

Research Article

Anthrax Lethal Toxin Inhibits the Production of Proinflammatory Cytokines

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In previous studies, we have found that anthrax lethal toxin (LeTx) induces apoptosis in both murine macrophages and human peripheral blood mononuclear cells (PBMCs). In this study, we further report that bacterial cell wall (CW) components of *Bacillus (B.) anthracis* are powerful inducers of proinflammatory cytokines from the PBMCs. These effects are deprived when the LeTx is present. The major causative element for this suppression is lethal factor (LF) rather than protective antigen (PA). These results indicate that the roles of LeTx in anthrax pathogenesis, particularly its effects on cytokine production, should be reevaluated as our findings and other reports are controversial to the conventional concept.

1. Introduction

Anthrax is initiated by infection with *B. anthracis*, a Gram-positive, spore forming bacterium. Infection occurs through three primary routes: cutaneous, ingestion, and inhalation of the spores. Systemic infection by inhalation of the spores has become a major research focus due to several reasons. First and foremost is the likelihood that *B. anthracis* spores would be used as a biological weapon due to its ease of growth, long shelf life, stability in the environment, and the ability to be effectively disseminated in a large area to inflict high mortality. Furthermore, the inhalation form can cause septic shock that has a mortality rate approaching 100% with death occurring within a few days after onset of symptoms if vigorous treatment is not given immediately after exposure. Lastly, to date, there is no effective treatment for the late-stage infection and it is usually fatal, even with aggressive antibiotic therapy [1]. Therefore, given these factors, in conjunction with the increased threat of a biological attack, it is imperative that the true pathogenesis of this microbe should be understood so that new successful treatment methods can be developed.

The virulence of *B. anthracis* depends on three plasmid-encoded factors: two secreted protein toxins and an antiphagocytic poly-D-glutamic acid capsule. The two toxins, collectively called anthrax toxin, are formed by the binary association of three proteins: PA (83 kDa), LF (90 kDa), and edema factor (EF, 89 kDa). PA either combines with LF to form lethal toxin (LeTx) or with EF to form edema toxin (EdTx) [2–4]. LeTx is considered to be responsible for the lysis of macrophages, which is accompanied by the release of cytokines, specifically tumor-necrosis-factor (TNF-) α and interleukin- (IL-) 1β . These cytokines are considered to be the major players responsible for septic shock, and sudden death in systemic anthrax infection [5–7]. EdTx toxin is thought to promote bacterial survival during early stages of anthrax infection since it inhibits neutrophil function *in vitro* by inhibiting phagocytosis of opsonized, avirulent *B. anthracis* spores [8]. EdTx is therefore acting synergistically with LeTx to promote the progression of the infection to perpetuate a fatal outcome.

The current view of anthrax pathogenesis suggests that LeTx is primarily responsible for the massive shock and

death associated with systemic anthrax infection. There is no doubt that these toxins are essential in anthrax infection. However, they may be less critical in the resultant sepsis, shock and death than once understood. PA is regarded as the principle protective element in natural or vaccine-induced immunity; though when administered alone, it induces a poor humoral response. Furthermore, a serum anti-PA level cannot be used to predict whether there is true protection [9]. In addition, the capability of LeTx to stimulate cytokine production is not certain [10–12]. These findings together with the observations that LeTx is highly unstable *in vivo* and lacks the capability to lyse many monocyte-macrophage cell lines suggest that other virulence factors, in addition to LeTx, may play important roles during the course of anthrax infection. The anthrax CW, which to date has not been studied extensively, could be one of those virulence factors. The concept of bacterial cell walls playing a role in the pathogenesis of bacterial infections is not new. It is well known that the lipopolysaccharide (LPS), constituent of Gram-negative bacteria cell walls, promotes sepsis and shock by inducing endogenous shock mediators [13]. Numerous proinflammatory cytokine-inducing molecules are present in Gram-positive bacteria such as staphylococcus, streptococcus, and mycobacterium [14]. The modulins found in Gram-positive organisms including Teichoic, Lipoteichoic Acids, and peptidoglycan stimulate the production of IL-1 β , TNF- α , IL-6, and granulocyte colony-stimulating factor [15, 16].

The objective of this work was to study the effects of the CW components on the production of the proinflammatory cytokines in human PBMCs and to compare the effects with those of LeTx. In addition, the interactive effects of CW components and LeTx on inducing production of the proinflammatory cytokines were evaluated. We show here that the causative agents for the cytokine production are from the bacterial CW rather than LeTx. Furthermore, the production of the proinflammatory cytokine induced by bacterial CW can be significantly inhibited in the presence of LeTx. When compared to studies on individual cell types in the literature, our results indicate that complex populations of human immune cells, such as PBMCs, may present an entirely different phenotype, and that this phenotype is more relevant to overall systemic anthrax disease. This study reveals that *B. anthracis* CW has a role in anthrax pathogenesis and may provide new clues in searching novel treatment and proper candidates for anthrax vaccine development.

2. Materials and Methods

2.1. Human Cells Preparation. Peripheral blood was collected from normal volunteer donors. The Department of Health and Human Services/NIH guidelines on protection of human subjects were followed with the authorization of the George Mason University Institutional Review Board. The blood was drawn into a 50 mL conical tube containing EDTA (1.8 mg/mL). Ten milliliter of blood was diluted with 20 mL of phosphate buffered saline (PBS) and layered on Ficoll/Lite-LymphoH (1.077, Atlanta Biologicals, Atlanta, GA, USA). The PBMCs were separated by a density centrifugation at 900 g for 30 min on Ficoll/Lite. The buffy coat was collected and

washed twice with RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals). The PBMCs were resuspended to 10⁶ cells/mL with phenol free RPMI-1640 medium.

2.2. Bacterial CW Preparation and Anthrax Toxin. An avirulent *B. anthracis* strain SNKE-FF-E308D was used for CW preparation. After growth, the CW was prepared according to Medvedev's protocol [17]. The amount of CW ranged between 30 and 35% of the dry weight. The CW preparations were checked for aerobic and anaerobic bacteria, endotoxin, and LPS contamination. Bacterial contamination was checked using various growth media. The endotoxin and LPS were tested using a Limulus amoebocyte lysate test kit (E-TOXATE, Sigma, St. Louis, MO, USA). No endotoxin and LPS were found in the preparations. PA, LF, and EF were purchased from List-Biologicals Inc. (Campbell, CA, USA). The activity of LeTx was determined by its cytolytic effect on murine macrophage cells RAW264.7. LPS was purchased from Sigma.

2.3. Activation of PBMCs with *B. anthracis* CW, LF, and PA. The cells were activated in 24-well plastic plates with different concentrations of anthrax CW and various combinations of the LF with PA for 24, 48, and 72 h. There was no visible cell lysis in LF, PA, and LeTx treated groups. The ratio of PA and LF used to form a LeTx *in vitro* is 5 : 1. Nonactivated cells were used as a negative control. The supernatants were harvested and tested for cytokine production using the ELISA assay and a flow cytometer.

2.4. Measurement of Cytokine Secretion. ELISA was used for quantitative measurement of released TNF- α , IL-1 β , IL-2, IL-6, IL-10, and INF- γ in the supernatants. ELISA kits were purchased from Endogen, Inc. (Woodburn, MA, USA). After treatment of the cells, the supernatants were collected and analyzed by using ELISA according to the protocols provided by the manufacturer. The BD-Pharmingen cytometric bead array (CBA) was used to test the cytokine panel of IFN- γ , TNF- α , IL-2, IL-4, IL-6, and IL-10 in the PBMC culture supernatants. This method uses flow cytometry to measure soluble analytes in a particle-based immunoassay as described previously [18]. Data was acquired using BD CellQuest software and analyzed using FCAP Array software provided by BD. The lower limit of detection for all cytokines in this assay was 20 pg/mL.

2.5. Statistical Analysis. All the experiments were repeated at least three times and GraphPad Prism software was used for statistical analysis. Student's *t*-test was used to compare the concentration of the tested cytokines under different conditions. A statistically significant difference was assumed at *P* < 0.05.

3. Results

3.1. Bacterial CW but Not LeTx Induces Production of Proinflammatory Cytokines. The release of the proinflammatory

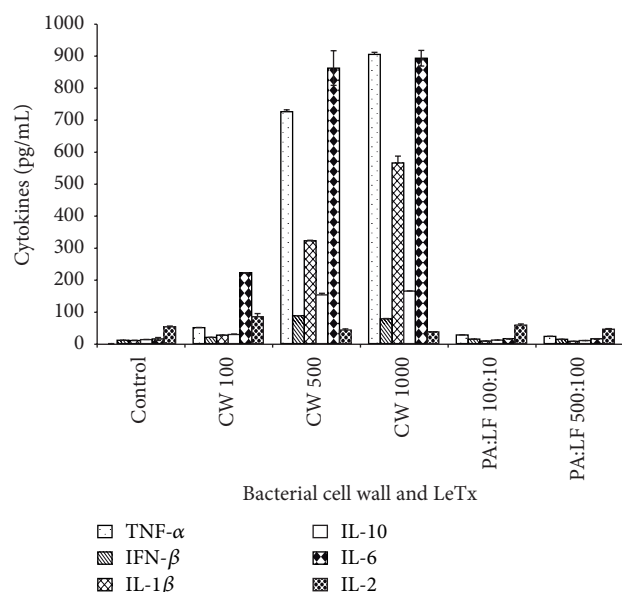


FIGURE 1: Cytokine release from human PBMCs after incubation with different amounts of *B. anthracis* cell wall preparation and LeTx. After 24 hours of incubation, the culture supernatants were collected and the cytokine concentration was measured by using ELISA. Data in the figure represents 6 donor repeats and is calculated as Mean \pm SE. Each sample was tested in triplicate.

cytokines from the human PBMCs in response to different concentrations of CW and LeTx is presented in Figure 1. PBMCs were treated with bacterial CW and LeTx at different concentrations for 24 h and the supernatants were harvested for testing the concentration of TNF- α , IFN- γ , IL-1 β , IL-10, IL-6, and IL-2 using ELISA assay. Among the tested groups, 1000 ng/mL of bacterial CW group induced the highest release of the cytokines compared to the control samples. To determine if the secretion of the cytokines from the PBMCs followed the CW dose dependent manner, up to 5000 ng/mL of the CW was tested. There were no significant changes in the cytokine production when the CW dose was increased from 1000 to 5000 ng/mL (data not shown). Since the production of IFN- γ at 24 h was relatively low, we further extended the incubation time. An increased production of IFN- γ was achieved with prolonged stimulation time (Figure 2). These results indicate that the expression of IFN- γ takes a longer time than other cytokines. Meanwhile, the PBMCs were treated with different concentrations of PA (100 ng and 500 ng/mL) and LF (20 ng and 100 ng/mL) at a ratio of 5:1. It is clear that there was no production of the cytokines following exposure to various concentrations of LeTx. In contrast, there was significant production of the proinflammatory cytokines by the PBMCs induced by the CW preparation. The highest release of the cytokines was observed in the group of 1000 ng/mL of the CW. In this group, the concentrations of TNF- α , IL-6, and IL-1 β were 900, 890, and 570 pg/mL, which were significantly higher than the control and LeTx treated

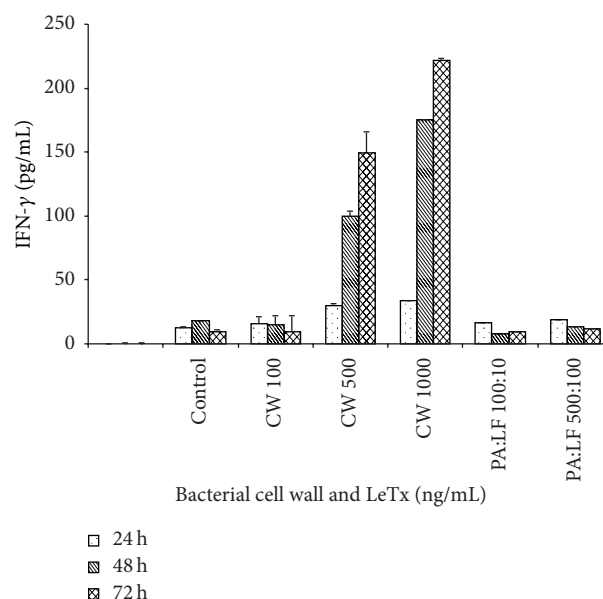


FIGURE 2: Time course of IFN- γ production from human PBMCs after incubation with different amounts of *B. anthracis* cell wall preparation and LeTx. Supernatants were harvested at 24, 48, and 72 hours, respectively, after the incubation. The amount of released IFN- γ was measured by using ELISA. Each sample was tested in triplicate. Data in the figure represent samples from 6 different donors.

groups ($P < 0.001$). The production of IL-10 (164 pg/mL) and IFN- γ (77 pg/mL) after 24 h incubation was relatively low but still significantly higher than the control group ($P < 0.001$, $P = 0.021$). The concentration of the tested cytokines in the control group was all below 30 pg/mL. No significant IL-2 release was observed in all groups as compared to control ($P = 0.42$). No difference in the viability of the cells was observed during the activation period.

3.2. Anthrax LeTx Inhibits the Production of the Proinflammatory Cytokines. To determine if there is synergic or antagonistic effect between CW and anthrax toxin in inducing cytokine production, human PBMCs were treated with CW, anthrax toxin components (PA, LF, and EF), either alone or in combination for 72 h. Human Th1/Th2 CBA kit was used to test the concentration of IL-2, IL-4, IL-6, IL-10, TNF- α , and IFN- γ in the supernatants. It was obvious that the bacterial CW could efficiently induce the release of IL-6, IL-10, TNF- α , and IFN- γ . However, the LeTx alone did not induce any production of the cytokines (Figure 3). Interestingly, the effects of the CW on the cytokine production were completely eliminated in the presence of the LeTx. We further tested the individual components of anthrax toxin. PA, EF, LF alone, and EdTx were all able to induce certain amount of the production. These results indicate that anthrax LeTx is only one strong inhibitor for the proinflammatory cytokine production in this study. The major causative element to block the cytokine release is LF. Figure 3 is a typical dot

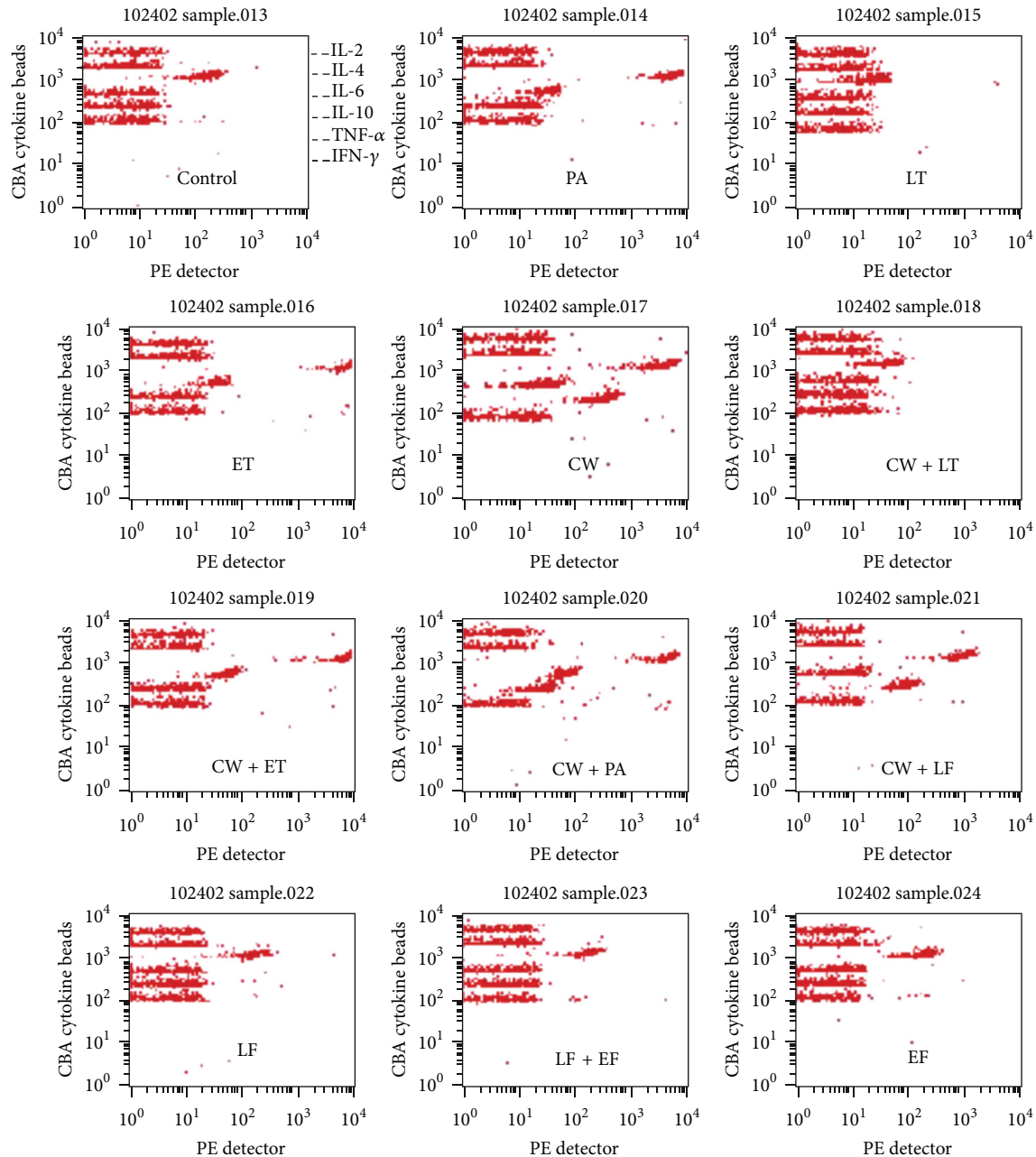


FIGURE 3: Th1/Th2 cytokine panel determination using cytometric bead array (CBA) assay. Human PBMCs were treated with *B. anthracis* cell wall preparation and various elements of anthrax toxin, either individually or in combination, for 72 h. Supernatants were harvested and assayed to determine the concentration of 6 different cytokines. The beads were conjugated with antibodies against corresponding cytokines. Secondary antibody conjugated with fluorescence dye PE was used as a detector. SSC versus FSC were used to locate the position of cytokine beads. The dots from top to bottom represent IL-2, IL-4, IL-6, IL-10, TNF- α , and IFN- γ , respectively. FL3 versus FL2 were used to determine the concentration of cytokines. With increased concentration, the dot plots move to right side. The intensity of the fluorescence is correlated with the concentration of tested cytokines. CW: cell wall, LT: lethal toxin, ET: edema toxin, LF: lethal factor, EF: edema factor, and PA: protective antigen.

plot of cytokine release from a healthy young male donor as measured by using CBA. The similar cytokine release pattern was observed from all 6 health donors. Figure 4 is the quantitative results of the cytokine production ($n = 6$) as determined by using CBA. There was no significant

production of IL-2 and IL-4 in any case. However, notable amounts of IL-6 and IL-10 were detected as compared to negative controls ($P < 0.001$). TNF- α was only observed in CW activation group. IFN- γ release was induced by CW and PA with a smaller amount compared to IL-6 and IL-10.

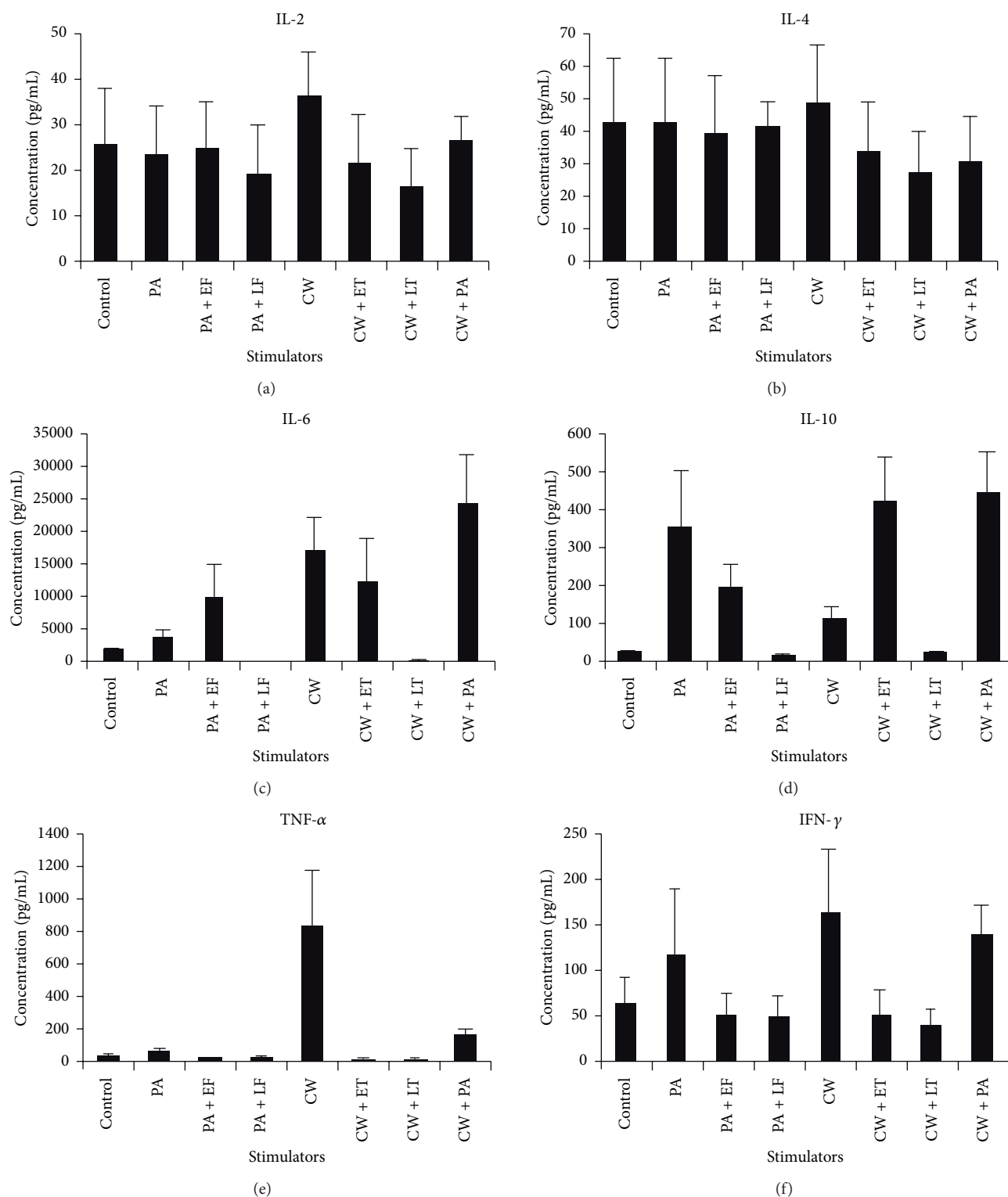


FIGURE 4: Quantitative analysis of Th1/Th2 cytokines produced by human PBMCs using BD-Pharmingen CBA kit. Human PBMCs were treated with *B. anthracis* CW and anthrax toxin components, either individually or in combination, for 72 h at 37°C. Supernatants were harvested, stained with antibody-conjugated beads, and analyzed with FACS Calibur. The concentration of each cytokine was calculated with BD CBA software. Data were derived from 6 different donors. (a): IL-2, (b): IL-4, (c): IL-6, (d): IL-10, (e): TNF- α , and (f): IFN- γ .

4. Discussion

It was believed that low levels of anthrax LeTx can induce the release of cytokines such as TNF- α and IL-1 β [1, 5, 10]. It was also presumed that LF only affects macrophages, causing a mass production of the proinflammatory cytokines, which in turn induced shock and death in the late stage of the infection. High concentration of LeTx resulted in cytolysis of macrophage [18]. This classical presumption of the pathogenesis of anthrax infection has been heavily challenged by recent studies. Several independent groups have reported that macrophages do not express the proinflammatory cytokines after exposure to anthrax LeTx [11, 12]. In this study, we presented the first evidence that anthrax LeTx inhibits the cytokine production by the human PBMCs.

The strong immune response of PBMCs to the released proinflammatory mediators such as TNF- α , IL-1 β , and IL-6 upon stimulation by the bacterial CW suggests potential mechanisms that underlie anthrax sepsis and shock. It has been hypothesized that detrimental effects observed in anthrax infections are primarily the result of a surge of TNF- α and IL-1 β released by the activated macrophages. High levels of IL-1 β and TNF- α are known to be major shock mediators and could contribute to the “dramatic death” seen in the late-stage anthrax infection. The fact that *B. anthracis* CW can activate the PBMCs to release the proinflammatory mediators indicates that the bacterial CW would play an important role in the pathogenesis of anthrax infection.

A homeostatic status of the cytokine network is critical in many different physical processes including cell growth and differentiation, development, and tissue repairing. One of the main functions of cytokines is to mediate interaction between the immune and inflammatory systems. Depending on the stage of inflammation or biological effect determined, the same cytokine might be anti-inflammatory [6]. During initial stages of infection, these inflammatory mediators at a low concentration (cytokines, chemokines, and small peptides) are essential as they enable the host to identify, target and finally eliminate pathogens. During the process of antigen presentation, the antigen processing cells (APC) secrete several mediators to stimulate T- and B-lymphocytes in order to develop specific immunity against the pathogen. CD4⁺ T lymphocytes exert their function primarily through secreted cytokines. Three distinguishable subpopulations of CD4⁺ cell have been defined. Th1 cells secrete IL-2, INF γ , TNF- α , and IL-12p35-40. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and Th3 cells express CD4/CD25 and release tumor necrosis factor- β [19, 20]. Generally, Th1 cells stimulate cellular mediated immunity and IgG2a production whereas Th2 cells favor the humoral immunity. This indicates that humoral antibodies against *B. anthracis* play an important role in controlling the spread of the pathogens. The cytokine panel used in this study revealed an initial polarization of the immune response to anthrax infection.

We have reported that LeTx can induce apoptosis in both murine macrophage and human PBMCs [21, 22]. The phagocytic function of macrophage is suppressed by LeTx.

In this study, we further show that LeTx inhibits the proinflammatory cytokine production induced by CW components. Several groups have reported that LeTx reduces the production of a number of inflammatory mediators including transcription factors, chemokines, and cytokines [23, 24]. The mechanism of the inhibition is not due to degradation of the mediators after release from the cells but rather suppression of expression of the mediators [25, 26]. Thus, we presume that immune response to the invading pathogen, particularly the function of macrophages, was overwhelmingly suppressed by LeTx during the process of the infection. This enables the *B. anthracis* organism to bypass the innate immunity by interrupting the defense mechanisms adapted in the early stage of infection. Thus, approaches to improve the function of macrophages should be considered for prophylaxis and early-stage treatment purposes in the future studies.

In summary, the following conclusions are derived from this study. First, CW rather than LeTx induces the production of the proinflammatory cytokines in anthrax infection. These results further confirm previous reports [11, 12]. Secondly, the unique and novel information we provide here is that LeTx inhibits the production of proinflammatory cytokine induced by CW components. The ability of LeTx to suppress cell wall induced cytokines has shed new light on functional properties of *B. anthracis* components.

Acknowledgments

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