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Immunomodulatory effect of poly-γ-glutamic acid derived from *Bacillus subtilis* on natural killer dendritic cells

Sung Won Lee [a,b,1], Hyun Jung Park [a,1], Se-Ho Park [b], Nayoung Kim [c,*], Seokmann Hong [a,*]

[a] Dept. of Bioscience and Biotechnology, Institute of Bioscience, Sejong University, Seoul 143-747, Republic of Korea
[b] School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
[c] Asan Institute for Life Sciences, Asan Medical Center, Seoul 138-736, Republic of Korea

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ABSTRACT

Bacillus subtilis-derived poly-γ-glutamic acid (γPGA) stimulates dendritic cells (DCs) to produce IL12, leading to $CD4^+$ T cell differentiation toward the Th1 phenotype, but DCs consist of heterogeneous subpopulations with a variety of immune functions. Among these, natural killer dendritic cells (NKDCs) play an important role in anti-tumor immune responses. Herein, we demonstrate the role of NKDCs in γPGA-meditated anti-tumor immune responses. $NK1.1^+$ $CD11c^+$ NKDCs were stimulated upon γPGA stimulation *in vitro* and *in vivo* to up-regulate lymphocyte activation markers, MHC class I and II, and co-stimulatory molecules. In particular, NKDCs were activated by γPGA to produce IFNγ and TNFα, like NK cells, as well as IL12, like DCs, implying that NKDCs have unique and multifunctional roles. Importantly, NKDCs stimulated by γPGA conferred stronger anti-tumor effects in mice and showed increased cytotoxicity against various tumor cell lines *in vitro*. In conclusion, NKDCs are one of the key players in anti-tumor immunity induced by γPGA.

1. Introduction

Poly-γ-glutamic acid (γPGA) is produced from several strains of *Bacillus subtilis* and is applied as the starter of natto and chungkookjang, which are fermented foods made from soybeans. As an immunomodulator, γPGA has therapeutic or preventive efficacy on asthma and atopic dermatitis mediated by Th2-biased immune deviation in mice [1,2]. High-molecular-weight (2000 kDa) γPGA induces strong anti-tumor immune responses that are mediated by NK cells in Toll-like receptor 4 (TLR4)- and dendritic cell (DC)-dependent manners [3,4]. Our recent study has shown that low-molecular-weight (50 kDa) γPGA induces Th1 differentiation through antigen presenting cell (APC)-dependent mechanisms. γPGA activates DCs and macrophages to produce IL12p40 and co-stimulatory molecules [5]. $CD11c^+$ splenic DCs and bone marrow-derived DCs express IL12p40, CD40, and CD86 in response to γPGA stimulation. In addition, γPGA activates $NK1.1^+$ cells to elicit anti-tumor activities mediated by IFNγ, suggesting that NK cells are the main cell population that responds to γPGA [4]. The effect of γPGA is mediated by TLR4-expressing DCs, implying that DCs might be a key mediator for the anti-tumor effect of γPGA [4]. γPGA stimulates DCs to produce IL12, which plays an important role in differentiating naive $CD4^+$ T cells toward Th1 [6,7].

DCs consist of heterogeneous subsets, such as plasmacytoid DC (pDC) and myeloid DC (mDC), which have differential roles. For example, $CD8^+$ lymphoid DCs are adept at cross-presenting exogenous antigens to cytotoxic T lymphocytes, whereas $CD8^-/CD11b^+$ mDCs are specialized in activating $CD4^+$ T cells [8]. In addition, $B220^+$ pDCs play a role in the defense against virus infections by producing type I interferons [9]. Natural killer dendritic cells (NKDCs) are a recent addition to the complexity of DC subsets. NKDCs express both NK cell (e.g., NK1.1 and CD49b) and DC (CD11c) markers; therefore, they play a significant role in anti-tumor immunity because they have antigen presenting and tumor-lytic functions via TRAIL- and FasL-dependent mechanisms. Upon stimulation, NKDCs secrete an ample amount of IFNγ [10,11]. Nevertheless, the role of NKDCs in γPGA-mediated anti-tumor immune responses has not been evaluated. It remains unclear whether $NK1.1^+$ cells responding to γPGA are *bona fide* NK or other $NK1.1^+$ immune cells, such as NKDCs. Herein, we provide evidence that $NK1.1^+$ $CD11c^+$ DCs were activated by γPGA to produce IFNγ and TNFα and to confer anti-tumor effects in mice. In conclusion, NKDCs are one of the key players in the anti-tumor immunity induced by γPGA.

* Corresponding authors. Fax: +82 2 465 4187.
E-mail addresses: naykim@amc.seoul.kr (N. Kim), shong@sejong.ac.kr (S. Hong).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Korea). IL12p40/YFP (yet40) and IFNγ/YFP reporter (yeti) mice were provided by Dr. R. Locksley (University of California at San Francisco, USA). MyD88 knockout (KO) mice were kindly provided by Dr. M. Lee (Sungkyunkwan University, Korea). All mice were of a B6 genetic background, maintained at Sejong University, and were used at 6–12 weeks of age. The animal experiments were approved by the Institutional Animal Care and Use Committee at Sejong University (SJ-20100401008).

2.2. Reagents

CpG oligodeoxynucleotides (CpG) were manufactured by Bioneer (Daejeon, Korea). Lipopolysaccharide (LPS) derived from *Escherichia coli* (serotype 0111:B4) was purchased from Sigma–Aldrich (St. Louis, MO, USA). *B. subtilis*-derived γPGA (50 kDa) was purchased from Bioleaders (Daejeon, Korea).

2.3. Cell isolation by magnetic activated cell sorting (MACS) and culture

A single-cell suspension of splenocytes was prepared and resuspended in RPMI 1640 (Gibco BRL, USA) media supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 units/mL penicillin–streptomycin, and 5 mM 2-mercaptoethanol, and we refer to this as RPMI complete medium. Total $CD11c^+$ splenic DCs were enriched using CD11c MACS beads (Miltenyi Biotec, Germany), and the DC population was >97% after MACS. NKDC-depleted splenic DCs were separated as follows. First, $NK1.1^+$ cell-depleted splenocytes were negatively selected by removing $NK1.1^+$ cells using anti-phycoerythrin (PE) MACS after staining total splenocytes with PE-conjugated anti-NK1.1 (clone PK-136) mAb. Second, the remaining $NK1.1^+$ cell-depleted splenocytes were subsequently loaded onto the CD11c MACS system (Miltenyi Biotec) to positively select $CD11c^+$ DCs to obtain NKDC-depleted splenic DCs. EL4, RMA, RMA-S, and YAC-1 cells (ATCC) were cultured in RPMI complete medium.

2.4. Flow cytometry

Cells were stained with fluorescence-conjugated monoclonal antibodies (mAbs) and washed with FACS buffer (PBS containing 1% FBS). The following mAbs from BD Biosciences (San Jose, USA) were used: fluorescence-conjugated anti-NK1.1 (clone PK-136), anti-CD69 (H1.2.F3), anti-TCRβ (H57-597), anti-MHC class II (M5/114.15.2), anti-MHC class I (KH95), anti-CD86 (GL1), anti-CD40 (3/23), anti-IL12RβI (114), anti-CD11b (M1/70), anti-CD11c (HL3), anti-NKG2D (C7), anti-Ly49A (A1), anti-FasL (MFL3), anti-IL12p40 (C15.6), anti-TNFα (MP6-XT22), and IgG1, κ as the isotype control (R3-34). The fluorescence-conjugated anti-IL23p19 antibody was from R&D systems (320244). The following mAbs from eBioscience (San Diego, CA, USA) were used: fluorescence-conjugated anti-IFNγRI (2E2), anti-TLR4 (MTS510), anti-IFNγ (XMG1.2), and anti-perforin (eBioOMAK-D). Biotin-conjugated anti-TRAIL mAb (N2B2) was purchased from Biolegend (San Diego, USA). All flow cytometric data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Intracellular cytokine staining

Purified $CD11c^+$ cells were stimulated with LPS or γPGA at the designated concentrations for 18 h *in vitro*. Alternatively, $CD11c^+$ cells were purified from the splenocytes of LPS- or γPGA-injected mice. Cells were harvested and restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 2 h in the presence of brefeldin A (10 μg/ml). The cells were then stained for cell surface markers, fixed with 4% paraformaldehyde, washed once with cold FACS buffer, and permeabilized with 0.5% saponin. The cells were then incubated with anti-IFNγ, anti-TNFα, anti-IL12, anti-perforin mAb, or the appropriate isotype control for an additional 30 min at 4 °C.

2.6. Cytokine assays

The quantity of IFNγ in the culture supernatant was determined using sandwich ELISA according to the manufacturer's instructions (BD PharMingen, USA). The optical density was measured using an Immunoreader (Bio-Tek ELX-800, USA).

2.7. Footpad injection and harvest of draining lymph nodes (DLN)

Mice were injected into the left hind footpad with 10 μg of γPGA in 50 μl of PBS. 2 days later, the mice were sacrificed, and the bilateral popliteal lymph nodes (LN) were harvested. Tissues and LN were processed as a single-cell suspension using 5 mg/ml collagenase type IV (Sigma, St. Louis, MO, USA) and 0.5 mg/ml DNase I (Promega, USA) for further analysis.

2.8. Tumor-infiltrating leukocyte isolation

Mice were injected subcutaneously with 1×10^6 EL4 B6-originated lymphoma cells. Single-cell suspensions were prepared from tumors 14 days after injection as described above. Mononuclear cells were isolated using Lympholyte-M (CedarLane Laboratories Ltd., Hornby, Ontario, Canada) by density gradient centrifugation.

2.9. Cytotoxicity assay

A flow cytometric CFSE/7-AAD cytotoxicity assay was performed as previously described [12] with minor modifications. In brief, EL4, RMA, RMA-S, and YAC-1 cells were labeled with 500 nM CFSE, and the CFSE-labeled target cells (20,000 cells) were used at the designated effector-to-target (E:T) ratios. After 10 h of incubation, cells were stained with 0.25 μg/ml of 7-AAD and incubated for 10 min at 37 °C in a CO_2 incubator. Cell death was analyzed by flow cytometry.

2.10. Statistical analysis

Statistical significance was determined using Excel (Microsoft, USA). To compare two groups, the Student's *t*-test was performed. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

3. Results

3.1. NKDCs are stimulated by γPGA

We examined whether NKDCs could be activated by γPGA. Splenic DCs were stimulated by γPGA *in vitro*, and the expression of CD69, an activation marker, was assessed. $CD11c^+$ DCs were gated into two subsets: $NK1.1^+$ NKDCs ($NK1.1^+$ $CD11c^+$) and $NK1.1^-$ conventional DCs ($NK1.1^-$ $CD11c^+$). CD69 expression increased upon γPGA stimulation in NKDCs and conventional DCs in a dose-dependent manner (Fig. 1A). When 3 mg/ml of γPGA