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The Poly-γ-D-Glutamic Acid Capsule of *Bacillus licheniformis*, a Surrogate of *Bacillus anthracis* Capsule Induces Interferon-Gamma Production in NK Cells through Interactions with Macrophages

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The poly-γ-D-glutamic acid (PGA) capsule, a major virulence factor of *Bacillus anthracis*, provides protection of the bacterium from phagocytosis and allows its unimpeded growth in the host. We investigated crosstalk between murine natural killer (NK) cells and macrophages stimulated with the PGA capsule of *Bacillus licheniformis*, a surrogate of the *B. anthracis* capsule. PGA induced interferon-gamma production from NK cells cultured with macrophages. This effect was dependent on macrophage-derived IL-12 and cell-cell contact interaction with macrophages through NK cell receptor NKG2D and its ligand RAE-1. The results showed that PGA could enhance NK cell activation by inducing IL-12 production in macrophages and a contact-dependent crosstalk with macrophages.

Keywords: *Bacillus anthracis*, poly-γ-D-glutamic acid, interferon gamma, NK cells, macrophages

Bacillus anthracis, the causative agent of anthrax, is a gram-positive, spore-forming bacterium, and is classified as a tier 1 select agent by the US Centers for Disease Control and Prevention [1, 2]. *B. anthracis* secretes exotoxins, edema toxin and lethal toxin, which cause edema or cell death during anthrax infection [3]. In addition to these two exotoxins, another virulence factor of *B. anthracis* is the capsule, which is composed of poly-γ-D-glutamic acid (PGA) and protects the bacterium from phagocytosis by the immune cells [4].

Natural killer (NK) cells are important immune effectors for preventing microbial invasion and dissemination. They perform a first line of defense against microbial invasion, through cytotoxicity and cytokine secretion [5]. Activation of NK cells during infection requires interactions with accessory cells, such as monocytes, macrophages, and dendritic cells. Accessory cell signals for NK cell activation include both cytokines, such as IL-12 and IL-18, and cell-to-cell physical contact, as indirect and direct signals, respectively [6].

Interferons (IFNs) secreted by NK cells play an important role in host defense against infections [7]. Type I (IFN-α and IFN-β) and Type II IFNs (IFN-γ) are prominent members of the host innate immune response to intracellular pathogens such as *Mycobacterium tuberculosis* [8] and *Francisella tularensis* [9]. In the case of anthrax, *B. anthracis* spores have been reported to efficiently drive IFN-γ production in NK cells [10]. IFNs protect mice against inhalational anthrax [11] and improve the viability of human macrophages from cell death by *B. anthracis* [12].

Accumulating studies have reported that NK cells-derived IFN-γ plays a pivotal role in protection against anthrax infection [10, 11]. However, whether PGA capsule can elicit protective immunity by inducing NK cells activation to produce IFN-γ has not been investigated yet. We used PGA from *Bacillus licheniformis* as a surrogate for *B. anthracis* PGA capsule, since *B. licheniformis* is a non-pathogenic bacterium which can be treated in a biosafety level 1 facility and it is able to produce high levels of PGA with D-enantiomer [14]. Thus, many researchers have used

B. licheniformis PGA as a surrogate for *B. anthracis* PGA including ours [13, 15–19]. In this study, we sought to investigate whether the PGA capsule of *B. licheniformis*, a surrogate of the *B. anthracis* capsule, can induce IFN-γ, and further examined underlying molecular mechanisms involved.

Experiments using animals were conducted according to the protocols (KCDC-007-13-1A) approved by the Institutional Animal Care and Use Committee of the Korea National Institute of Health. For cell preparation, 6- to 10-week-old female C57BL/6 mice were purchased from Central Lab. Animal Inc. (Korea). Splenocytes were prepared by disruption with a cell strainer (BD Biosciences, USA) and bone marrow cells were isolated from femora of mice in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, USA) and antibiotics (Invitrogen, USA). Bone marrow-derived macrophages (BMDMs) were prepared as described previously [13]. NK cells from splenocytes were purified using the NK cell isolation kit II (Miltenyi Biotec Inc., USA) according to the manufacturer's protocol. Non-NK cells were used as NK-depleted cells. The purity of NK cells was determined to be more than 90% by flow cytometry. Cell activation was performed by incubating 5×10^6 splenocytes, 5×10^6 NK-depleted splenocytes, 5×10^5 BMDMs, or 5×10^6 purified NK cells with or without 5×10^5 BMDMs in the presence of 100 μg/ml PGA in 96-well plates.

For PGA preparation, *B. licheniformis* ATCC 9945a was grown in E medium, which contained L-glutamic acid 20 g, citric acid 12 g, glycerol 80 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3 \cdot 6H_2O$ 0.04 g, $CaCl_2 \cdot 2H_2O$ 0.15 g, and $MnSO_4 \cdot H_2O$ 0.104 g per liter to maximize the production of D-glutamate capsule [14]. Usually, under these condition, *B. licheniformis* ATCC 9945a produces PGA with approximately 80–90% D-enantiomer [14]. PGA was purified from the culture supernatant as described previously [15]. The purity and structure of PGA were verified by ^1H nuclear magnetic resonance spectroscopy.

Concentrations of mouse IFN-γ and IL-12 p70 were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (BioLegend, USA) according to the manufacturer's instructions. For immunofluorescence microscopy, splenocytes were seeded onto a chamber slide (Nalge Nunc, USA) and then the cells were stimulated with 250 μg/ml PGA for 3 h. The cells were then stained with Alexa Fluor 647-conjugated anti-NK 1.1 antibody (BioLegend, USA) and FITC-conjugated anti-IFN-γ antibody (eBioscience, USA). Samples were analyzed using an FV1000 confocal microscope (Olympus, Japan). Detailed procedures for immunofluorescence microscopy were described previously [13].

For IL-12 blocking experiments, NK cells co-cultured with BMDMs or splenocytes were treated with anti-IL-12 antibody (2.5 μg/ml; BD Biosciences) or its isotype control (2.5 μg/ml; BD Biosciences). In some experiments, NK cell activation was conducted in 96-well transwell plates (8 μm pore diameter; Corning Life Sciences, USA). BMDMs (5×10^5 cells/well) were seeded into the lower chamber and NK cells (5×10^6 cells/well) were placed into the upper chamber followed by stimulation with 100 μg/ml PGA for 48 h. For receptor function blocking experiments, NK cells co-cultured with BMDMs were pretreated with anti-NKG2D antibody (5 μg/ml, BioLegend, USA), anti-RAE-1 antibody (5 μg/ml, BioLegend, USA), or their isotype control (5 μg/ml, BioLegend, USA). Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software,

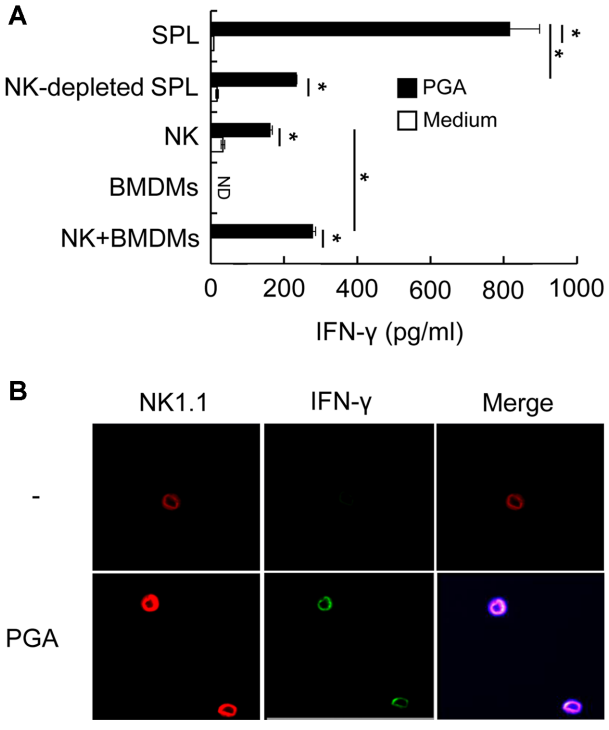

Fig. 1. Poly-γ-D-glutamic acid (PGA) induces IFN-γ production in murine NK cells.

(**A**) Splenocytes (SPL), NK-depleted SPL, bone marrow-derived macrophages (BMDMs), and purified NK cells with or without BMDMs were stimulated with 100 μg/ml PGA for 48 h. Levels of IFN-γ in culture supernatants were measured by ELISA. *, $p < 0.05$, compared with the untreated control group. ND, not detected. (**B**) SPL were left untreated (-) or treated (PGA) with 250 μg/ml PGA for 3 h and then stained with Alexa Fluor 647-conjugated anti-NK 1.1 and FITC-conjugated anti-IFN-γ antibodies. Fluorescence images were obtained by confocal microscopy.

USA). The mean value and standard deviation were obtained from triplicate samples for each treatment group. Statistical significance was examined using the *t*-test. Differences were considered significant when the *p* value was <0.05.

To examine whether PGA capsules can induce IFN-γ production in murine splenocytes, the cells were stimulated with 100 μg/ml PGA for 48 h, and then the IFN-γ concentration was measured by ELISA. As shown in Fig. 1A, PGA significantly augmented IFN-γ production compared with the level in untreated cells. Since the NK cell has been reported as the main producer of IFN-γ [20–22], we examined whether NK-depleted splenocytes retain the ability to produce IFN-γ in response to PGA stimulation. As shown in Fig. 1A, the PGA-induced IFN-γ production from splenocytes was significantly reduced in NK-depleted cells ($p = 0.009$). Because it has been known that interactions between NK cells and macrophages are important for IFN-γ production in response to bacterial infection [22, 23], we investigated whether PGA can induce IFN-γ production in BMDMs alone or in purified NK cells with or without BMDMs. PGA induced IFN-γ production in NK cells but not in BMDMs. However, the PGA-induced IFN-γ production from NK cells was significantly augmented when the cells were co-cultured with BMDMs (Fig. 1A). To further confirm whether NK cells are the main source of IFN-γ produced in PGA-stimulated splenocytes, we examined the expression of IFN-γ in response to PGA by confocal microscopy analysis. As shown in Fig. 1B, PGA induced IFN-γ expression in NK 1.1-positive NK cells only. These results indicate that NK cells are the main producer of IFN-γ in PGA-stimulated splenocytes.

It has been known that IFN-γ production by NK cells requires cytokines and contact-dependent receptor signals by accessory cells such as macrophages [6, 22–24]. In addition, macrophage-secreted IL-12 has been known to play a key role in *B. anthracis*-induced IFN-γ production by NK cells [10]. Thus, we first examined whether PGA can induce IL-12 production in splenocytes and BMDMs. As shown in Fig. 2A, PGA significantly induced IL-12 production both in splenocytes and BMDMs compared with unstimulated cells. To evaluate the role of macrophage-secreted IL-12 in regulating PGA-induced IFN-γ production by NK cells, purified NK cells with BMDMs (Fig. 2B) and splenocytes (Fig. 2C) were incubated with isotype control IgG or anti-IL-12 neutralizing antibody. The IFN-γ production by PGA was significantly reduced by addition of anti-IL-12 neutralizing antibody both in NK cells with BMDMs and splenocytes, whereas these inhibitory effects were not observed in control IgG-treated cells. Taken together, these

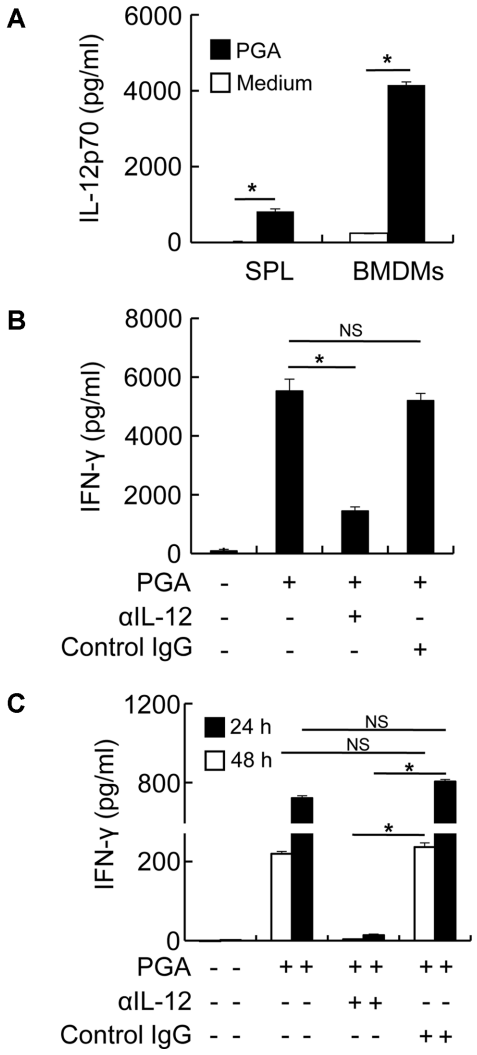

Fig. 2. IL-12 secreted by poly-γ-D-glutamic acid (PGA) stimulated macrophages is involved in NK cell activation to produce IFN-γ.

(**A**) Splenocytes (SPL) and bone marrow-derived macrophages (BMDMs) were stimulated with 100 μg/ml PGA for 48 h. IL-12 p70 concentrations were determined by ELISA. NK cells with BMDMs (**B**) or SPL (**C**) were pretreated with anti-IL-12 neutralizing antibody (2.5 μg/ml) or control IgG (2.5 μg/ml) for 1 h, followed by stimulation with 100 μg/ml PGA for an additional 48 h. IFN-γ concentrations were measured by ELISA. *, $p < 0.05$. NS, not significant.

results indicate that PGA induction of IL-12 secretion in macrophages plays a pivotal role in IFN-γ production by NK cells. Next, to investigate whether cell-cell contact between NK cells and macrophages is involved in PGA-induced IFN-γ production, we used conditioned medium from PGA-stimulated BMDMs and transwells to prevent direct cellular interactions between the NK cells and macrophages. As shown in Fig. 3A, conditioned medium

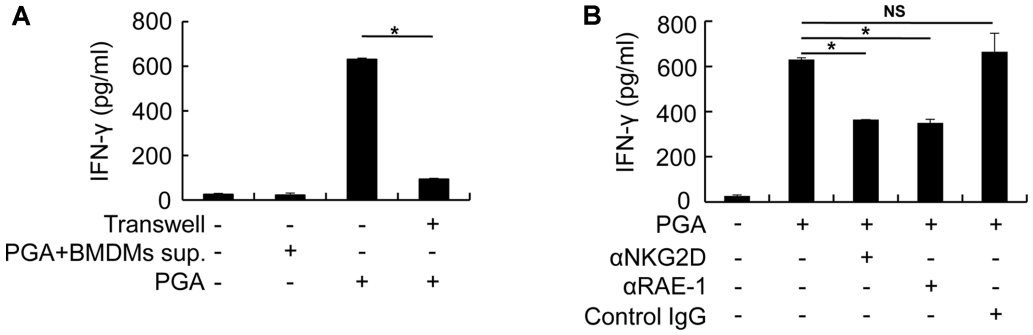

Fig. 3. NK cell activation by poly-γ-D-glutamic acid (PGA) requires cell contact-dependent co-stimulation by macrophages. (**A**) Purified NK cells with bone marrow-derived macrophages (BMDMs) were stimulated with 100 μg/ml PGA for 48 h in the different conditions as indicated. (**B**) Purified NK cells with BMDMs were pretreated with control IgG, anti-NKG2D (5 μg/ml), or anti-RAE1 (5 μg/ml) neutralizing antibodies for 1 h and then the cells were stimulated with 100 μg/ml PGA for 48 h. IFN-γ concentrations in the culture supernatants were determined by ELISA. *, $p < 0.05$. NS, not significant.

from PGA-stimulated BMDMs did not induce IFN-γ production in the NK cells, and PGA-induced IFN-γ production was almost completely impaired by preventing NK cells-macrophages contact using transwells. The NK activating receptor, NKG2D, has been known to play a key role in IFN-γ production in NK cells [25, 26]. Thus, we examined whether the NKG2D receptor is involved in IFN-γ production through the interactions between NK cells and PGA-stimulated BMDMs. Pretreatment of NKG2D neutralizing antibody partially abrogated the PGA-induced IFN-γ production by 55% ($p = 0.01$). Furthermore, neutralizing antibody for RAE-1, one of the NKG2D ligands, also decreased IFN-γ production by 57% ($p = 0.01$, Fig. 3B). These results indicate that PGA-induced activation of NK cells and following IFN-γ production are dependent on cell-to-cell contact interaction with macrophages through NK cell receptor NKG2D and its ligand RAE-1.

In the present study, we examined NK cell activation through crosstalk between NK cells and macrophages stimulated with PGA. We found that interaction between macrophages and NK cells through NKG2D-RAE-1 as well as IL-12 secreted by activated macrophages was required for PGA-induced IFN-γ production. However, it has been reported that not only soluble factors (including IL-18, IL-15, and type I IFNs) but also another NK-activating receptors (including NKp30 and NKp46) are also involved in IFN-γ production by NK cells [20, 22]. Thus, further study is needed to identify whether these cytokines or receptors are involved in PGA-induced activation of NK cells. In our experiments, we used *B. licheniformis* PGA as a surrogate for *B. anthracis* capsule to examine the IFN-γ stimulatory activity of PGA. It has been known that *B. anthracis* capsule made up of only D-glutamate was first polymerized on the surface to the high molecular weight polymer molecules (>100 kDa) and then capsule depolymerase degraded the capsule to the lower-molecular mass capsule (<50 kDa), releasing from the cell surface [27]. Thus, we maximized *B. licheniformis* to produce D-glutamate capsule using E medium and the PGA purified from *B. licheniformis* was then fragmented to the molecular mass of <50 kDa by acid hydrolysis. In addition, *B. licheniformis* PGA has been used as a surrogate of *B. anthracis* PGA for development of vaccine [16], therapeutic antibodies [17], and diagnostic assay [18]. Thus, our results using *B. licheniformis* PGA would be applicable to studies on *B. anthracis*.

It has been known that NK cells are a main producer of IFN-γ and play a significant role in host defense to bacterial infection [28]. However, a detrimental role of NK cells in bacterial immunity has also been reported. In fact, depletion of NK cells in *scid* mice reduced bacteremia and inflammatory cytokine responses in a *Streptococcus pneumoniae* infection model [29]. Depletion of NK cells led to enhanced splenic bacterial clearance in *Pseudomonas aeruginosa*-infected mice [30]. NK cell depletion improved the survival rate in *Escherichia coli*-infected mice [31]. In late-stage anthrax, *B. anthracis* has been known to reach 10^7–10^8 organisms per milliliter of blood, inducing high amount of cytokines in an experimental animal model [32, 33]. Moreover, a sepsis model has been proposed as an emerging hypothesis for the lethality of anthrax [34]. IFN-γ is associated with various inflammatory diseases such as sepsis [35]. In fact, exogenous IFN-γ increased mortality in mice with inhalation anthrax [36]. Therefore, NK cells might be involved in severe inflammatory responses to *B. anthracis* infection by overproducing IFN-γ. Further study will be required to elucidate the role of IFN-γ secretion by PGA on anthrax

pathogenesis.

To the best of our knowledge, our current study is the first to demonstrate that the PGA-induced IFN-γ production is dependent on IL-12 produced by PGA-activated macrophages and cell-cell contact interaction between NK cells and macrophages through NKG2D-RAE-1.

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