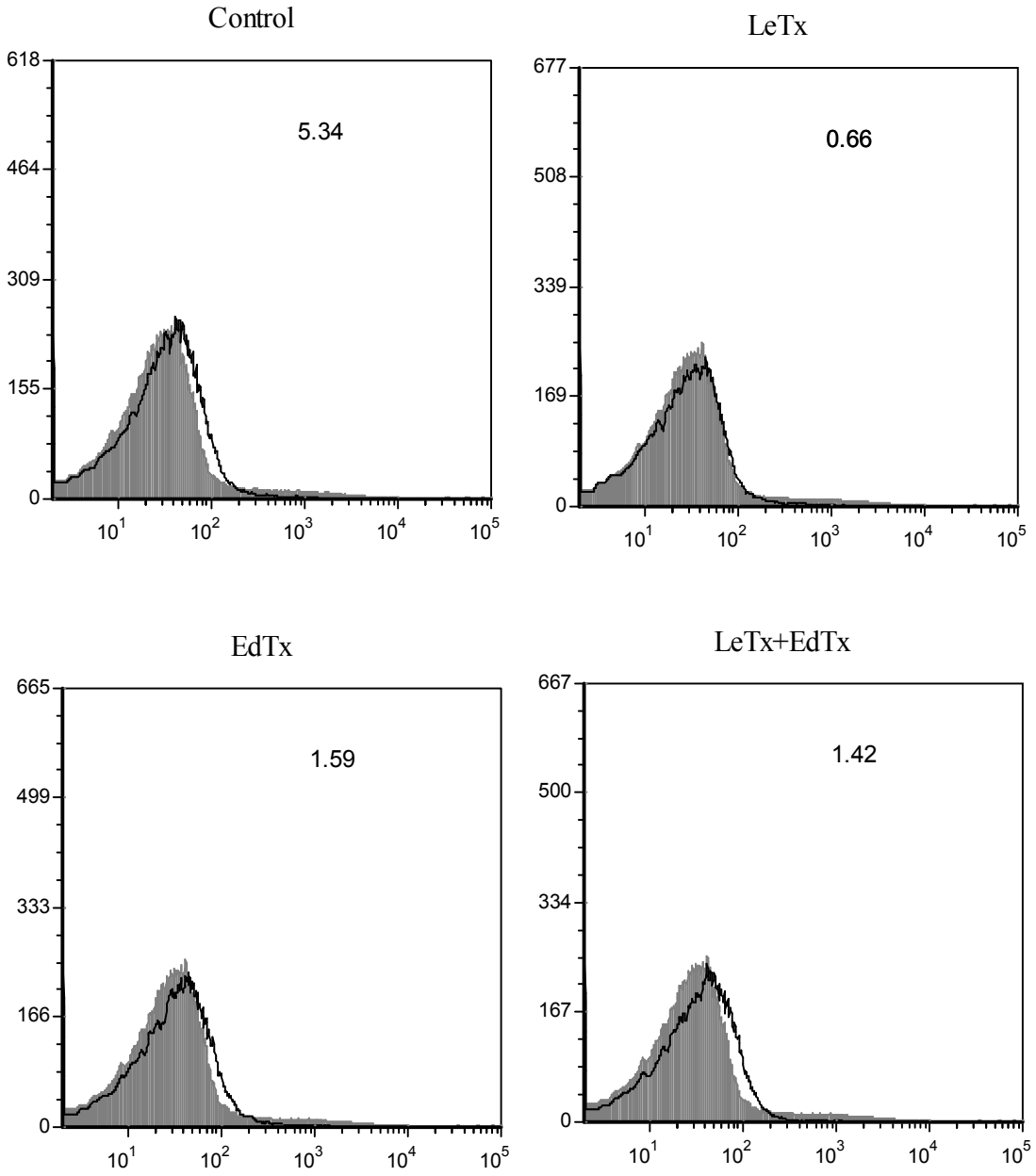

Figure 3.13 CD69 expression on anthrax toxin treated anti-IgM stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5µg/ml anti-IgM. After 24h, cells were stained with fluorochrome labeled anti-CD69 and CD69 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

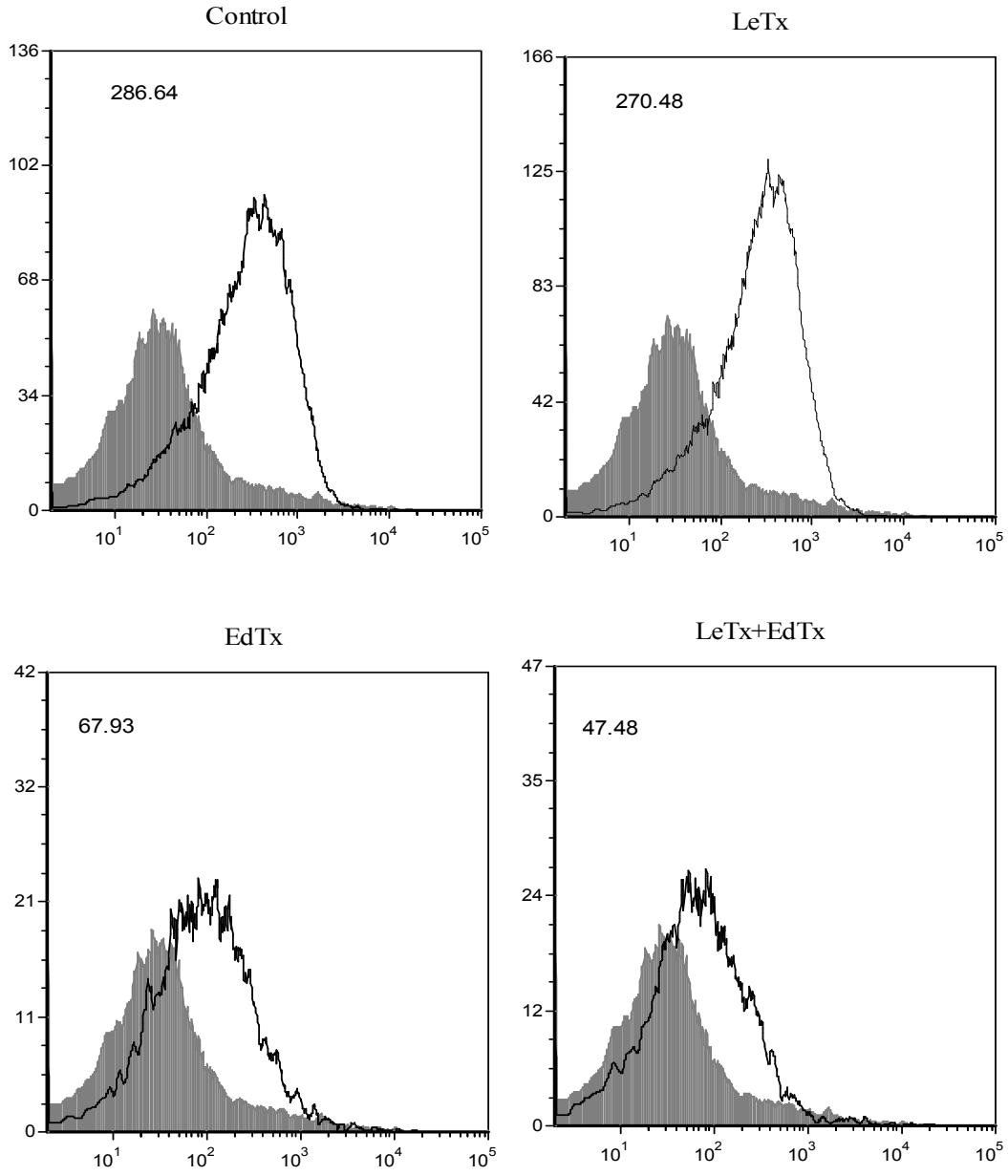

Figure 3.14 CD23 expression on anthrax toxin-treated anti-IgM-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5 μg/ml anti-IgM. After 24 h, cells were stained with fluorochrome labeled anti-CD23 and CD23 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

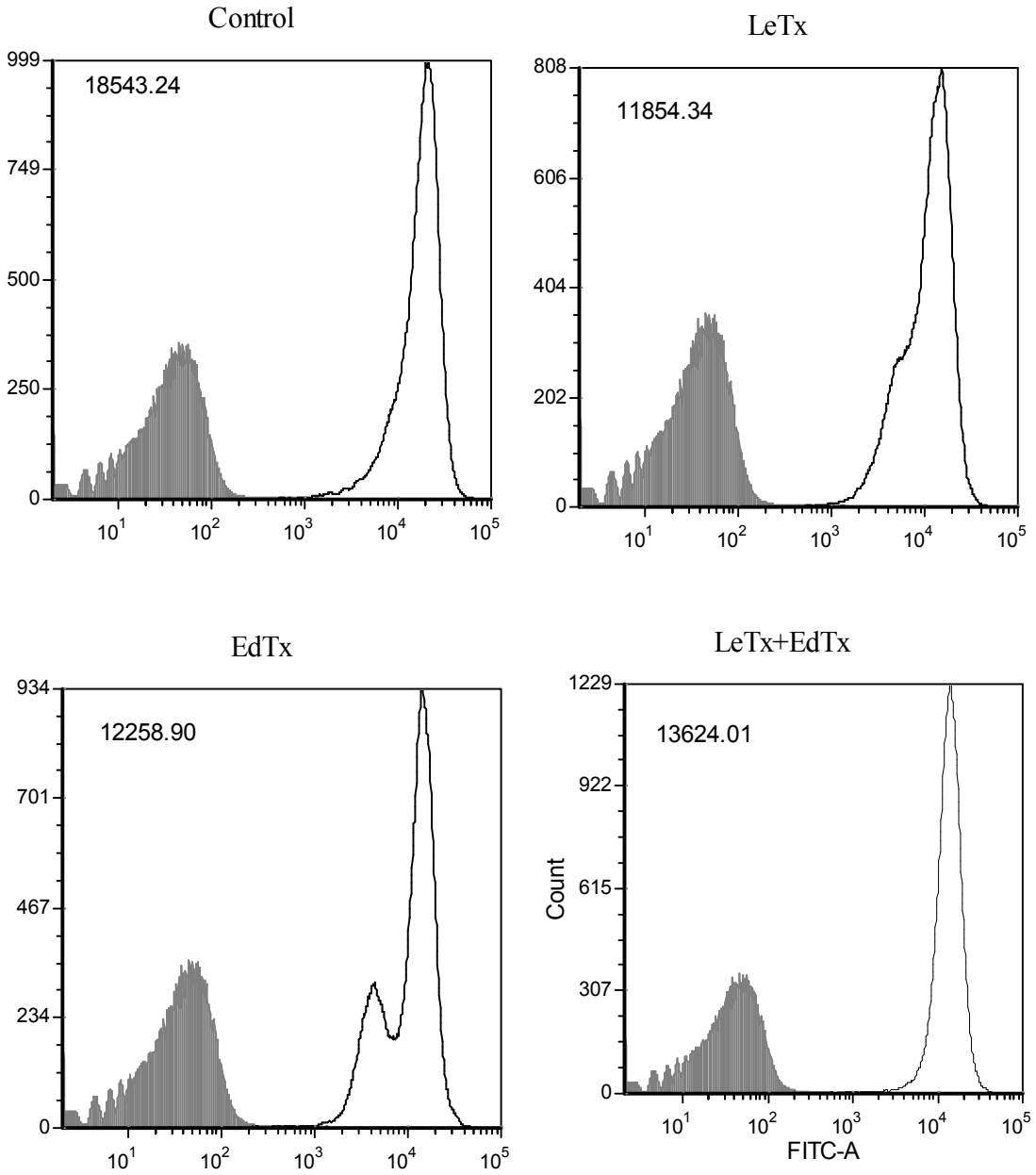

Figure 3.15 MHCII expression on anthrax toxin-treated anti-IgM-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4h. Cells were then stimulated with 5µg/ml anti-IgM. After 24h, cells were stained with fluorochrome labeled anti-MHCII and MHCII surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

3.6 Activation markers on LPS-stimulated, toxin-treated B lymphocytes are altered.

To determine the effects of EdTx and LeTx on TLR4-mediated B lymphocyte activation, naïve B cells were exposed to 2.5μg/ml of PA and 1.0μg/ml of EF, LF or both and incubated for 4 h. Cells were then stimulated with 25 μg/ml of LPS for 24 h. Surface expression of activation markers was measured by staining cells with fluorochrome-labeled antibodies to CD86, CD69, CD23, and MHCII, and the cells were analyzed on a flow cytometer. Cells were gated on forward- and side-scatter to eliminate dead cells and cell debris. EdTx treatment resulted in an increase in CD86-surface expression while LeTx impaired it **(Figure 3.16)**. EdTx treatment had no effect on CD69 expression, while LeTx treatment resulted in decreased expression **(Figure 3.17)**. CD23 expression was not affected by either toxin **(Figure 3.18)**. EdTx had no effect on MHCII expression while LeTx impaired it **(Figure 3.19)**.

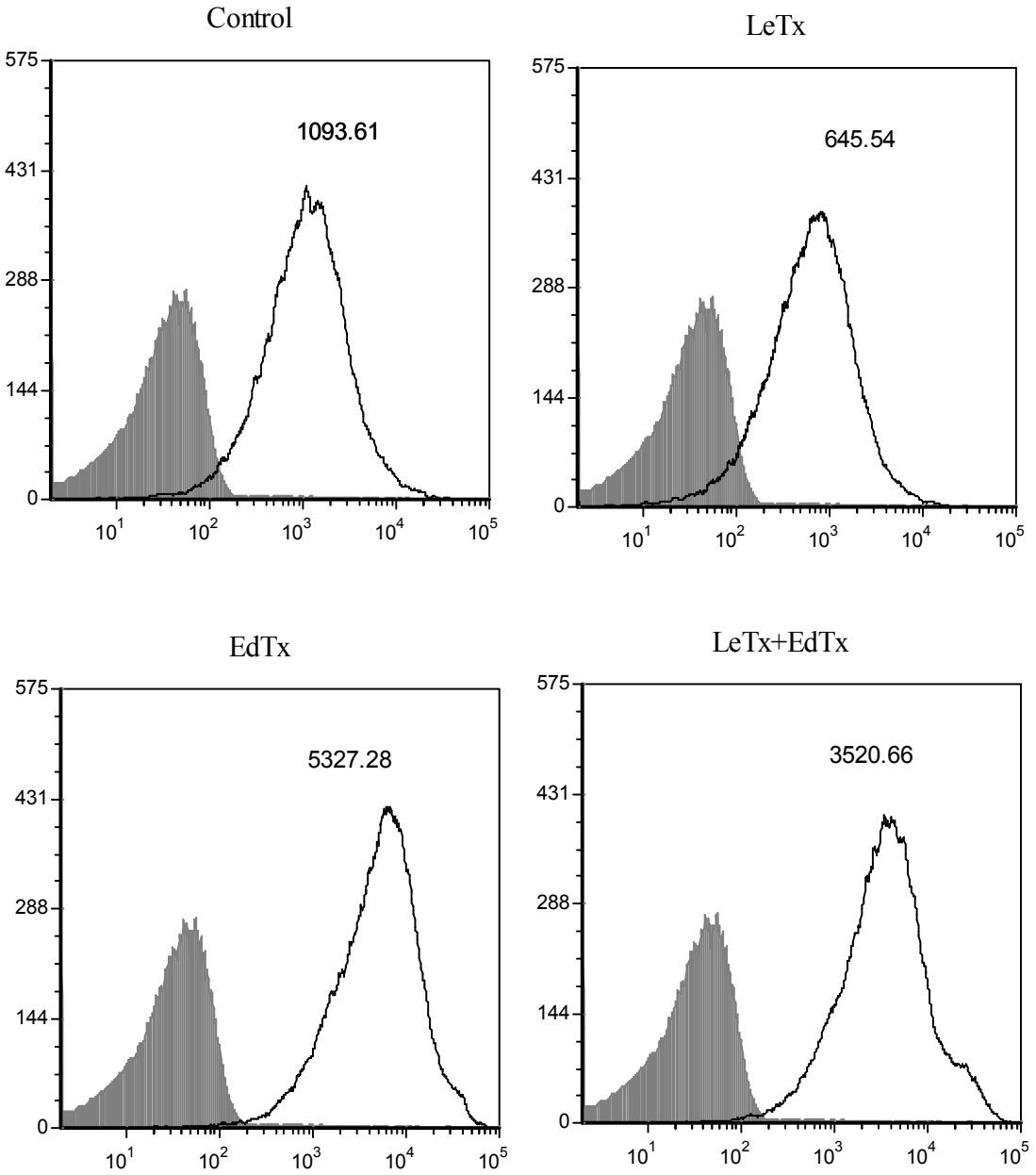

Figure 3.16 CD86 expression on toxin-treated LPS-stimulated B lymphocyte. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 25 µg/ml LPS. After 24h, cells were stained with fluorochrome-labeled anti-CD86 and CD86 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

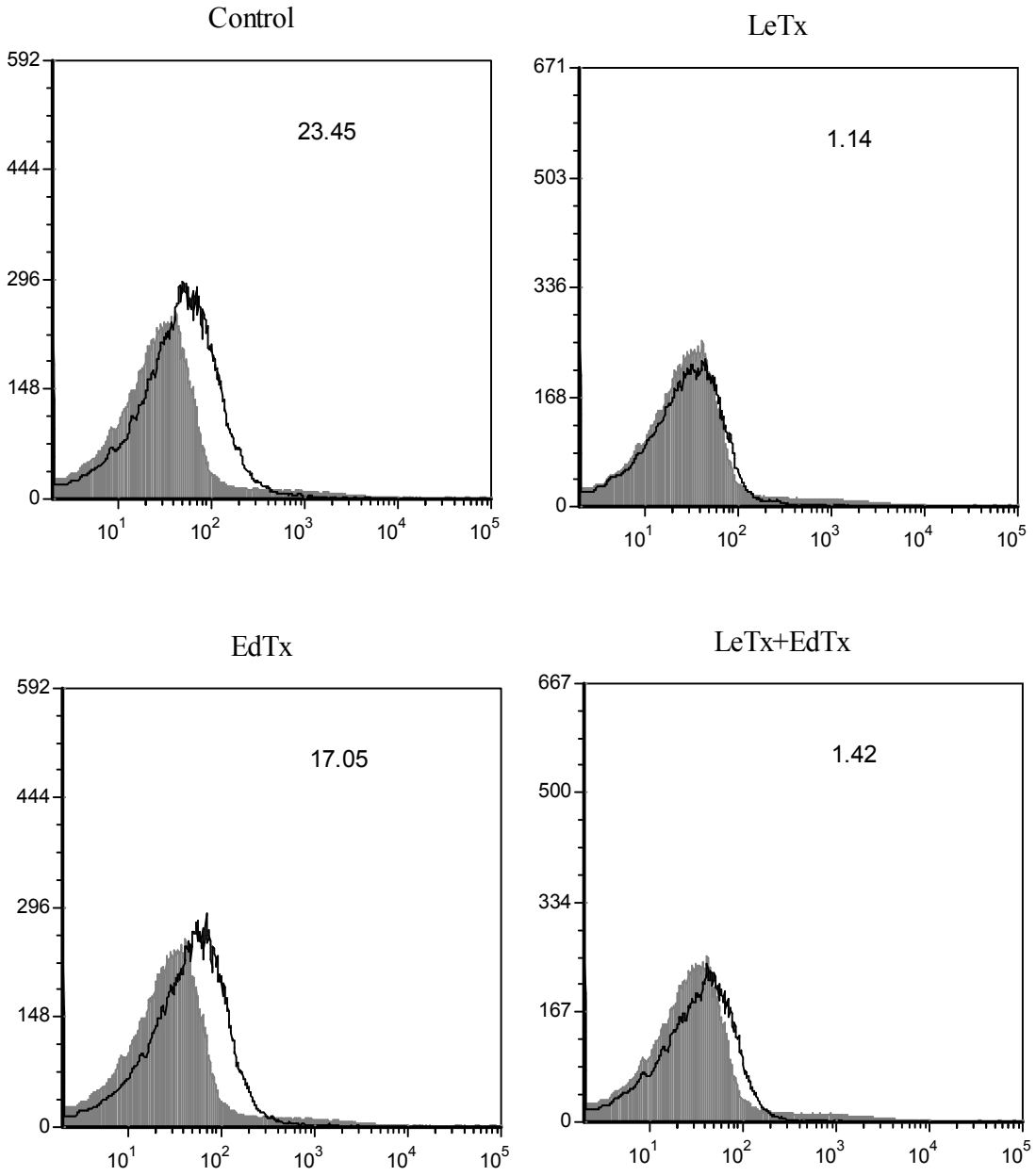

Figure 3.17 CD69 expression on anthrax toxin-treated LPS-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. The cells were then stimulated with 25 µg/ml LPS. After 24 h, cells were stained with fluorochrome-labeled anti-CD69, and CD69 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

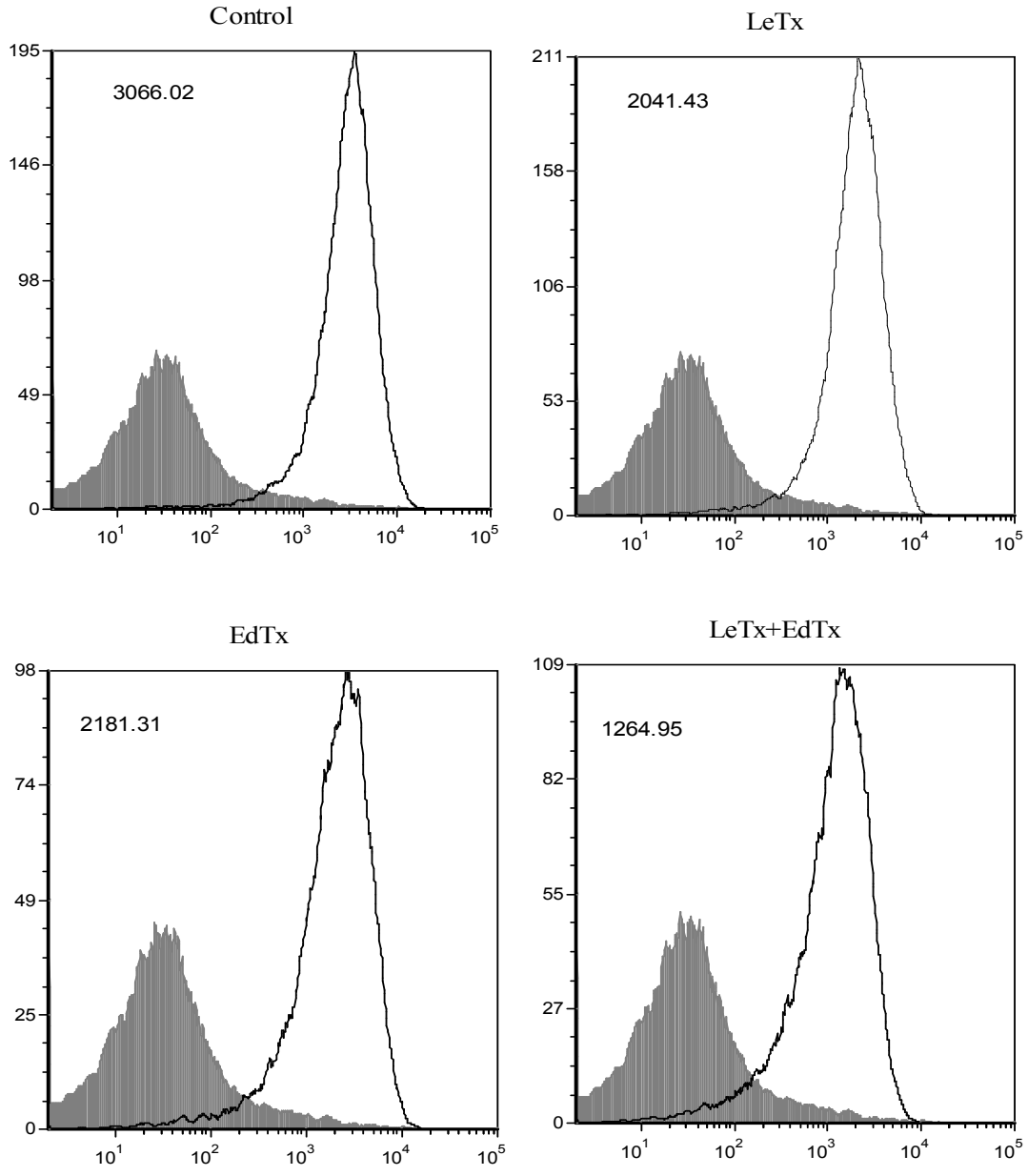

Figure 3.18 CD23 expression on anthrax toxin-treated LPS-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4h. Cells were then stimulated with 25 μg/ml LPS. After 24 h, cells were stained with fluorochrome-labeled anti-CD23 and CD23 surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

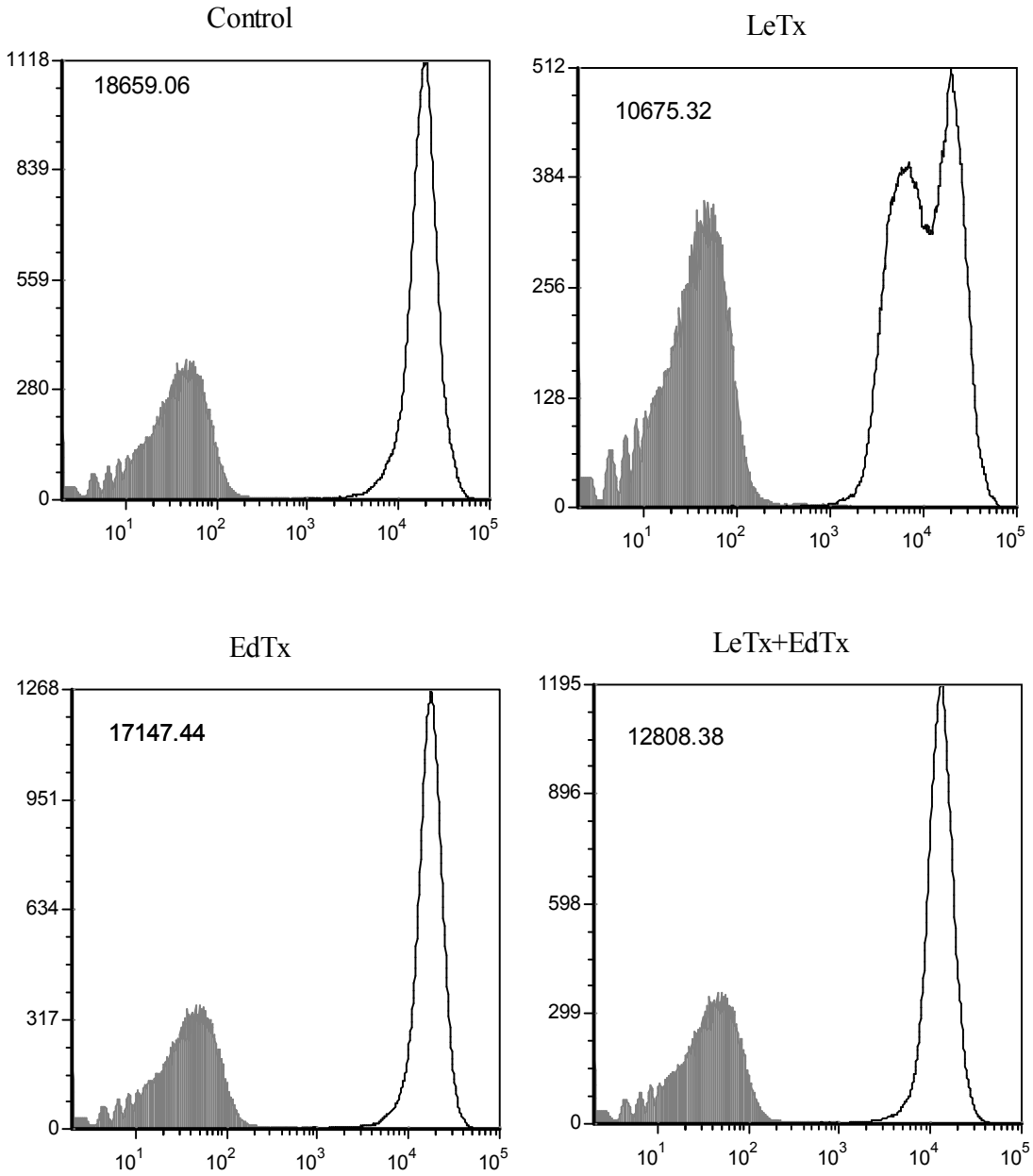

Figure 3.19 MHCII expression on anthrax toxin-treated LPS-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 μg/ml of PA and 1.0 μg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 25 μg/ml LPS. After 24 h, cells were stained with fluorochrome labeled anti-MHCII and MHCII surface expression was measured using flow cytometry (solid black line). The shaded area is the isotype control. The inserted numbers indicate median fluorescence intensity. The data are from one experiment and are representative of at least three independent experiments.

3.7 Anthrax edema toxin but not LeTx inhibits B cell migration.

To determine the effects of anthrax toxins on B cell migration, B lymphocytes were collected and treated with the anthrax toxins for 4 h. Complete B cell media with or without chemokines MIP-3β or BCA-1, 500ng/ml and 1000ng/ml respectively, was added to the lower well of a transwell chamber and toxin-treated or control cells were added to the upper wells, and incubated for 3 h at 37°C with 5% CO_2. The cells in the lower chamber were then counted using a hemocytometer. EdTx decreased basal migration by 53%, BCA-1-mediated migration by 63% and MIP-3β-mediated migration 53%, while LeTx had no effect on migration **(Figure 3.20)**.

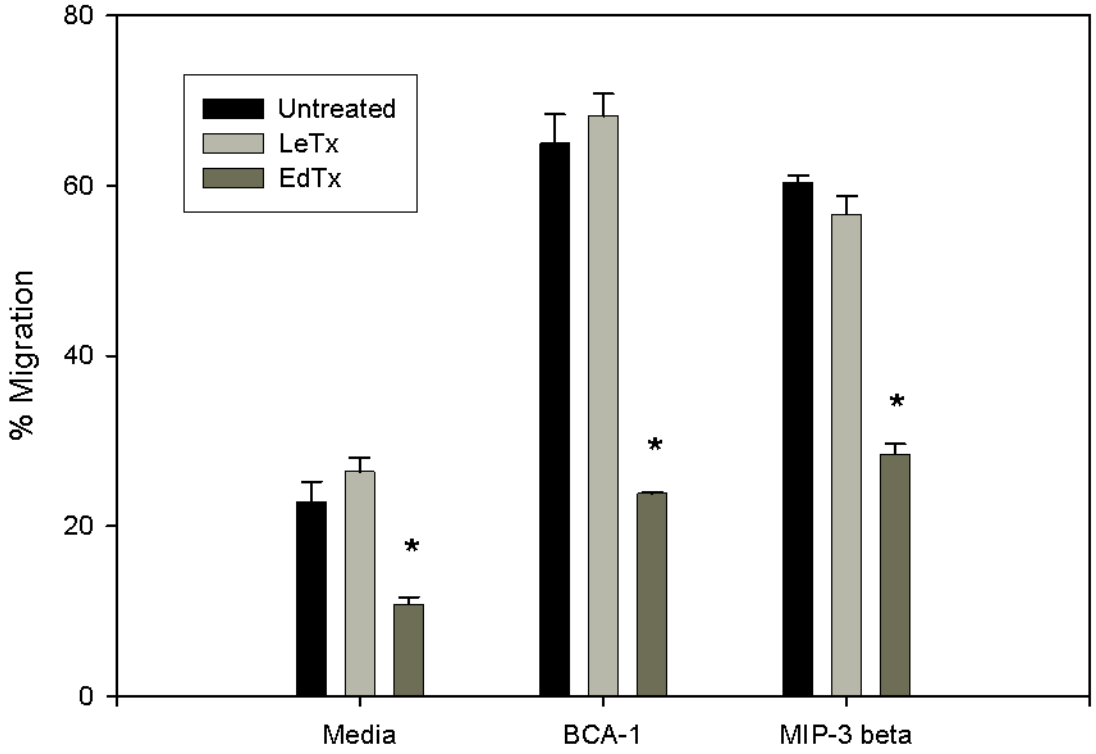

Figure 3.20 Migration of EdTx-treated naïve murine B lymphocytes. Naïve Balb/c B lymphocytes were incubated with EdTx (2.5 ug/ml PA, 1.0 ug/ml EF) for 4 h at 37°C and 5% CO_2. Cells were added to the upper wells of 5 μm pore transwell migration chambers. The lower wells contained media alone or media with BCA-1 and MIP-3β. Cells were incubated for 3 h and migrated cells were counted using trypan blue and a hemocytometer. Values are the % of cells added to the upper wells that migrated to the lower wells. The bars represent the mean of two values ± standard deviation. The data are from one experiment and are representative of three independent experiments. Asterisks denote a statistically significant difference between untreated and EdTx-treated B lymphocytes ($p<0.05$ by the Student's t-test).

To determine whether this impaired migration was due to a change in chemokine receptor expression, treated cells were stained for CCR7 and CXCR5, the receptors for MIP-3β and BCA-1 respectively. Despite the EdTx-induced decrease in migration towards both chemokines, EdTx-treated cells had an increase in CCR7 expression **(Figure 3.21, 3.22, 3.23, 3.24),** but no effect on CXCR5 expression **(Figure 3.25, 3.26, 3.27, 3.28)**.

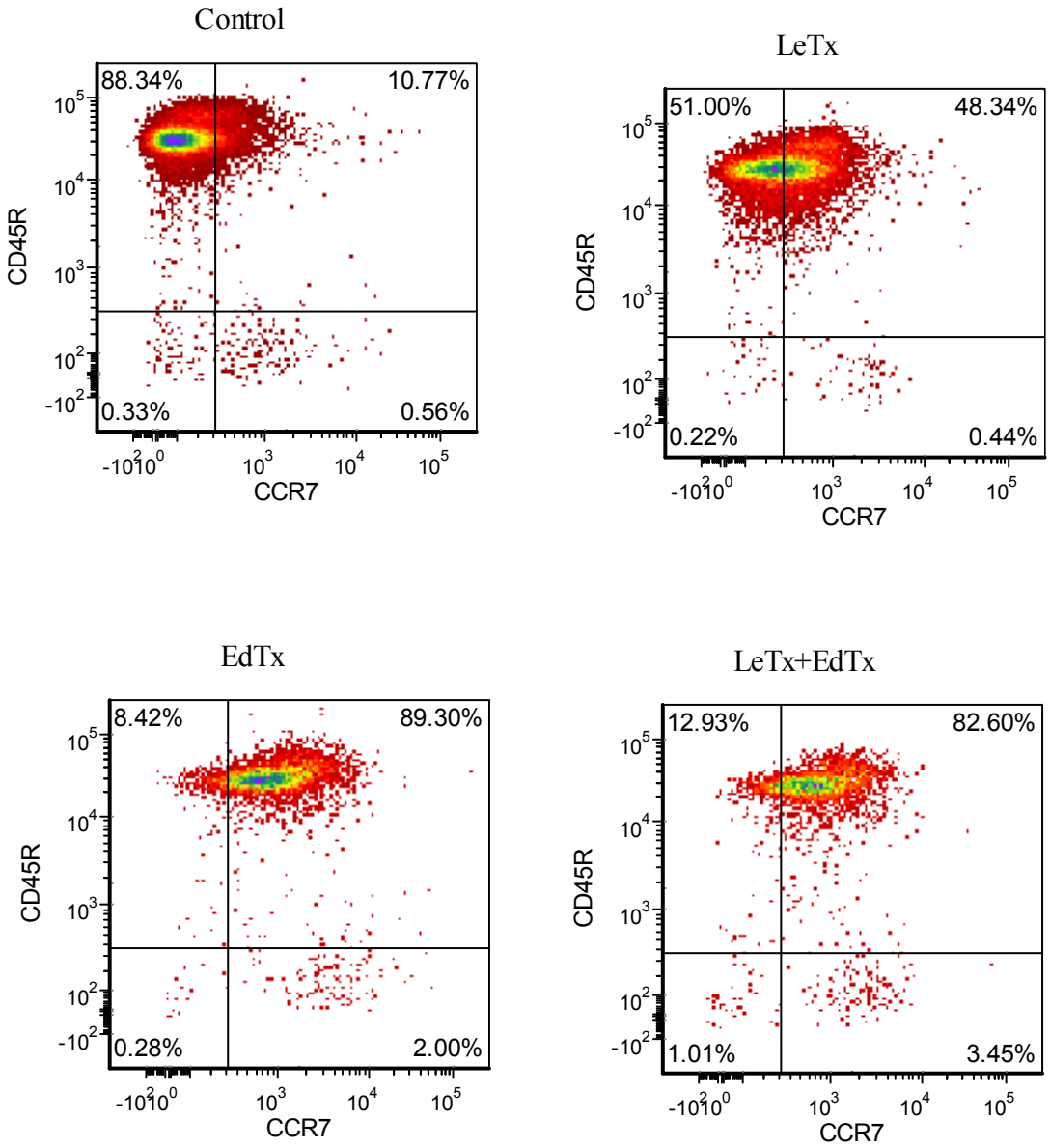

Figure 3.21 CCR7 expression on toxin-treated naïve B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF or EF, and incubated at 37°C with 5% CO_2 for 24h. CCR7 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

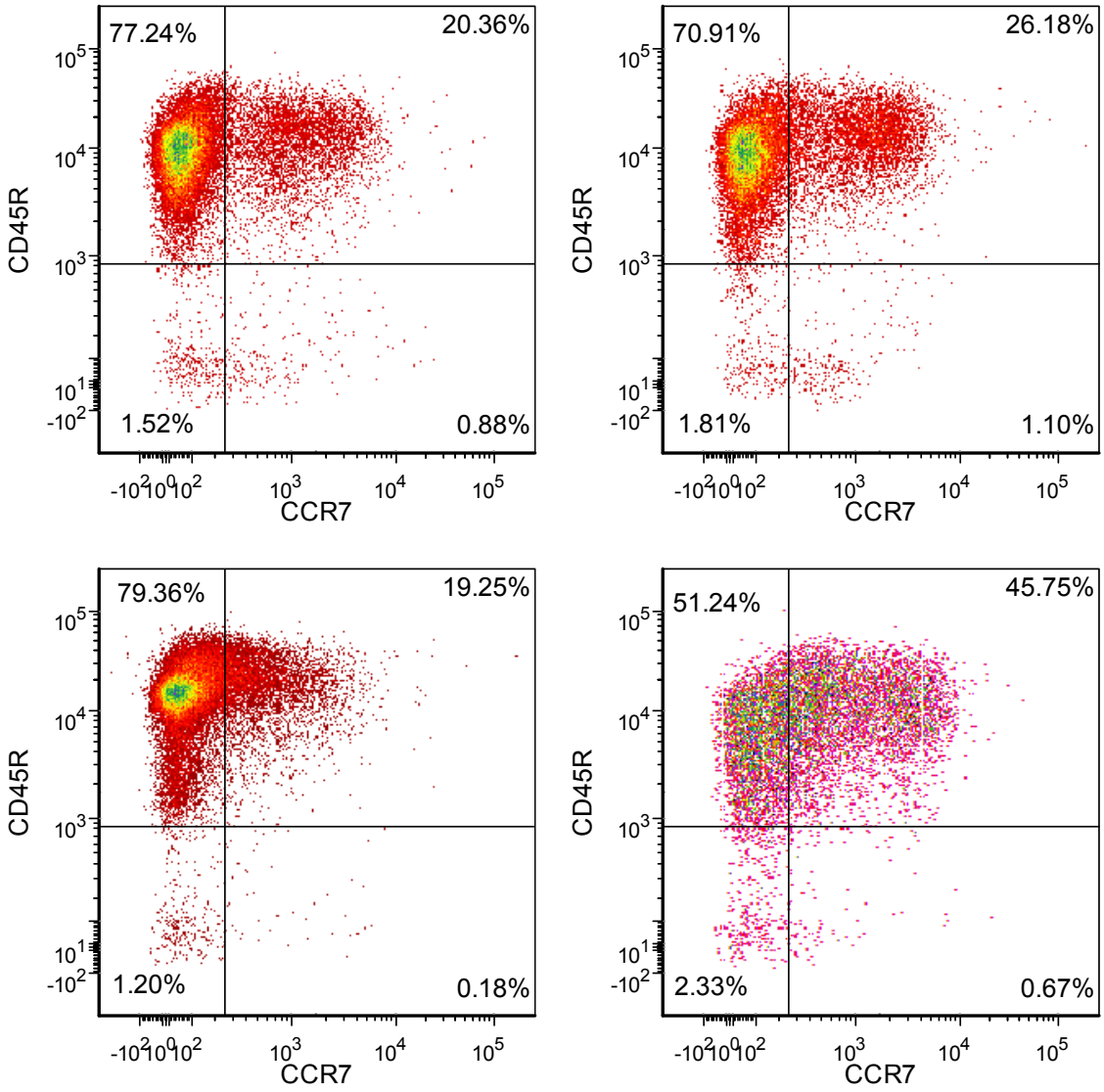

Figure 3.22 CCR7 expression on anthrax toxin-treated and anti-CD40/IL-4-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5 µg/ml anti-CD40 and 5 ng/ml IL4. After 24h, cells were stained with fluorochrome-labeled anti-CCR7 and CCR7 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

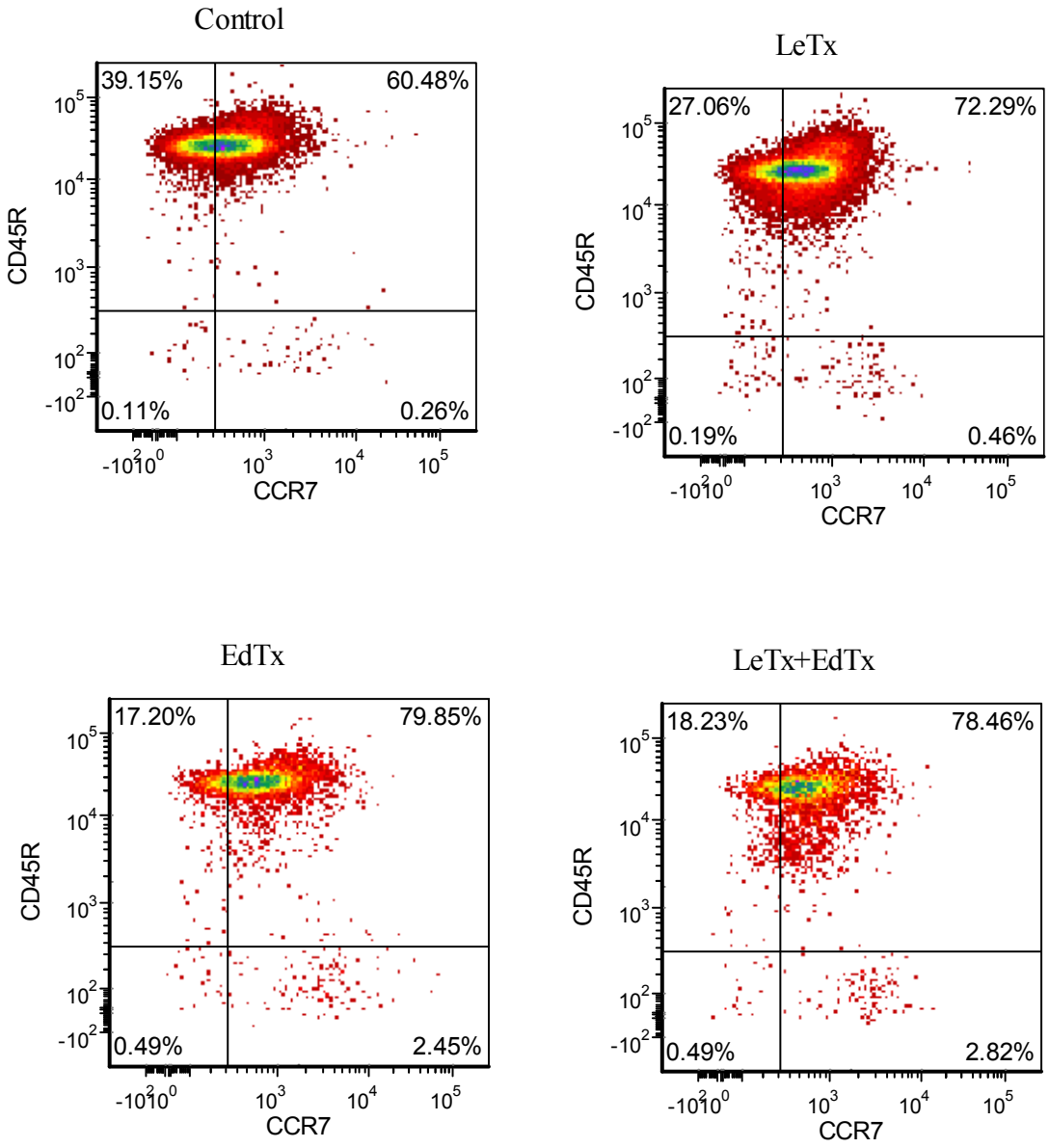

Figure 3.23 CCR7 expression on anthrax toxin-treated, anti-IgM-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5µg/ml anti-IgM. After 24 h, cells were stained with fluorochrome-labeled anti-CCR7, and CCR7 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

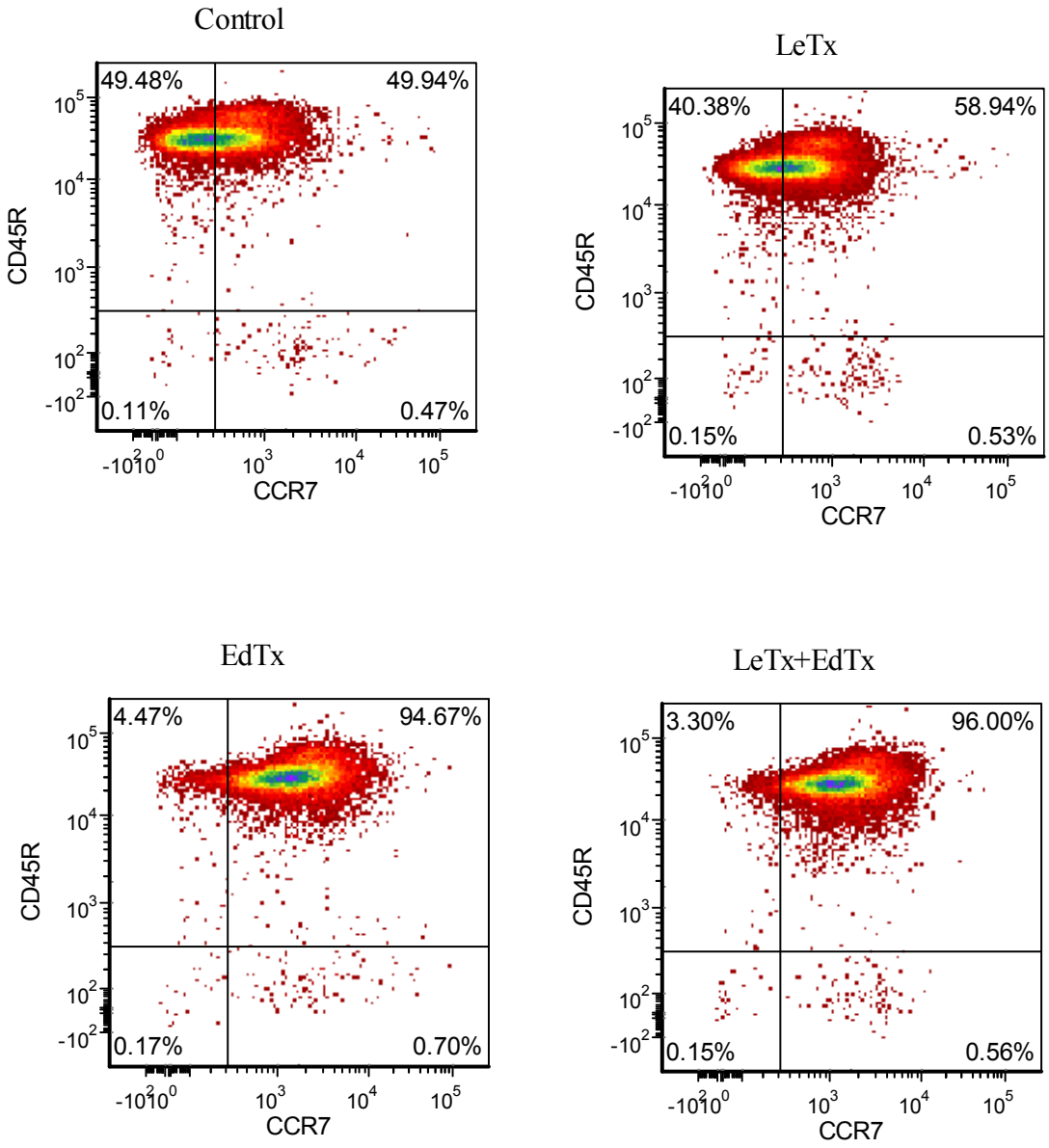

Figure 3.24 CCR7 expression on anthrax toxin-treated LPS stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 25 µg/ml LPS. After 24 h, cells were stained with fluorochrome labeled anti-CCR7 and CCR7 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

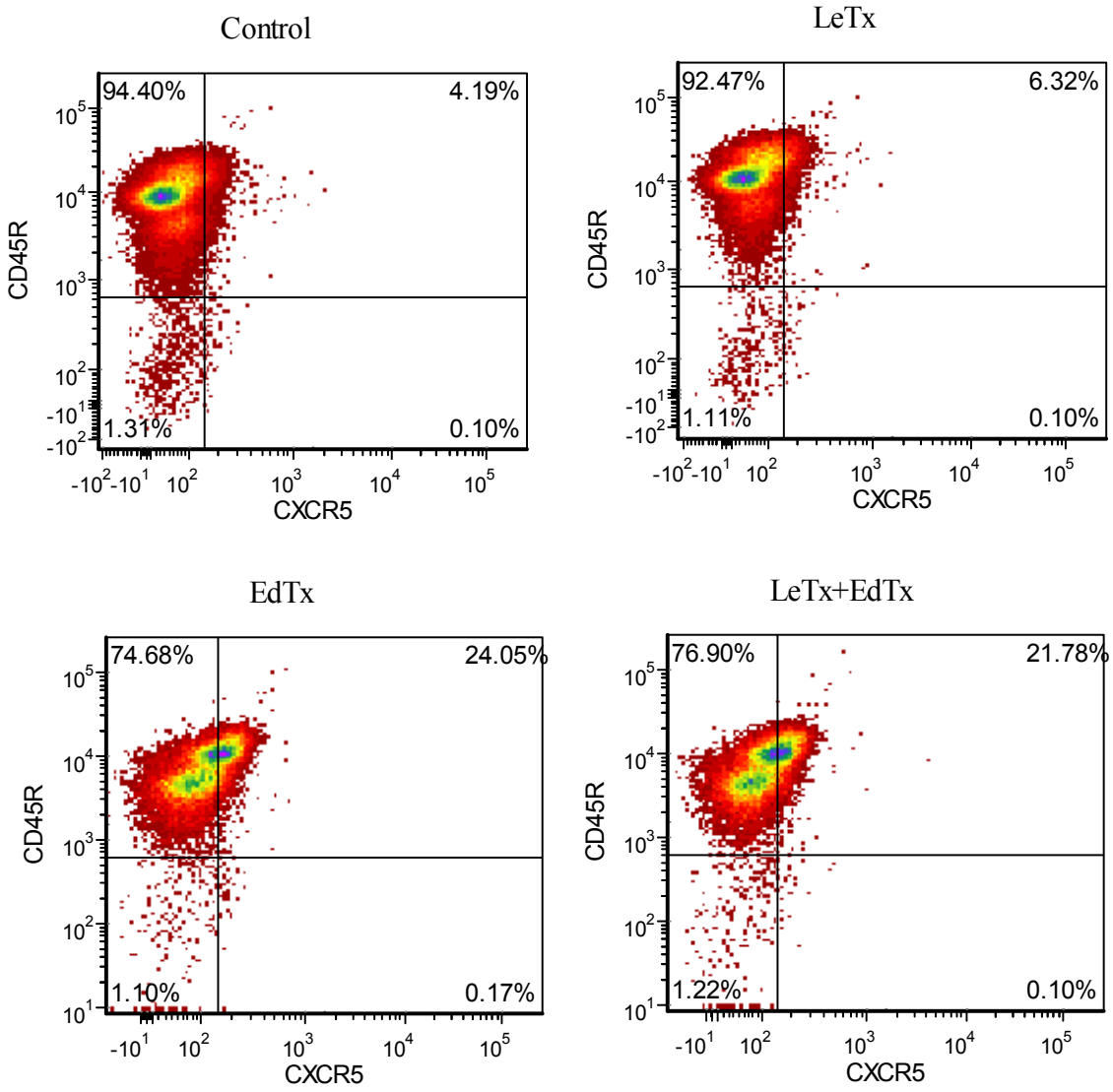

Figure 3.25 CXCR5 expression in toxin-treated naïve B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C and 5% CO_2 for 24 h. After 24 h, cells were stained with fluorochrome-labeled anti-CXCR5, and CXCR5 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

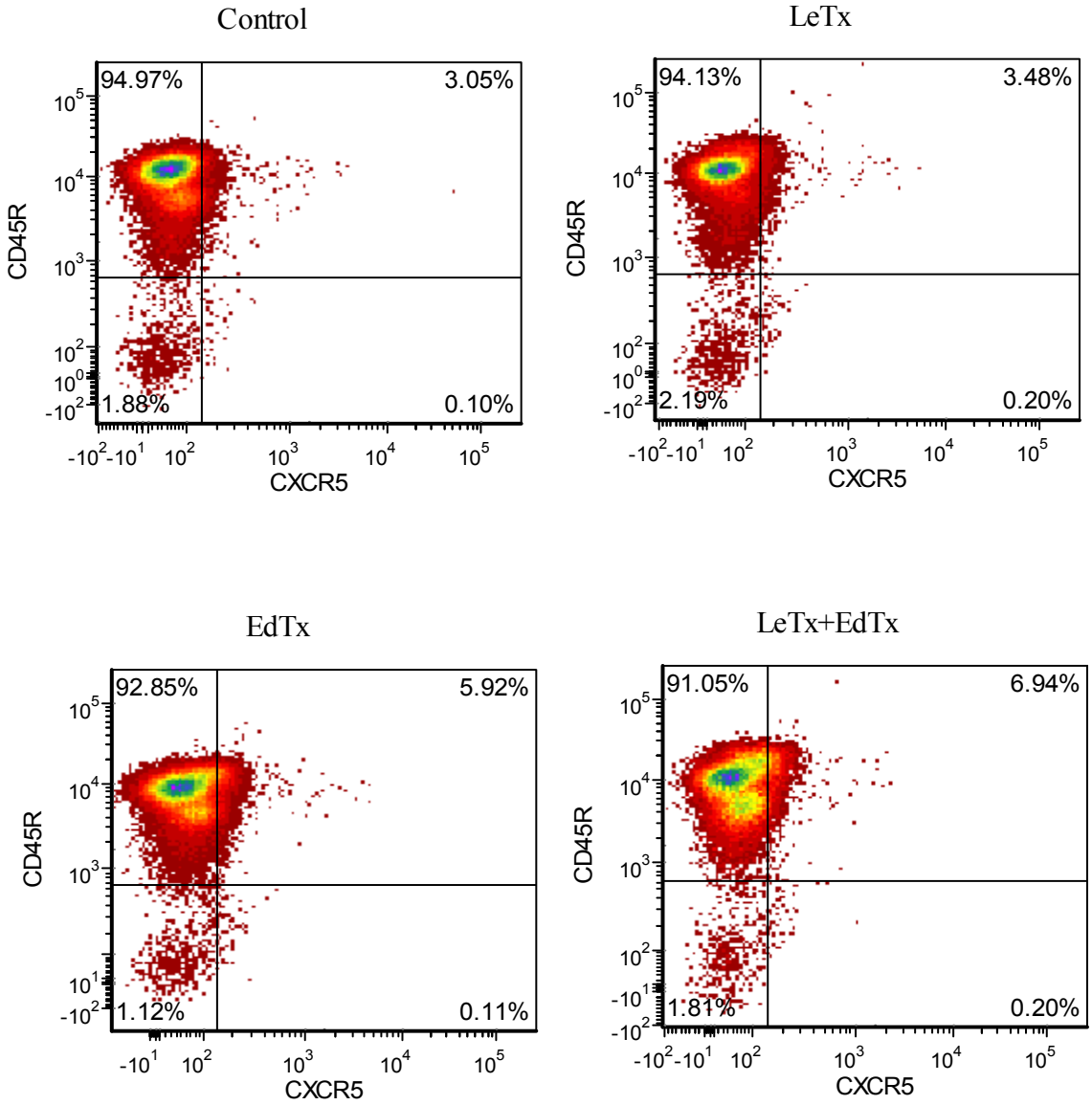

Figure 3.26 CXCR5 expression on anthrax toxin-treated anti-CD40/IL-4-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C and 5% CO_2 for 4 h. Cells were then stimulated with 5 µg/ml anti-CD40 and 5 ng/ml IL4. After 24 h, cells were stained with fluorochrome-labeled anti-CXCR5, and CXCR5 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

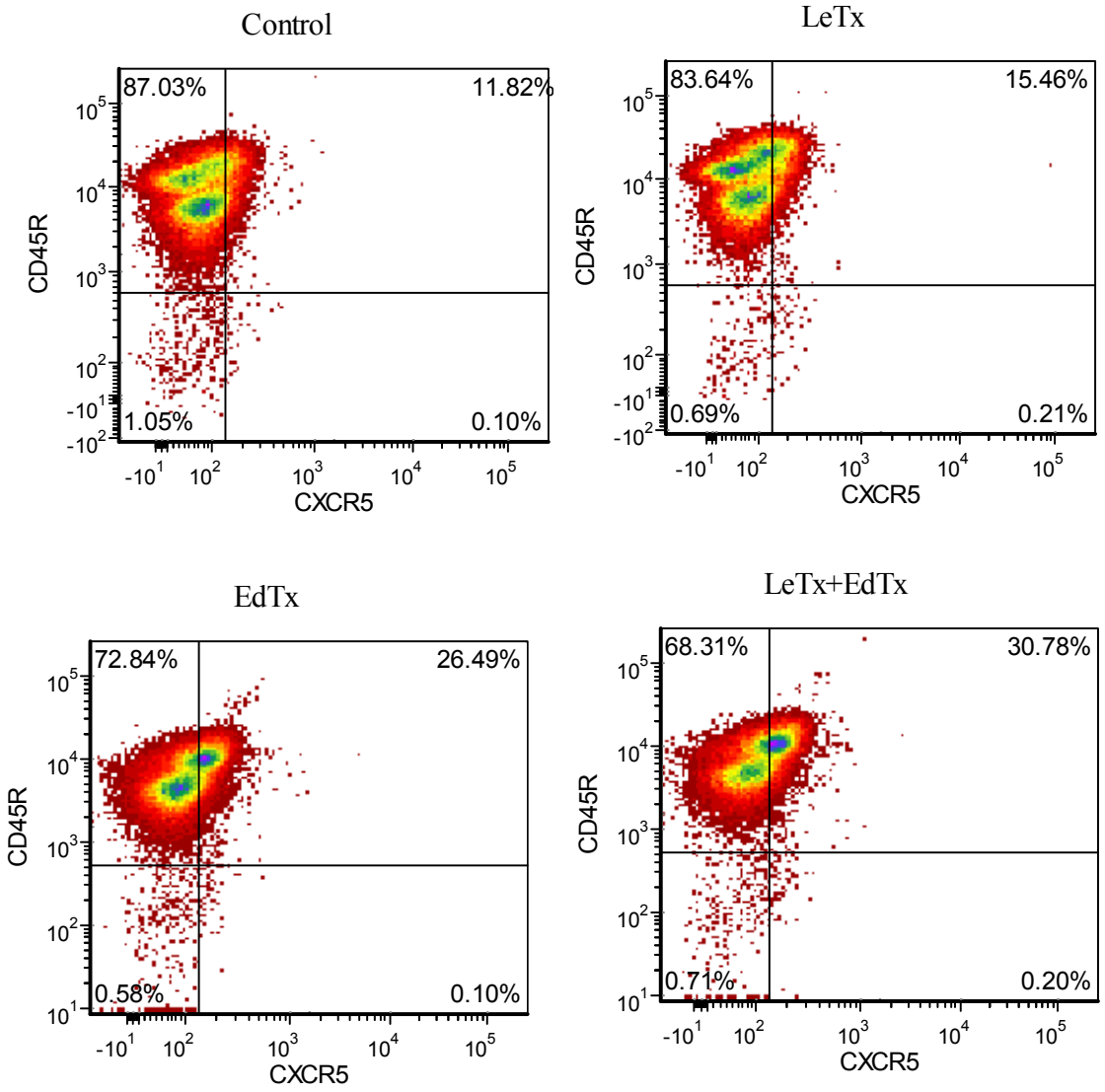

Figure 3.27 CXCR5 expression on anthrax toxin-treated anti-IgM-stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C and 5% CO_2 for 4h. Cells were then stimulated with 5µg/ml anti-IgM. After 24 h, cells were stained with fluorochrome-labeled anti-CXCR5, and CXCR5 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

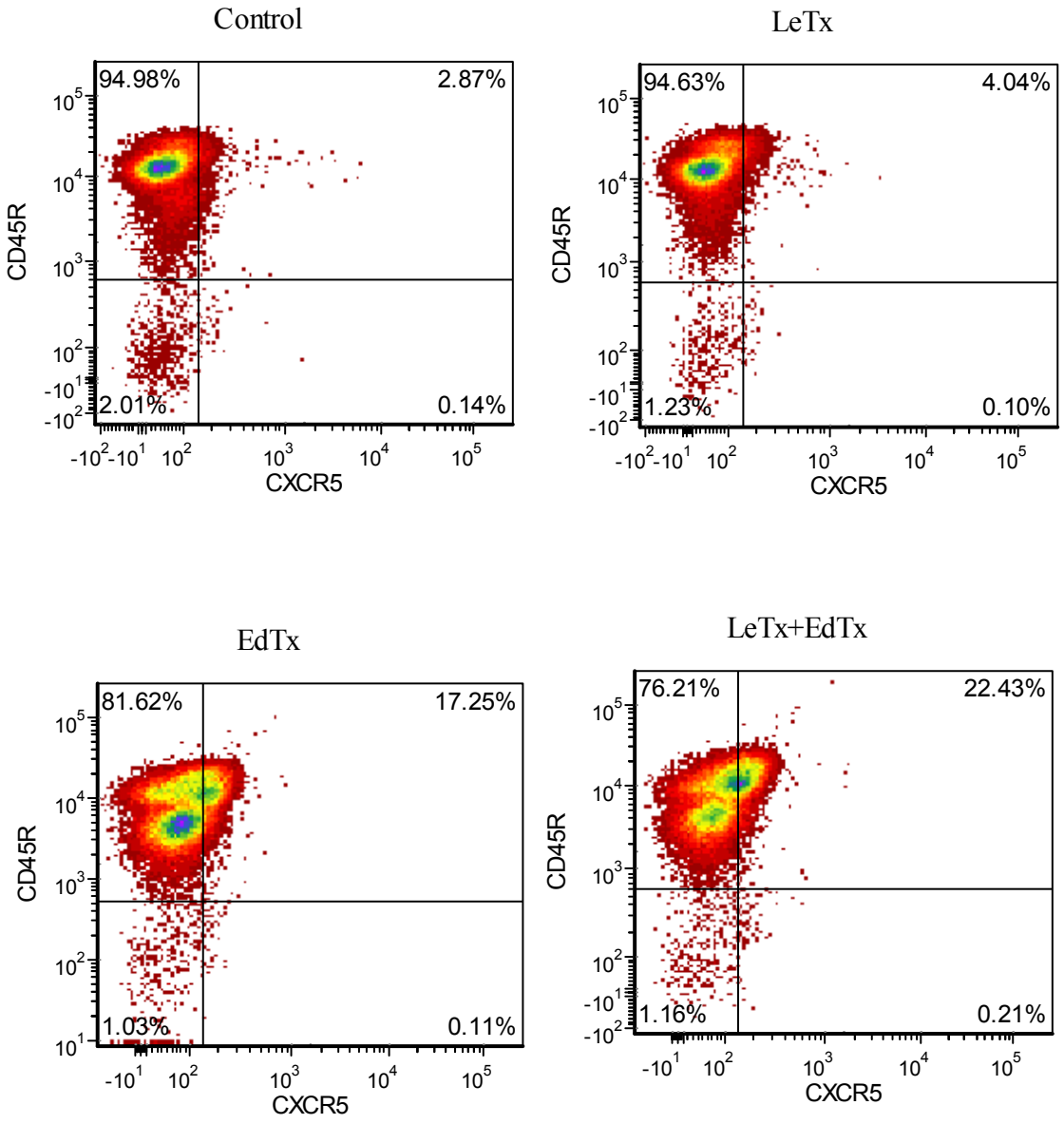

Figure 3.28 CXCR5 expression on anthrax toxin-treated LPS stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C and 5% CO_2 for 4h. Cells were then stimulated with 25 µg/ml LPS. After 24 h, cells were stained with fluorochrome-labeled anti-CXCR5 and CXCR5 expression was measured using flow cytometry. The numbers indicate % positive. The data are from one experiment and are representative of at least three independent experiments.

3.8 Anthrax lethal toxin and edema toxin impair proliferation of stimulated B lymphocytes.

To determine the effects of anthrax toxins on B cell proliferation, B lymphocytes were labeled with CFSE, treated with toxins as described, and then stimulated with 5 μg/ml anti-CD40 and 5 ng/ml IL-4 to induce proliferation. CFSE binds to cellular proteins and is divided equally between each daughter cell resulting in discernable peaks for each round of division. After three days, cells were analyzed with a FACSCanto to determine the proliferative response. At three days, 77.73% of control cells had divided at least once while only 14.82% of LeTx-treated cells had undergone division. This confirms the results previously described by Fang *et al*. using the thymidine incorporation assay. EdTx did not impair proliferation in B cells, but caused a slight increase with 80.75% of the cells undergoing at least one division. Additionally, EdTx resulted in a similar Division index, 1.57 and proliferation index, 1.95 as the untreated control B cells, 1.48 and 1.90 **(Figure 3.29)**.

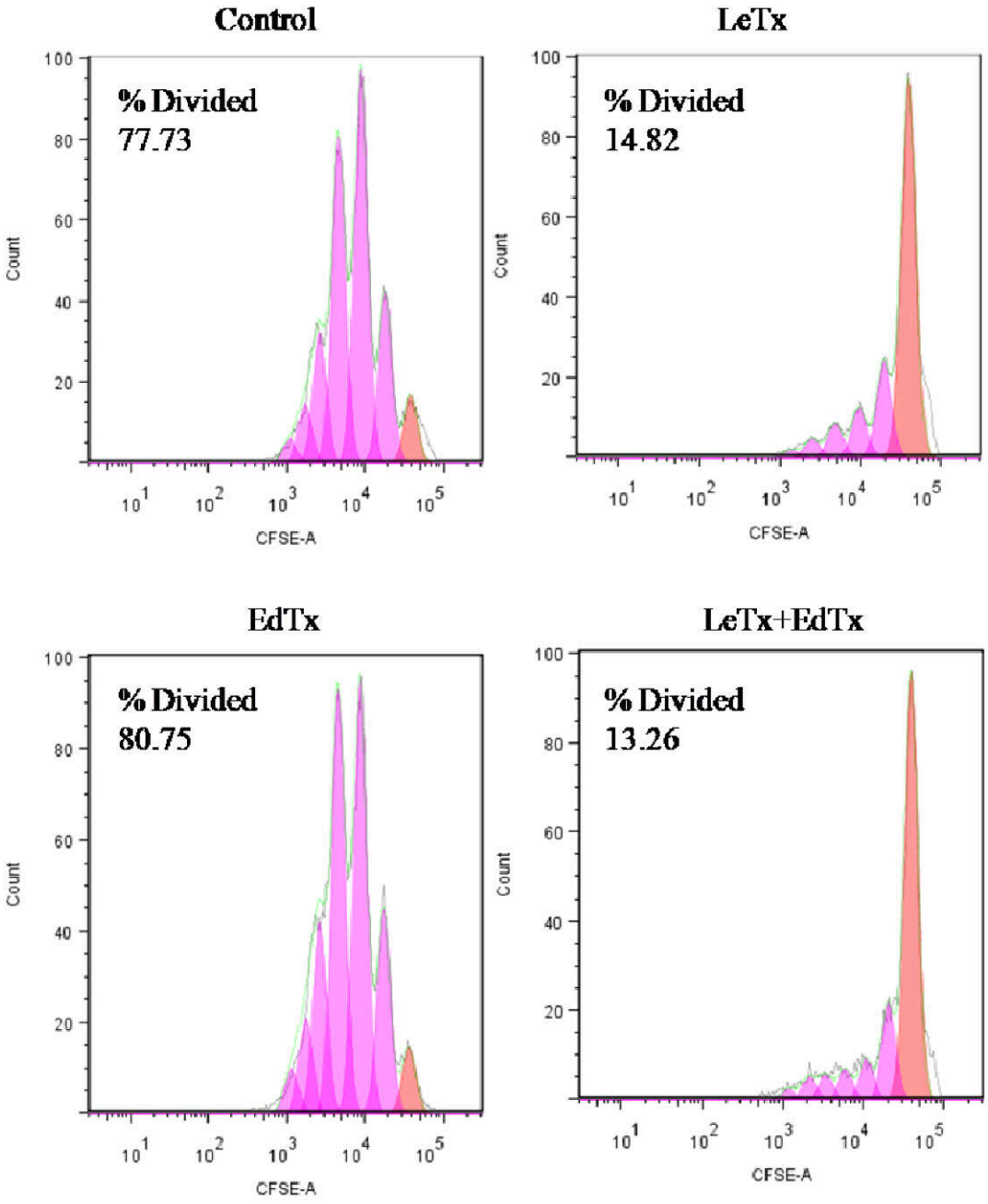

Figure 3.29 Anthrax toxins' effects on B lymphocyte proliferation. Naïve B lymphocytes were isolated from female BALB/c mice, labeled with CFSE, treated with 2.5 µg/ml of PA and 1.0 µg/ml LF, EF or EF and LF, and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5µg/ml anti-CD40 and 5ng/ml IL4. After 72 h, cell divisions were measured using flow cytometry. The numbers indicate % cells divided. The data are from one experiment and are representative of at least three independent experiments.

3.9 Anthrax edema toxin impairs cytokine secretion in stimulated B lymphocytes.

To determine the effect of anthrax toxins on chemokine and cytokine production by activated B lymphocytes, B cells were collected and treated with EdTx as described for 4 h and stimulated with 5 μg/ml anti-CD40, 5 ng/ml IL4 and 5 μg/ml anti-IgM for 24, 48, or 72 h. MIP-1α and MIP-1β in EdTx-treated, activated B lymphocytes was significantly decreased, compared to untreated controls at both 48 and 72 h ($p<0.005$) **(Fig 3.30),** but not at 24 h (data not shown). MIP-1α and MIP-1β in LeTx-treated, activated-B lymphocytes were significantly decreased compared to untreated controls at 72 h ($p<0.005$) **(Fig 3.30).** IL-6 production in EdTx-treated, activated B lymphocytes was significantly increased over untreated B cells at 48 h and 72 h, but not at 24 h ($p<0.005$) **(Figure 3.31).** No increase in IL6 production was observed in the LeTx treatment group. IL10 and TNF-α levels were below the detection limits of the assay in all experimental groups.

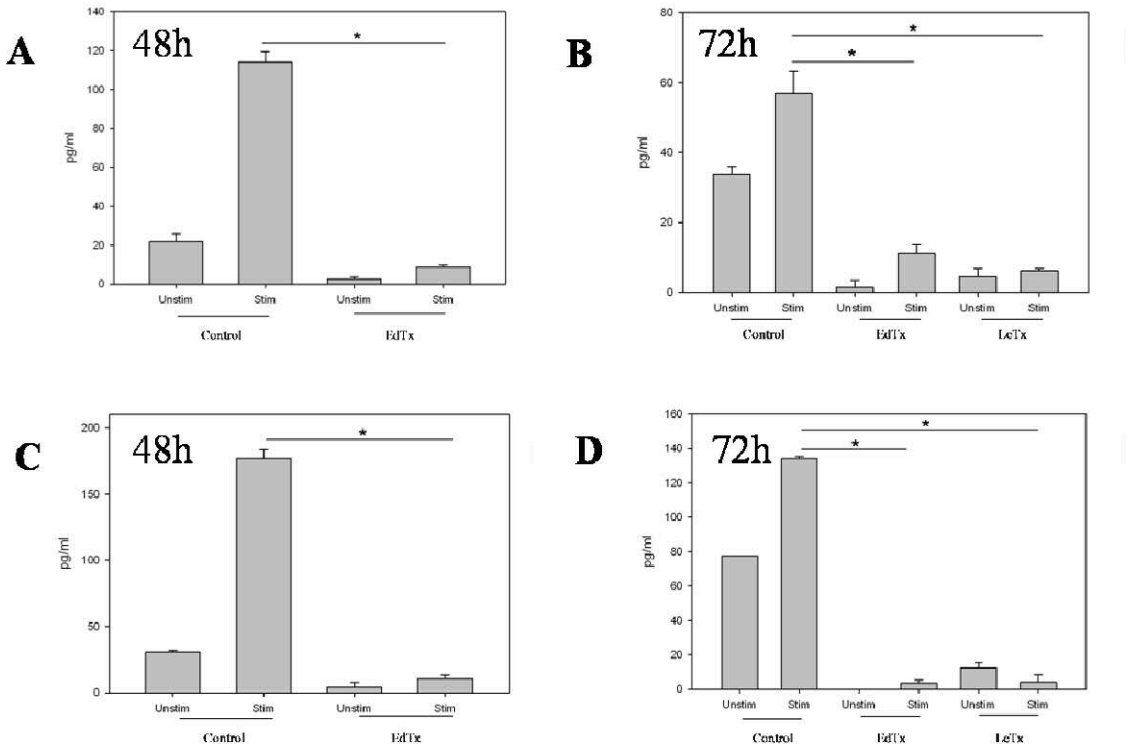

Figure 3.30 Edema toxin effect on chemokine production in stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml EF and incubated at 37°C and 5% CO_2 for 4 h. Cells were then stimulated with 5 µg/ml anti-CD40 and 5 ng/ml IL4 and 5 µg/ml anti-IgM. After 48 h, supernatants were collected and MIP-1α (A and B) and MIP-1β (C and D) measured. The data are expressed as means ± standard deviation of triplicate values and are representative of two independent experiments. Asterisks denote a statistically significant difference between untreated and edema toxin-treated cells ($p<0.05$ by the Student's t-test).

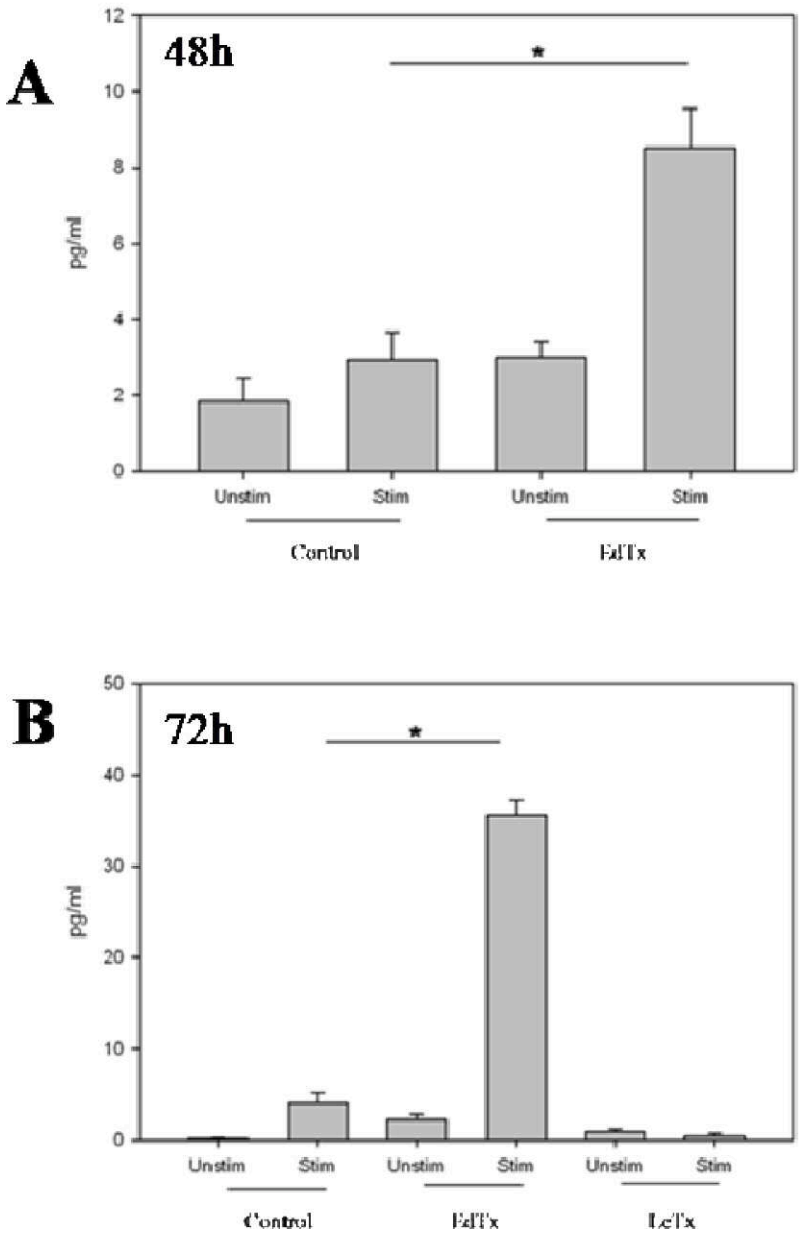

Figure 3.31 IL6 production in edema toxin-treated stimulated B lymphocytes. Naïve B lymphocytes were isolated from female BALB/c mice and treated with 2.5 µg/ml of PA and 1.0 µg/ml EF and incubated at 37°C with 5% CO_2 for 4 h. Cells were then stimulated with 5 µg/ml anti-CD40 and 5 ng/ml IL4 and 5 µg/ml anti-IgM. After 48 h, supernatants were collected and cytokines measured. The data were expressed as means ± standard deviation of triplicate values and were representative of two independent experiments. Asterisks denote a statistically significant difference between untreated and edema toxin treated cells ($p<0.05$ by the Student's t-test).

EdTx	Unstim	αCD40/IL4	αIgM	LPS
CD86	▲	▲	▲	▲
CD69	NC	NC	NC	NC
CD23	▼	NC	▼	▼
MHCII	▲	▼	▼	NC
CCR7	▲	NC	▲	▲
CXCR5	▲	NC	▲	▲

LeTx	Unstim	αCD40/IL4	αIgM	LPS
CD86	NC	NC	▼	▼
CD69	NC	▼	NC	▼
CD23	▼	▼	NC	▼
MHCII	NC	▼	▼	▼
CCR7	▲	▲	▲	▲
CXCR5	**NC**	**NC**	**NC**	**NC**

Table 3.1 Summary of surface expression of activation markers on toxin-treated B lymphocytes. Summary of surface expression results from section 3.3, 3.4, 3.5 and 3.6

	Viability	cAMP	Proliferation	IL-6	Chemokines	Migration
LeTx	N.C.	N/A	-	N.C.	-	N.C.
EdTx	-	+	N.C.	+	-	-

Table 3.2 Summary of toxin effects on B cell function

Chapter 4: Discussion

The anthrax toxins are major virulence factors in the pathogenesis of *B. anthracis* infections, enabling the bacteria to impair and evade the immune system of the host. Previous reports have shown that PA binds to the surface of B lymphocytes, and LeTx cleaves MAPKKs [61]. Fang *et al*. [61] also showed that LeTx impaired B cell proliferation but was not lethal to the cells. Numerous other indicators of B cell function were not assayed in these early studies, and the effect of EdTx on B cell function was ignored completely. To date, no reports of cytotoxicity in any cell type due to EdTx exist in the literature. In this dissertation, we confirm Fang's finding that LeTx does not kill B cells **(Figure 3.1)**. We also demonstrate for the first time that EdTx is cytotoxic to murine B lymphocytes in a dose- and time-dependent manner **(Figure 3.2, Figure 3.1)**. Additionally, the results in this report indicate that both LeTx and EdTx are capable of impairing various B cell functions and altering B cell activation markers.

These findings about the impact of the anthrax toxins on murine B cells are important for understanding the mechanism by which *B. anthracis* evades the immune system. Functional B lymphocytes are needed in order to mount an effective adaptive immune response, in addition to their role in the innate immune response.

In this study, B cell survival was measured using Annexin V and PI staining. Lymphocytes were exposed to LeTx or EdTx for 4-24 h with no change in viability, as

compared with untreated control cells. In contrast, an increase in cytotoxicity in EdTx-treated B lymphocytes was observed beginning at 8 h after treatment.

As expected, cells treated with EdTx, a bacterial adenylyl cyclase, increased intracellular cAMP levels compared to controls. This increase was detectable as early as 1 h after EdTx treatment and peaked 12 h after treatment **(Figure 3.3)**. While EdTx has not been shown to be cytotoxic in other cell types, it has been reported that increases in cAMP induce B cell apoptosis [62]. To determine if the cytotoxic effects observed here were due to apoptosis, EdTx-treated cells were stained with Annexin V and PI with $AnnV^+/PI^+$ cells becoming necrotic, $AnnV^+/PI^-$ cells being apoptotic, and $AnnV^-/PI^-$ cells remaining healthy. In these studies, we observed only a small increase in $AnnV^+/PI^-$ cells at 8 h, indicating that the cytotoxic effects of EdTx on B lymphocytes were not due to apoptosis.

A number of B cell surface markers are up-regulated when B lymphocytes are activated, including CD69, CD86, CD23 and MHCII. All of these surface markers were up-regulated by anti-CD40/IL4 and LPS, while only CD86 and MHCII were up-regulated due to anti-IgM stimulation in untreated control cells. The up-regulation of these markers is important for T cell/B cell interactions and antigen presentation.

While LeTx had no effect on these surface markers in naïve B cells, CD86, important for co-stimulation of T cells, as well as MHC II surface expression, important for antigen presentation, were increased in EdTx-treated naïve B cells **(Figure 3.4 and Figure 3.7)**. This apparent enhancement of activation markers on naïve B cells was

unexpected. However, cAMP has been shown to induce CD86 (B7-2) expression in macrophages and B7 expression was shown to be elevated in cAMP-treated B cells and is a possible explanation for the observed CD86 increase in naïve, as well as activated B cells [63]. This could also be due to a decreased susceptibility to the toxic effects of EdTx by cells with a higher initial level of CD86 expression; however, the level of CD86 expression seen in the EdTx-treated cells would seem to be too high for this to be the case. Additionally, CD86 expression was elevated in all EdTx treatment groups regardless of the mode of activation **(Figure 3.4, Figure 3.8, Figure 3.12, and Figure 3.16).** Despite the down regulation of this indicator of B cell activation, we found CD86 to be up-regulated in all EdTx-treated B cells.

MHCII is required for antigen presentation to T cells during B cell-T cell interactions. It has been demonstrated previously that cAMP analogs enhance CD40-mediated B cell activation, while impairing BCR-mediated activation [64]. Our results seem to be partially at odds with this observation as EdTx-treated B cells had impaired MHCII expression when stimulated by either anti-CD40/IL4 or anti-IgM **(Figure 3.11 and Figure 3.15)**; however, LeTx impaired all modes of activation-induced MHCII expression **(Figure 3.7, Figure 3.11, Figure 3.15 and Figure 3.19)**. This disturbance in MHCII expression could have dramatic effects on the formation of T cell B cell conjugates. This interaction is needed to induce the appropriate signals such as cytokines and up-regulation of CD40L from the T cell needed for B cell proliferation and differentiation into plasma cells and memory B cells [65].

CD69 is an early activation marker that has been shown to play a pivotal role in the inflammatory response. Cross-linking of CD69 has been shown to promote ERK activation [66]. EdTx had no effect on CD69 expression **(Figure 3.5, Figure 3.9, Figure 3.13, and Figure 3.17),** while LeTx impaired anti-CD40/IL4-induced CD69 expression **(Figure 3.9)**.

CD23 expression is up-regulated after CD40-receptor stimulation, enhancing T cell - B cell contact, as well as being involved B cell survival in germinal centers [67, 68]. In these studies, we demonstrated that CD23 was impaired by LeTx treatment in anti-CD40/IL-4-stimulated B lymphocytes, possibly impairing important T cell-B cell interactions and disrupting the composition of germinal centers. EdTx was shown to have no effect on anti-CD40/IL4-induced CD23 expression **(Figure 3.10)**. Both toxins resulted in a small decrease in LPS-induced CD23 expression **(Figure 3.18).**

Migration of B lymphocytes is vital for an effective humoral response following bacterial challenge. In the lymphoid organs, B lymphocytes localize to the B cell follicles due to CXCR5 surface expression and local CXCL13 expression [69]. CCR7-mediated migration is required for B cell accumulation in the lymph nodes and migration to the B cell - T cell boundary [70, 71]. Hong *et al*. [72] demonstrated that EdTx inhibited migration of endothelial cells and Paccani [73] showed impaired migration of macrophages and PBMCs that had been treated with LeTx or EdTx. In contrast to Paccani's PBMC findings, we observed that LeTx did not affect B cell migration towards either CXCL13 (BCA-1) or CCL19 (MIP-3β) **(Figure 3.20)**. This may be due to

differing experimental design, as Paccani [73] used PBMCs, and the change in migration may be attributed to the non B cell fraction of this population. EdTx, however, resulted in a significant reduction in migration. This decrease in migration is observed in BCA-1- and MIP-3β-mediated migration as well as basal migration **(Figure 3.20)**. This impairment of B cell migration was independent of the presence or type of chemokine suggesting that it was not due to a decrease in chemokine receptor expression. We determined this by measuring CCR7 and CXCR5 surface expression on toxin-treated B cells. We observed that CCR7 and CXCR5 expression were not impaired in toxin-treated B lymphocytes, but were, on the contrary, enhanced by EdTx in naïve B cells, as well as anti-IgM- and LPS-stimulated B cells **(Figure 3.21, Figure 3.23, Figure 3.24, Figure 3.25, Figure 3.27, and Figure 3.28)**. Previous reports of migration impairment in the HL-60 cell line showed a decrease in F-actin content in EdTx-treated cells, and it is a possible explanation for the migration impairment observed here.

B cell proliferation is an important step in the humoral immune response allowing for the clonal expansion of antigen-specific B cells. Fang *et al*. [58, 60, 61] demonstrated that B cell proliferation was impaired by LeTx, and Paccani [58] and Comer [60] both observed that T cell proliferation is impaired by both LeTx and EdTx [58, 61]. Here, we observed the same impaired proliferation in LeTx-treated B cells observed by Fang; however, we observed that B cell proliferation was not impaired by EdTx **(Figure 3.29)**.

The progression of the B cell response is dependent on B cell - T cell interactions. Antigen-stimulated B lymphocytes have been shown to release important T cell

chemotractants, MIP-1α and MIP-1β [74]. These chemotractants play an important role in facilitating the B/T interactions that interrupt the B cell receptor-induced cell death program. Further development of high-affinity antibodies and memory B lymphocytes is dependent on the interaction of these B lymphocytes and antigen-specific T cells, as well as follicular dendritic cells. Here, we showed that MIP-1α and MIP-1β production by stimulated lymphocytes was impaired by EdTx at 48 and EdTx or LeTx at 72 h **(Figure 3.30)**. This could impair the migration of T cells to antigen-stimulated B lymphocytes resulting in the absence of a CD40/CD40L cell interaction, and subsequently, programmed cell death in these B lymphocytes without producing memory B lymphocytes and high-affinity antibodies. It has been shown that survival of Balb/c mice to *B. anthracis* challenge does not result in increased survival to subsequent challenge. This may be due to a possible impairment of memory B cell production caused by the direct disruption of B cell migration by EdTx, as well as the impaired chemokine production by both toxins, or a lack of proliferative response due to LeTx.

IL-6 production in stimulated B cells was enhanced by EdTx treatment at 48 and 72 h, but not by LeTx **(Figure 3.31)**. It has been previously demonstrated that cAMP-inducing agents, including EdTx induce IL-6 production in monocytes [75, 76]. Additionally, cAMP has been shown to increase IL-6 gene expression, as well as secretion of biologically active IL-6 [77]. We believe that this increase in IL-6 production is a result of the increase in intracellular cAMP caused by the adenylyl cyclase activity of EdTx. It has been shown that excess IL-6 during infection can impair TNFα production, an important cytokine in the early innate immune response. This

induction of IL-6 by EdTx could cause dysregulation of the early innate response allowing the bacteria a foothold.

The results presented here demonstrate that both anthrax toxins directly inhibit B lymphocyte function. LeTx was shown to impair expression of surface markers normally up-regulated by stimulation. Proliferation as well as chemokine expression was also impaired by LeTx. EdTx also impaired expression of select cell surface markers associated with activation *via* the B cell receptor and T cell interaction *via* the CD40 receptor. Additionally, EdTx impaired both B cell migration and chemokine production. EdTx also induced a significant increase in IL6 production in activated B cells. These findings support the hypothesis that anthrax toxins directly impair B cell function.

The results presented here indicate that the anthrax toxins have the capacity to disrupt the immune response in multiple ways. The receptor-mediated activation of B cells is impaired by the anthrax toxins in a number of ways, which can have far reaching effects on the innate, as well as the adaptive immune response. The inhibitory effects of LeTx and EdTx on B cell function is also of importance in the treatment of *B. anthracis* infection, as well as in vaccine development. The currently licensed vaccine requires an initial dose followed by boosters at 2 and 4 weeks, 6, 12 and 18 months followed by yearly boosters. This cumbersome vaccination schedule as well as manufacturing difficulties can result in inadequate protection if the boosters are not maintained. A study showed that only 30% of vaccinated individuals had detectable antibody levels 18-24 months after vaccination [78]. Infection with toxin producing *B. anthracis* Ames could

result in impairment of memory B cells in vaccinated individuals with waning antibody titers. This could potentially allow the bacterium to overwhelm the host. Additionally, the development of therapeutics such as antibodies designed to block toxin effects are of great interest currently. Identifying these toxin effects further establishes the importance of the development of these treatment modalities.

Chapter 5: Conclusions

In conclusion, our results indicate that both anthrax toxins directly inhibit murine B cell function. This impairment is felt across the spectrum of B cell activities when the effects of both toxins are considered. B cell migration is critical to the formation of B cell - T cell conjugates. This impairment was seen despite the up-regulation of CCR7 in EdTx- and LeTx-treated cells and CXCR5 in EdTx-treated B cells. Additionally, MIP-1α and MIP-1β were impaired by both toxins potentially affecting the migration of T cells and further compounding the effects seen on B cell migration. When considered with previous works demonstrating the impairment of migration by numerous immune cell types including neutrophils, macrophages and T cells, cell trafficking appears to be a major target of anthrax toxins [50, 73]. IL6 was up-regulated by EdTx in stimulated B cells which could contribute to an imbalance in cytokines altering the inflammatory response. While migration was not impaired in LeTx-treated B cells, we did see a near complete abrogation of proliferation indicating that should B cell - T cell interactions take place, it would likely not result in a subsequent clonal expansion or antibody production. It would seem that the combination of these toxins produced by *B. anthracis* do a remarkable job of impairing B cell functions. Where one toxin fails to have an observed effect, the other toxin seems to compensate to achieve immune suppression.

Additional research is needed to determine the mechanism of EdTx mediated B cell death. Since this is the first report of EdTx-mediated cell death known to the author,

the mechanism that would induce B cell-specific toxicity are of considerable interest. Determining whether the cytotoxic effect of EdTx extends to memory B cells and plasma cells could be important. Additional *in vitro* experiments should be performed to determine whether these toxins inhibit antibody production and class switching and to determine if these effects are seen across mammalian species. The effects of these toxins on B cells *in vivo* are of interest as well. The use of EF^- and LF^- mutants in an infection model could elucidate the impact of these toxins on B cells during *B. anthracis* infection.

While the underlying mechanism behind these effects has not been defined, the research clearly demonstrates that anthrax toxins have an inhibitory effect on murine B cells. This is in line with the overall body of literature prescribing the effects of anthrax toxins to be primarily immunoevasive. This insight is important in further elucidating the scope of immune evasion wrought by *B. anthracis* and its impact on treatment and vaccine development

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