Bacillus anthracis Capsule Activates Caspase-1 and Induces Interleukin-1β Release from Differentiated THP-1 and Human Monocyte-Derived Dendritic Cells[▽][†]

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The poly-γ-D-glutamic acid (PGA) capsule is one of the major virulence factors of *Bacillus anthracis*, which causes a highly lethal infection. The antiphagocytic PGA capsule disguises the bacilli from immune surveillance and allows unimpeded growth of bacilli in the host. Recently, efforts have been made to include PGA as a component of anthrax vaccine; however, the innate immune response of PGA itself has been poorly investigated. In this study, we characterized the innate immune response elicited by PGA in the human monocytic cell line THP-1, which was differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) and human monocyte-derived dendritic cells (hMoDCs). PGA capsules were isolated from the culture supernatant of either the pXO1-cured strain of *B. anthracis* H9401 or *B. licheniformis* ATCC 9945a. PGA treatment of differentiated THP-1 cells and hMoDCs led to the specific extracellular release of interleukin-1β (IL-1β) in a dose-dependent manner. Evaluation of IL-1β processing by Western blotting revealed that cleaved IL-1β increased in THP-1 cells and hMoDCs after PGA treatment. Enhanced processing of IL-1β directly correlated with increased activation of its upstream regulator, caspase-1, also known as IL-1β-converting enzyme (ICE). The extracellular release of IL-1β in response to PGA was ICE dependent, since the administration of an ICE inhibitor prior to PGA treatment blocked induction of IL-1β. These results demonstrate that *B. anthracis* PGA elicits IL-1β production through activation of ICE in PMA-differentiated THP-1 cells and hMoDCs, suggesting the potential for PGA as a therapeutic target for anthrax.

Anthrax is a highly lethal infectious disease caused by the spore-forming bacterium *Bacillus anthracis* (24). After entering the host, anthrax spores are rapidly exposed and phagocytosed by antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), and carried to regional lymph nodes (25, 27). Normally, at this stage, APCs act as the first line of defense against microbial pathogens by engulfing and killing infectious agents. In addition to macrophages, immature DCs have then reported to capture anthrax spores by phagocytosis and migrate to lymphoid organs, and the lymph nodes, where they liaise with and activate antigen-specific T cells (2). Macrophages and immature DCs both produce numerous modulators of the inflammatory response to recruit and activate additional cells of the immune system, which link to the adaptive immune response (5). During this process, however, anthrax spores are able to survive, germinate into vegetative bacilli, multiply, and escape the control of the innate immune system (25, 27). The vegetative form of *B. anthracis* then penetrates into the circulatory system by disrupting these cells, secretes high levels of exotoxin, and spreads systemically, reaching 10^8 organisms per ml of blood (20, 25, 27, 39). Because APCs are

used to bypass the host immune system in *B. anthracis* infection, it is important to define the interactions between APCs and spore as well as the virulence factors of *B. anthracis*.

The major virulence factor of *B. anthracis*, exotoxin, is composed of three distinct proteins—protective antigen (PA), edema factor (EF), and lethal factor (LF)—which are secreted separately as nontoxic monomers (24). The binding of LF or EF to PA results in the formation of active lethal toxin (LT) or edema toxin, respectively (24). LF is a metalloprotease that cleaves most isoforms of mitogen-activated protein kinase kinase (MAPKK) (15), and EF is a calmodulin-dependent adenylate cyclase that causes a prolonged increase of cytosolic cyclic AMP (22, 31). *B. anthracis* contains another virulence factor, the capsule, which is composed of poly-γ-D-glutamic acid (PGA) (24). The weakly immunogenic and antiphagocytic PGA capsule disguises the bacilli from immune surveillance in a similar manner to that of the capsular polysaccharides that protect pathogens such as pneumococci and meningococci (12, 23). Like other T-cell-independent polysaccharide antigens, the immunogenicity of PGA is enhanced when it is conjugated with other proteins such as PA (4, 35, 38). Recently, oral administration of high-molecular-weight PGA to C57BL/6 mice revealed natural killer (NK) cell-mediated antitumor activity and gamma interferon (IFN-γ) secretion (18), which indicates a role for PGA in the immune response. However, the immunogenic effect of PGA on APCs, including downstream effectors such as cytokines, remains to be elucidated.

In the present study, we have characterized the innate im-

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mune response to PGA in the human monocytic cell line THP-1, which was differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) and ex vivo using human monocyte-derived dendritic cells (hMoDCs). PGA capsules were isolated from the culture supernatant of either the pXO1-cured strain of *B. anthracis* H9401 (37) or *B. licheniformis* ATCC 9945a (35). PGA treatment of differentiated THP-1 cells and hMoDCs induced significant amount of the proinflammatory cytokine IL-1β in a dose-dependent manner through the activation of caspase-1, also known as IL-1β-converting enzyme (ICE). The production of other proinflammatory cytokines such as interleukin-4 (IL-4), IL-6, and IL-18 was not affected by PGA treatment. These results indicate that PGA, as well as exotoxin, involves the regulation of the innate immune response by macrophages and DCs. The present study is the first to examine the immunological effects of *B. anthracis* PGA both in vitro using human macrophage cell line and ex vivo with hMoDCs.

MATERIALS AND METHODS

Cell lines and culture conditions. THP-1, a human monocytic cell line, was obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in RPMI 1640 medium (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% penicillin-streptomycin (BioSource International, Camarillo, CA) at 37°C in a humidified incubator containing 5% CO_2. Cellular differentiation into the macrophage-like phenotype was induced by the addition of PMA (Sigma-Aldrich, St. Louis, MO) at a final concentration of 100 nM. After incubation for 72 h, PMA-containing medium was aspirated, and adherent cells were resuspended in fresh culture medium and incubated for an additional 24 h.

Generation and characterization of hMoDCs from peripheral blood mononuclear cells (PBMC). According to Korea National Institute of Health Guidelines, PBMC were prepared by density centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden) from buffy coats (Korean Red Cross Blood Service). CD14+ cells from PBMC were separated by using MACS magnetic cell sorting (Miltenyi Biotec, Inc., Sunnyvale, CA). Purity of separated monocytes was >90% as determined by flow cytometry. Monocytes were cultured for 6 days in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin, 20 ng of human IL-4 (BD Biosciences, San Jose, CA)/ml, and 100 ng of human granulocyte-macrophage colony-stimulating factor (BD Biosciences)/ml. Differentiated hMoDCs were washed with phosphate-buffered saline (PBS) and stained with fluorochrome-conjugated antibodies on ice for 30 min to analyze the immunophenotype of DCs. The anti-mouse antibodies used were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-CD11c, FITC-conjugated anti-CD14, FITC-conjugated anti-CD40, FITC-conjugated anti-CD80, FITC-conjugated anti-CD83, FITC-conjugated anti-CD86, and FITC-conjugated anti-HLA-DR. Mouse FITC-conjugated anti-immunoglobulin G2a (IgG2a) was used as isotypic control. After three washes in PBS, the stained cells were fixed with 1% paraformaldehyde in PBS until subjected to flow cytometric analysis using a FC500 flow cytometer (Beckman Coulter, Krefeld, Germany). (See also the supplemental material.)

Purification of PGA. *B. licheniformis* ATCC 9945a was obtained from the ATCC and grown in E broth medium (35). PGA was purified from the cell culture supernatant as described previously (35). In brief, the highly viscous bacterial culture was centrifuged at 4°C (6,500 × g, 20 min) to remove bacteria. The supernatant was collected and precipitated with 3 volumes of ethanol at 4°C overnight. PGA precipitate was collected by centrifugation and dialyzed against deionized water. PGA solution was acidified to pH 1.5 with 6 M HCl and immediately precipitated with 3 volumes of 1-propanol at −20°C. PGA was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified PGA was then dissolved in water, dialyzed extensively, and lyophilized. Because of the large size of PGA from *B. licheniformis* (>500 kDa), direct addition of isolated PGA to THP-1 cell or hMoDC cultures would result in an increase in viscosity, which might be undesirable for determining immune responses. Therefore, PGA was degraded to molecular sizes ranging from 15 to 30 kDa (30-kDa PGA) by using acid hydrolysis. Fragmented PGA was verified by ^{1}H nuclear magnetic resonance spectroscopy to be 99% pure (data not shown).

B. anthracis H9401 is a clinical isolate from a Korean cutaneous anthrax patient (37) and grown in brain heart infusion medium (Difco Laboratories, Detroit, MI). *B. anthracis* H9401 is fully virulent and possesses both pXO1 and pXO2 plasmids. For the purification of PGA, pXO1 of *B. anthracis* H9401 was cured as described previously (14). The PGA from pXO1 cured strain of *B. anthracis* H9401 was purified from the cell culture supernatant by using the same method for *B. licheniformis* ATCC 9945a.

Reagents. The ICE inhibitor Z-WEHD-FMK (R&D Systems, Minneapolis, MN) was reconstituted in dimethyl sulfoxide to make a stock solution of 20 mM. ICE inhibitor was applied 30 min prior to PGA application. Trypan blue staining showed that Z-WEHD-FMK had no effect on THP cell and hMoDC viability (data not shown). Antibodies for ICE, IL-1β, and β-actin were obtained from Cell Signaling Technology (Danvers, MA). Peroxidase-conjugated goat anti-rabbit IgG antibodies were obtained from Jackson Immunoresearch (West Grove, PA).

ELISA. IL-1β cytokine levels were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). Culture supernatants from differentiated THP-1 cells and hMoDCs stimulated with PGA were harvested, and absorbance readings were generated by using a Techan absorbance microplate reader (Phoenix Research Products, Hayward, CA). Each culture was assayed in triplicate and averaged. ELISA results are expressed in pg/ml.

Western blotting. To produce cell lysates, the cell pellet was resuspended in 100 μl of 1× lysis buffer (Sigma-Aldrich) supplemented with protease inhibitor mixture (Sigma-Aldrich), followed by incubation on ice for 30 min. The cell lysates were clarified by microcentrifugation, and 50 μg of each clarified lysate was loaded on a 10% NuPage gradient gel (Invitrogen). The lysates were electrophoretically separated and then transferred to 0.2-μm nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). Blots were blocked in TBS-T (100 mM Tris-HCl [pH 7.5]; 0.9% NaCl; 0.1% Tween 20) containing 5% skim milk for 1 h. Blots were rinsed three times in TBS containing 0.05% Tween 20 for 5 min and subsequently incubated with the diluted primary antibodies according to the manufacturer's instructions: rabbit polyclonal anti-caspase-1 (1:1,000), anti-cleaved IL-1β (1:1,000), and anti-β-actin IgG (1:1,000). Horseradish peroxidase-conjugated polyclonal anti-rabbit IgG (1:5,000; Jackson Immunoresearch) was used as the secondary antibody. After three washes with TBS-T, the blots were developed with enhanced chemiluminescence substrate (Pierce, Rockford, IL) and exposed to X-ray film (Kodak XAR5; Eastman Kodak, Rochester, NY).

RESULTS

PGA induces specifically IL-1β production in differentiated THP-1 cells and hMoDCs. First, we investigated the effects of PGA isolated from culture supernatants of either the pXO1-cured strain of *B. anthracis* or *B. licheniformis* ATCC 9945a on cytokine production in PMA-differentiated THP-1 cells. Differentiated THP-1 cells were treated for either 24 or 48 h with isolated PGA at the concentration of 100 μg/ml, and then supernatants were collected and analyzed for production of a panel of proinflammatory cytokines, IL-4, IL-6, IL-18, and IL-1β. PGA did not increase extracellular IL-4, IL-6, and IL-18 levels produced by differentiated THP-1 cells (data not shown). The IL-1β level was, however, increased by treatment with PGA from both sources. The treatment of THP-1 cells with various concentrations of PGA (0, 10, 20, 40, 80, and 100 μg/ml) for either 24 or 48 h was tested, and this moment increased production of IL-1β in a dose-dependent manner (Fig. 1). The extracellular IL-1β level from THP-1 cells treated with PGA from *B. anthracis* was an average of 4.5-fold greater than the baseline level at the concentration of 100 μg/ml after 48 h treatment (Fig. 1A). Similarly, the extracellular IL-1β level from THP-1 cells treated with PGA from *B. licheniformis* 9945a was an average of 4.2-fold greater than the baseline level (Fig. 1B).

To test ex vivo relevance of the IL-1β production by PGA treatment in DCs, we studied the effect of PGA on IL-1β production in hMoDCs. Similarly, the treatment of hMoDCs with various concentrations (0, 10, 20, 40, 80, and 100 μg/ml) of
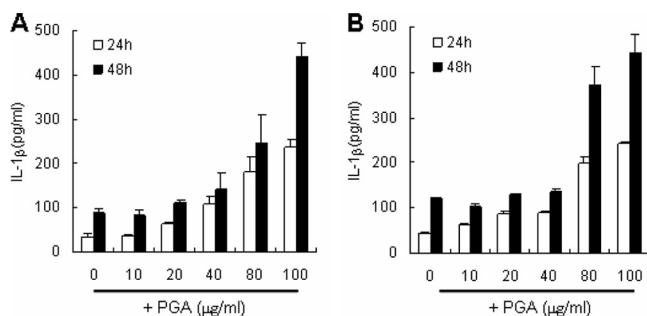
FIG. 1. Dose-dependent cytokine induction in THP-1 cells treated with PGA. Differentiated THP-1 cells were treated with various concentrations (0, 10, 20, 40, 80, and 100 μg/ml) of PGA from the pXO1-cured strain of *B. anthracis* H9401 (A) or from *B. licheniformis* ATCC 9945a (B). Extracellular levels of IL-1β were measured in triplicate by ELISA after 24 or 48 h of treatment. Each bar represents the average concentration from triplicate ELISAs. Bars represent the interassay standard deviations.

FIG. 2. Dose-dependent cytokine induction in hMoDCs treated with PGA. hMoDCs were treated with various concentrations (0, 10, 20, 40, 80, and 100 μg/ml) of PGA from the pXO1-cured strain of *B. anthracis* H9401 (A) or from *B. licheniformis* ATCC 9945a (B). Extracellular levels of IL-1β were measured in triplicate by ELISA after 24 or 48 h of treatment. Each bar represents the average concentration from triplicate ELISAs. Bars represent the interassay standard deviations.

PGA for either 24 or 48 h also increased the production of IL-1β in a dose-dependent manner (Fig. 2). The extracellular IL-1β level from hMoDCs treated with PGA from *B. anthracis* was an average of 10.5-fold greater than the baseline level at the concentration of 100 μg/ml after 48 h of treatment (Fig. 2A). Similarly, the extracellular IL-1β level from hMoDCs treated with PGA from *B. licheniformis* 9945a was an average of 12.5-fold greater than the baseline level (Fig. 2B).

PGA increases the processing of IL-1β through the activation of ICE in differentiated THP-1 cells and hMoDCs. IL-1β is an potent mediator of inflammation that is responsible for various effects associated with host responses to microbial invasion and tissue damage (9, 10). IL-1β is expressed as an inactive form (33 kDa), pro-IL-1β, which is processed by the cytosolic cysteine protease ICE to generate its active form (17 kDa) (11, 13). The production of extracellular IL-1β is regulated at several levels, including posttranslational cleavage of its proform and extracellular release of its active form (36). To further investigate the effect of PGA on IL-1β regulation, the effect on IL-1β processing by PGA was studied by Western blot analysis. A commercial antibody that detects the cleaved form of IL-1β (17 kDa) was used for Western blotting, with β-actin as a control. The level of cleaved IL-1β (17 kDa) started to increase ca. 2 to 3 h after PGA treatment in differentiated THP-1 cells (Fig. 3A and B) and hMoDCs (Fig. 4A and B). These findings indicate that PGA increases the processing of the IL-1β proform both in vitro and ex vivo.

ICE is an enzyme that proteolytically cleaves precursor forms of the proinflammatory cytokines such as IL-1β and IL-18 into active mature peptides (8). The resulting cleavage products of IL-1β and IL-18 are then available for secretion in their active forms. ICE itself also exists as a proform (p45) and, upon activation, the proform of ICE is ultimately cleaved into two bioactive forms, p20 and p10 (3, 40). Because the amount of processed IL-1β increased in response to PGA, we tested whether PGA treatment can also lead to activation of ICE. Thus, lysates from PGA-treated THP-1 cells and hMoDCs were analyzed by Western blotting. After treatment with PGA, the level of cleaved p20 subunit started to increase rapidly within ca. 30 to 45 min and decreased to the basal level after
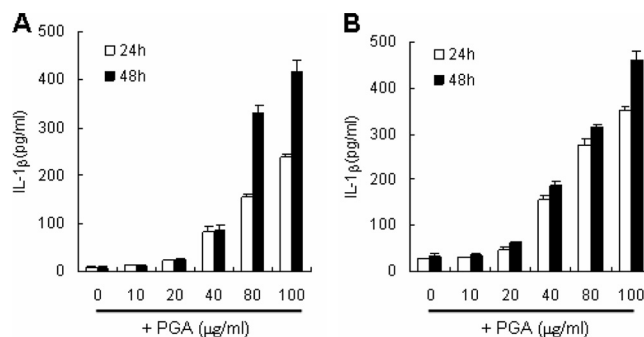
2 h. (Fig. 3C and D and Fig. 4C and D). These results show that PGA leads to the activation of ICE.

To confirm that ICE was required for secretion of IL-1β in PGA-treated THP-1 cells and hMoDCs, we used an ICE-specific inhibitor, Z-WEHD-FMK. PGA treatment alone led to induction of IL-1β in THP-1 cells and hMoDCs after 48 h of treatment, whereas the administration of Z-WEHD-FMK prior to PGA treatment blocked induction of IL-1β in a dose-dependent manner (Fig. 3E and F and Fig. 4E and F). At a dose of 100 μM, Z-WEHD-FMK blocked nearly 80% of the IL-1β induced by 100 μg of PGA/ml. Taken together, with Western blot assays, these results indicate that PGA-induced IL-1β secretion occurs via ICE in PMA-differentiated THP-1 cells and hMoDCs.

DISCUSSION

A PGA capsule is produced primarily by *Bacillus* strains but is also infrequently produced by other strains, including *Staphylococcus epidermidis* (19). In general, the biological function of the PGA capsule in *B. anthracis* has been unclear except for the role of sheltering the bacteria from phagocytosis (24). In the case of *S. epidermidis*, PGA has been reported to efficiently shelter the bacteria from key components of the innate host defense (antimicrobial peptides and neutrophil phagocytosis) and from high salt concentrations, a common feature of its natural environment, the human skin (19). Recently, oral administration of high-molecular-mass PGA (2,000 kDa) isolated from *B. subtilis* generated NK cell-mediated anti-tumor activity in mice bearing major histocompatibility complex class I-deficient tumors (18). Nanoparticles made of PGA are being developed as an adjuvant for vaccines and are capable of inducing strong cellular and humoral immune responses (41). These results suggest that PGA is able to induce an innate immunological reaction in the host, and thus it may be useful as a novel adjuvant for cancer immunotherapy as well as for vaccines for infectious diseases.

The capsule of *B. anthracis* is produced as the high-molecular-mass capsule (>100 kDa) which is first polymerized on the bacterial cell surface in vivo and then degraded to the
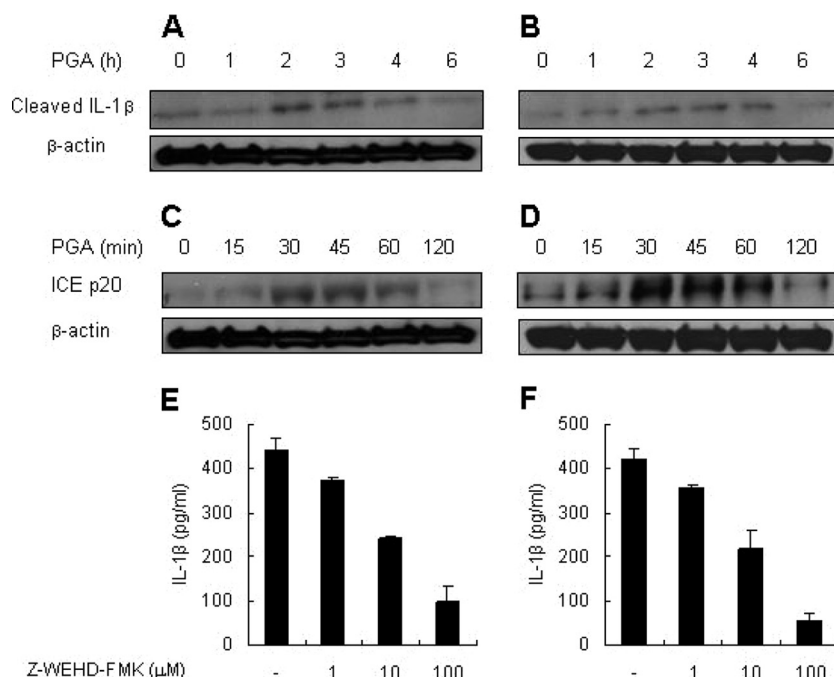
FIG. 3. Processing of IL-1β, activation of ICE, and dose-dependent inhibition of IL-1β production by an ICE inhibitor in PGA-treated THP-1 cells. Cultures of THP-1 cells were treated with a fixed dose of PGA (100 μg/ml) from the pXO1-cured strain of *B. anthracis* H9401 (A and C) or from *B. licheniformis* ATCC 9945a (B and D) for increasing periods as indicated. Protein lysates generated from these cultures were analyzed by Western blotting (50 μg of total protein per lane) for the presence of cleaved IL-1β (17 kDa) (A and B, upper panels), the activated cleavage product of ICE, p20 (C and D, upper panels), and β-actin (A, B, C, and D, lower panels). Shown are the results of one representative experiment from three separate experiments. For the inhibitor experiments, cultures of THP-1 cells were pretreated with or without increasing concentrations of Z-WEHD-FMK. After 30 min of pretreatment, cultures were treated with or without 100 μg of PGA/ml from the pXO1-cured strain of *B. anthracis* H9401 (E) or from *B. licheniformis* ATCC 9945a (F) for 48 h. Values represent the average extracellular cytokine concentrations of IL-1β measured in triplicate by ELISA. Bars indicate the interassay standard deviations.

lower-molecular-mass capsule (<14 kDa) (25). The lower-molecular-mass capsule is released from the bacterial cell surface into the culture medium and is known to act as a decoy to protect bacteria from complement (25). However, lower-molecular-mass PGA was not detected in sera of infected mice, which might be either due to a failure of depolymerization of the high-molecular-mass form in vivo or due to fast clearance from serum (20). Although the production of PGA by *B. anthracis* at each stage of anthrax infection is not thoroughly studied, a high level of serum PGA reaching up to 500 to 1,000 μg/ml was observed in the later stage of murine infection, in which actively produced virulence factors of *B. anthracis* stimulate cytokine production (20, 39). Similar levels of serum PGA were observed in rabbit and nonhuman primate models of pulmonary anthrax (39). In our experiment, we used PGA concentration up to 100 μg/ml, which might be a five times lower than the physiological concentration at the later stage of infection in vivo. Our experimental results strongly suggest that the high concentration of PGA at a later stage of infection will be sufficient to induce IL-1β production through ICE activation, which possibly contributes to the induction of septic shock in vivo.

LT of *B. anthracis* has been reported to acts as a positive regulator of ICE-dependent IL-1β secretion (6). However, LT also elicits a negative effect on the production of proinflammatory cytokines and chemokines, including IL-1β and TNF-α (1, 7, 30, 33). LT cleaves MAPKK and blocks p38 MAPK and

NF-κB activation, resulting in apoptosis of activated macrophages (16, 28, 29). LT-dependent apoptosis is also associated with the activation of various caspases, including ICE (6, 33). Selective apoptosis of activated macrophages by LT prevents secretion of cytokines and chemokines that alert the remainder of the immune system (28). Thus, anthrax infection can proceed undetected to the terminal stage. These findings indicate that LT is involved both in the induction and in the prevention of proinflammatory cytokine and chemokine secretion through ICE activation and in the apoptosis of activated macrophages (42). Similarly, the activation of ICE is subject to both the proinflammatory and the proapoptotic pathways in the anthrax infection (33). The balance among these processes might be important for the progression of the anthrax infection. Our results show that PGA also functions as an important virulence factor for anthrax infection by activating ICE. In addition, PGA might also affect MAPK or other signaling pathways and could thus potentially contribute to the expression of IL-1β and/or other cytokines. This possibility as well as the role of PGA in the apoptosis of APCs, including activated macrophages, is currently under investigation.

Because IL-1β is one of the key host modulators of septic shock (6), regulation of ICE activation by both anthrax LT and PGA at the late stage of infection can be considered as a potential target for therapeutic intervention. Actually, although anthrax spore-induced ICE-mediated IL-1β production at early stage of infection is required for host defense
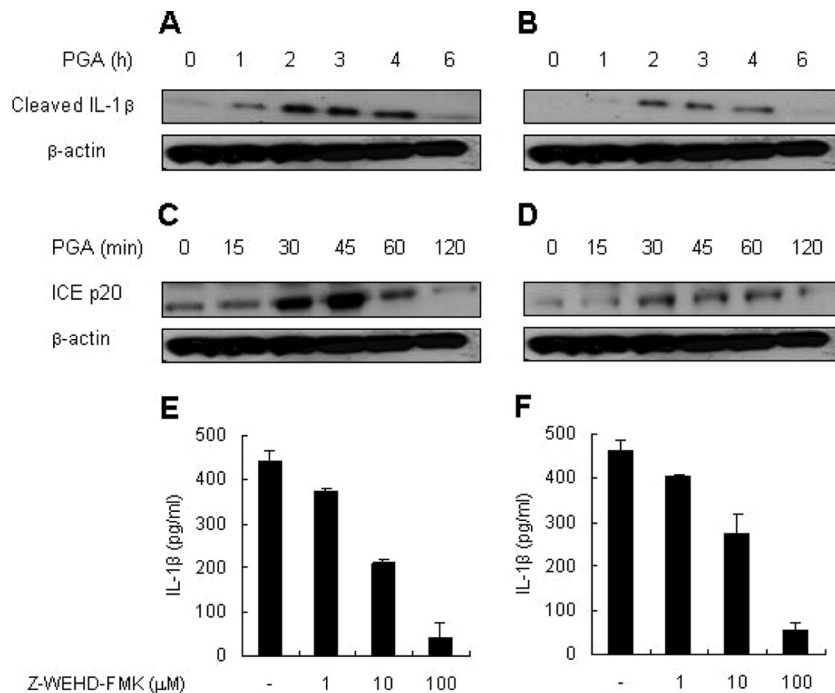
FIG. 4. Processing of IL-1β, activation of ICE, and dose-dependent inhibition of IL-1β production by an ICE inhibitor in PGA-treated hMoDCs. hMoDCs were treated with a fixed dose of PGA (100 μg/ml) from the pXO1-cured strain of *B. anthracis* H9401 (A and C) or from *B. licheniformis* ATCC 9945a (B and D) for increasing periods as indicated. Protein lysates generated from these cultures were analyzed by Western blotting (50 μg of total protein per lane) for the presence of cleaved IL-1β (17 kDa) (A and B, upper panels), the activated cleavage product of ICE, p20 (C and D, upper panels), and β-actin (A, B, C, and D, lower panels). Shown are the results of one representative experiment from three separate experiments. For the inhibitor experiments, cultures of hMoDCs were pretreated with or without increasing concentrations of Z-WEHD-FMK. After 30 min of pretreatment, cultures were treated with or without 100 μg of PGA/ml from the pXO1-cured strain of *B. anthracis* H9401 (E) or from *B. licheniformis* ATCC 9945a (F) for 48 h. Values represent the average extracellular cytokine concentrations of IL-1β measured in triplicate by ELISA. Bars indicate the interassay standard deviations.

against *B. anthracis* infection (17), LT-induced IL-1β production has been reported to account for lethality of *B. anthracis* infection (32). ICE inhibitors have already been proposed as a potential therapeutic agent for anthrax infection (33). In addition to IL-1β, IL-18 (which is also activated by ICE) is another potential target for therapy of an anthrax infection. In our study, the induction of IL-18 secretion by PGA was not, however, observed. Usually, THP-1 cells are likely to express low levels of pro-IL-18 without lipopolysaccharide priming, similar to human monocytes and peripheral blood mononuclear cells (21, 26, 34). In our experiment, it was difficult to detect the level of IL-18 induced by PGA both in differentiated THP-1 cells and in hMoDCs. The potential of an ICE inhibitor as a target for therapy requires confirmation in vivo using animal models through the examination of the host response to anthrax PGA (and/or LT) and to live anthrax spore infection in ICE$^{-/-}$ compared to wild-type mice.

Our study is the first to show that PGA produced by *B. anthracis* infection is capable of inducing IL-1β production through activation of ICE both in vitro using THP-1 cells and ex vivo with hMoDCs. Similarly, the treatment of human CD14$^+$ monocytes with PGA also increased the production of IL-1β with ICE activation (data not shown). A complete understanding of how PGA of *B. anthracis* with or without LT interacts with host against infection at each stage of infection

may lead to novel therapeutic strategies to improve the efficiency of the immune response to anthrax infection.

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