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

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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.

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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

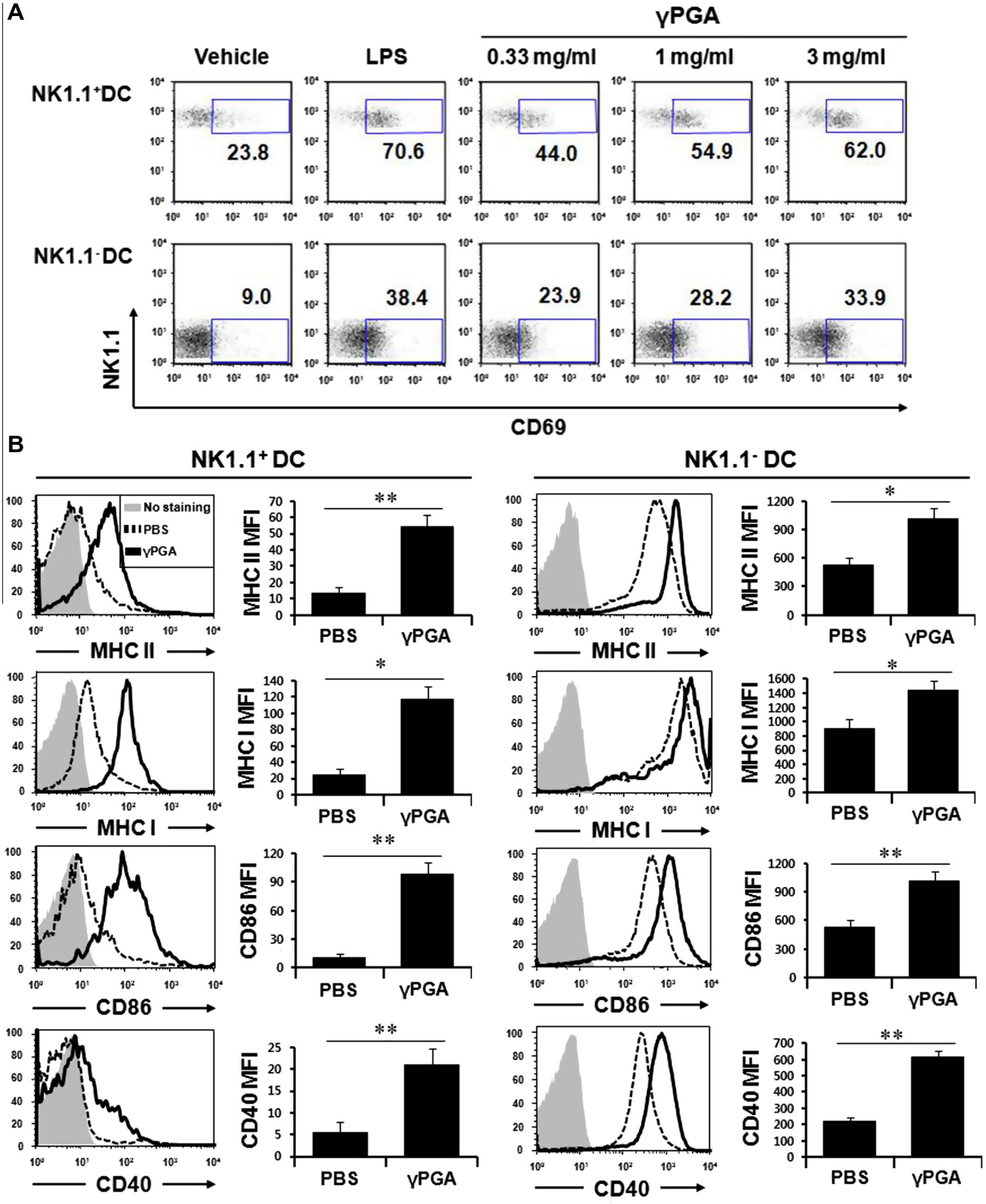


Fig. 1. NKDC stimulation by γPGA. (A) CD11c$^+$ splenic DCs from B6 mice were enriched and cultured in the presence of γPGA (0.33, 1, or 3 mg/ml) or LPS (1 μg/ml) for 16 h. Surface expression of CD69 on NK1.1$^+$ CD11c$^+$ cells (NK1.1$^+$ DC) and NK1.1$^-$ CD11c$^+$ cells (NK1.1$^-$ DC) were analyzed by flow cytometry. Representative results from four independent experiments are shown. (B) B6 mice were injected i.p. with γPGA (2 mg) or PBS as a vehicle control. Spleens were harvested 16 h later and splenic CD11c$^+$ DCs were enriched. Expression of MHC class II or I, CD86, and CD40 on NK1.1$^+$ DCs and NK1.1$^-$ DCs was evaluated by flow cytometry. The graphs on the right represent the mean fluorescence intensities (MFI). The mean values ± SD are shown ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

was added, the CD69$^+$ NKDC population comprised 62% of the total NKDCs, compared to 34% of conventional DCs. It should be noted that the basal level of CD69$^+$ NKDCs (24%) was higher than that in conventional DCs (9%) in the vehicle (PBS) control. To confirm the stimulatory effect of γPGA on NKDCs *in vivo*, we injected γPGA into B6 mice i.p. *In vivo* γPGA stimulation resulted in the up-regulation of MHC and co-stimulatory molecules on NKDCs (Fig. 1B). Considering the lower basal expression of MHC and co-stimulatory molecules on NKDCs by mean fluorescence intensities (MFI), the stimulatory effect of γPGA appeared to be more effective on NKDCs than on conventional DCs. In summary, γPGA stimulated NKDCs *in vitro* and *in vivo*.

Because the TLR4 has been suggested as a receptor for γPGA [3,4], its expression in DC subsets was determined. CD11c$^+$ DCs robustly express TLR4, whereas NK cells express TLR4 at a low level. NKDCs expressed a relatively low level of TLR4 when compared to conventional DCs (data not shown), although NKDCs were well stimulated by γPGA. Alternative receptors for γPGA, other than TLRs, were first presumed. However, NKDCs from MyD88-deficient mice failed to up-regulate CD69 in response to γPGA, implying that other TLRs could be involved (data not shown).

3.2. NKDCs produce IFNγ, rather than IL12, in response to γPGA

The next question was whether NKDCs could produce IFNγ in response to γPGA. Total DCs were treated with γPGA for 16 h, and IFNγ in the culture supernatant was measured by ELISA. Treatment with γPGA induced IFNγ production in a dose-dependent manner in total DCs (Fig. 2A). Because NKDCs are the major IFNγ producer in total DCs, as shown previously [13], we investigated which DC subset was responsible for producing IFNγ. Upon *in vitro* γPGA stimulation, intracellular IFNγ was assessed in conventional DCs (NK1.1$^-$ CD11c$^+$) and NKDCs (NK1.1$^+$CD11c$^+$). As expected, enhanced IFNγ production was observed in NKDCs but not in conventional DCs (Fig. 2B).

TLR agonists induce distinct profiles of cytokine production in NKDCs. CpG preferentially induces the secretion of IFNγ, while LPS induces those of TNFα, IL12, and IL6, but not IFNγ [14]. Therefore, it was interesting to know which cytokines NKDCs would produce when stimulated with γPGA. Total splenocytes from yet40 (YFP co-expressed with IL12p40) and yeti (YFP co-expressed with IFNγ) mice were stimulated with γPGA for 16 h, and cytokine-producing (YFP-positive) cells were measured by flow cytometry. YFP$^+$ cells from yet40 mice were nearly exclusively NK1.1$^-$ CD11c$^+$ (97%), while the majority of YFP$^+$ cells from yeti mice were NK1.1^{+-} CD11c$^+$ (84%), suggesting that conventional DCs produce IL12, while NKDCs produce IFNγ, in response to γPGA (Fig. 2C). Because NK cells also produce IFNγ, we confirmed that IFNγ-producing NK1.1$^+$ DCs were not NK cells based on the findings that YFP$^+$ NK1.1$^+$ DCs expressed MHC class II and CD86 molecules, which NK cells lack (data not shown).

3.3. In vivo administration of γPGA increases pro-inflammatory cytokine production in NKDCs and the accumulation of DCs in the DLN

To determine which cytokines NKDCs produce upon γPGA *in vivo* stimulation, CD11c$^+$ total DCs from either γPGA- or PBS-treated B6 mice were analyzed for intracellular cytokine production. As observed in Fig. 3A, significantly more NKDCs (14.6 ± 1.7%) produced IFNγ than conventional DCs (1.2 ± 0.2%). These data suggest that NKDCs were a major proportion of the IFNγ-producing subset in response to γPGA stimulation *in vivo* and *in vitro*. NKDCs and conventional DCs produced IL12 upon γPGA stimulation, but the percentage of IL12-producing NKDCs was only one-third of that of conventional DCs (Fig. 3A). NKDCs and conventional DCs produced TNFα upon stimulation with γPGA, although the percentages of TNFα$^+$ NKDCs were lower than those of conventional DCs regardless of γPGA treatment. Interestingly, IL23 production was up-regulated in NKDCs by γPGA, but not in conventional DCs, implying a role of γPGA in inflammation (Fig. 3A). In summary, these results indicate that NKDCs and conventional DCs might play differential roles in controlling immune responses by secreting distinct cytokines upon γPGA stimulation.

Footpad injection of LPS enhances DC accumulation in the draining popliteal LN [15]. Thus, it was investigated whether *in vivo* administration of γPGA could induce the accumulation of NKDCs in the DLN. CD11c$^+$ DCs were identified, and IL12 production was assessed by YFP expression in CD11c$^+$ DCs in the DLN from γPGA-injected yet40 mice, as observed in the upper right panels of Fig. 3B. γPGA injection resulted in an increase of DCs and YFP$^+$ DCs in the DLN by approximately 310% and 360%, respectively, compared to vehicle control. Absolute numbers of total lymphocytes, NK1.1$^+$ DC, and NK1.1$^-$ DC per popliteal LN are shown in the lower panels of Fig. 3B. Additionally, the total lymphocytes, NKDC, and conventional DC populations increased in the DLN at similar proportions, although the actual number of conventional DCs was 10-fold higher. These results suggest that *in vivo* administration of γPGA increases the activation status and actual numbers of DCs, including NKDCs and conventional DCs, in the DLN.

3.4. γPGA increases tumor-infiltrating NKDC numbers and cytotoxicity

High-molecular-weight γPGA induces NK cell-mediated anti-tumor immunity [3], but NKDCs also have a quintessential role in tumor surveillance [14,16]. Therefore, an *in vivo* tumor model was established to test whether low-molecular-weight γPGA also enhanced anti-tumor immunity through NKDCs. EL4 cells were subcutaneously injected into B6 mice, and from day 7, γPGA (2 mg/injection) was i.p. injected three times per week. On day 14, tumor-infiltrating lymphocytes were analyzed by flow cytometry (Fig. 4A). γPGA increased NK1.1$^+$ TCRβ$^-$ cells in spleens and the tumors (Fig. 4B). Among the NK1.1$^+$ TCRβ$^-$ cells, the ratio of NK1.1$^+$ CD11c$^+$ NKDCs vs NK1.1$^+$ CD11c$^-$ NK cells did not appear to change, meaning that NK cells and NKDCs increased by γPGA in tumor-bearing mice. The numbers of NKDCs increased approximately 3-fold in the spleens and tumors of γPGA-treated tumor-bearing mice compared to PBS-treated tumor-bearing mice.

We then examined how γPGA affects the cytotoxicity of NKDCs. Several molecules are responsible for natural cytotoxicity in NK cells: perforin, granzyme, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL). The expression of perforin, TRAIL, and FasL in NKDCs was examined to assess the cytotoxicity of NKDCs upon stimulation with γPGA *in vivo*. Perforin-expressing NKDCs in γPGA-stimulated NKDCs were substantially increased by 2.5-fold ($P < 0.01$), and the expression of FasL and TRAIL was significantly up-regulated by 3-fold ($P < 0.05$) and 2-fold ($P < 0.01$), respectively (Fig. 4C). The cytotoxicity of CD11c$^+$ total DCs, including NKDCs, against various tumor cell lines was increased by *in vivo* treatment with γPGA (Fig. 4D). Because conventional DCs do not express cytolytic molecules or death ligands, this cytotoxicity of total DCs should be attributed to NKDCs. The increase of cytotoxicity by γPGA against EL4 and RMA was comparable to that of CpG. As observed in the right panel of Fig. 4D, γPGA-stimulated total DCs could kill RMA-S cells, which are deficient for MHC class I molecules, and are thus sensitive to NK cytotoxicity. This result suggests that NKDCs adopt similar mechanisms as NK cells to initiate cytotoxicity, notably, by recognizing missing-self. Indeed, NKDCs express NK-activating receptors, such as NK1.1 and NKG2D, and NK-inhibitory receptors, such as Ly49A, whose ligands are MHC class I molecules (data not shown). Intriguingly, cytotoxicity against RMA, which is resistant to NK cells, was enhanced by CpG

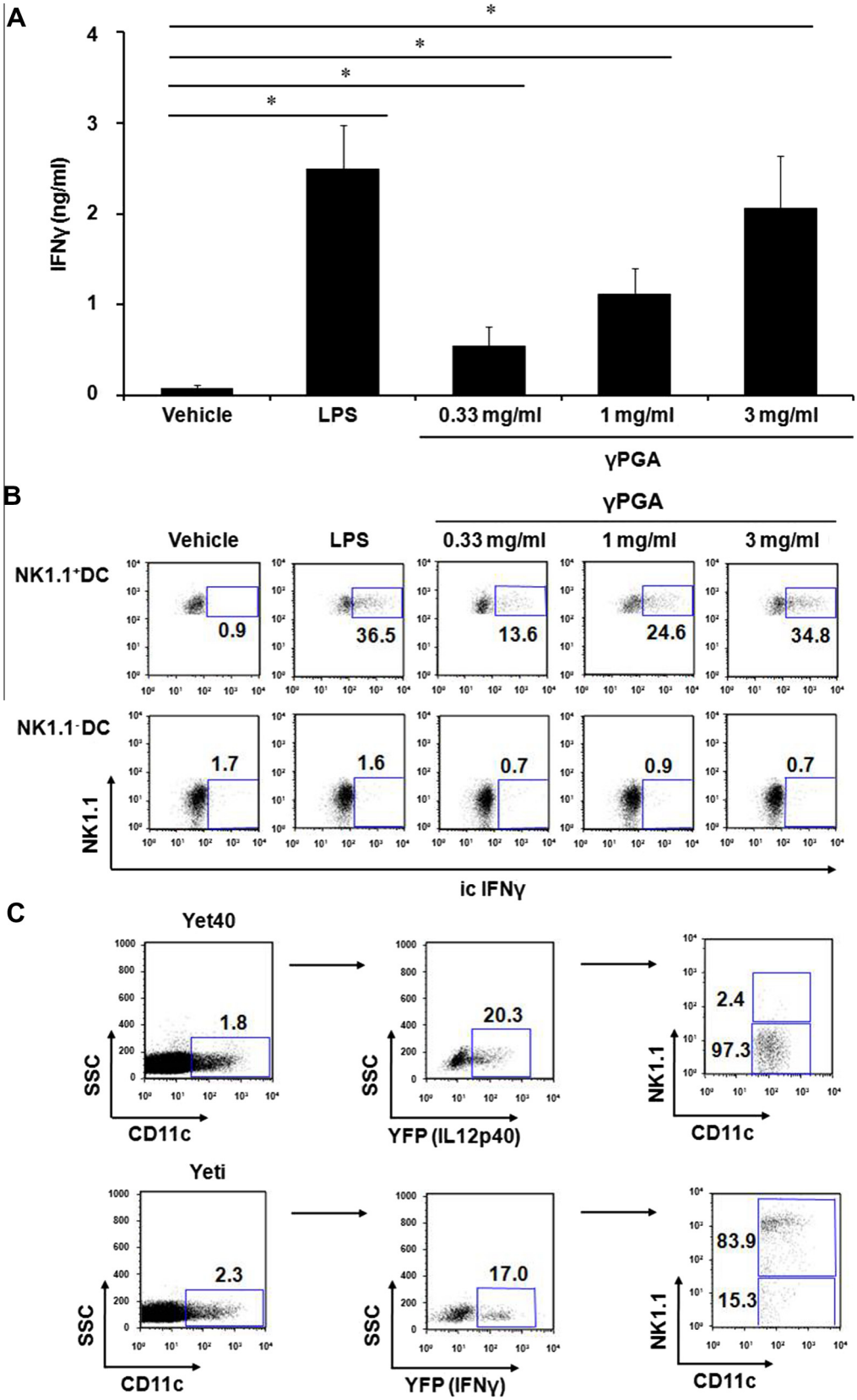
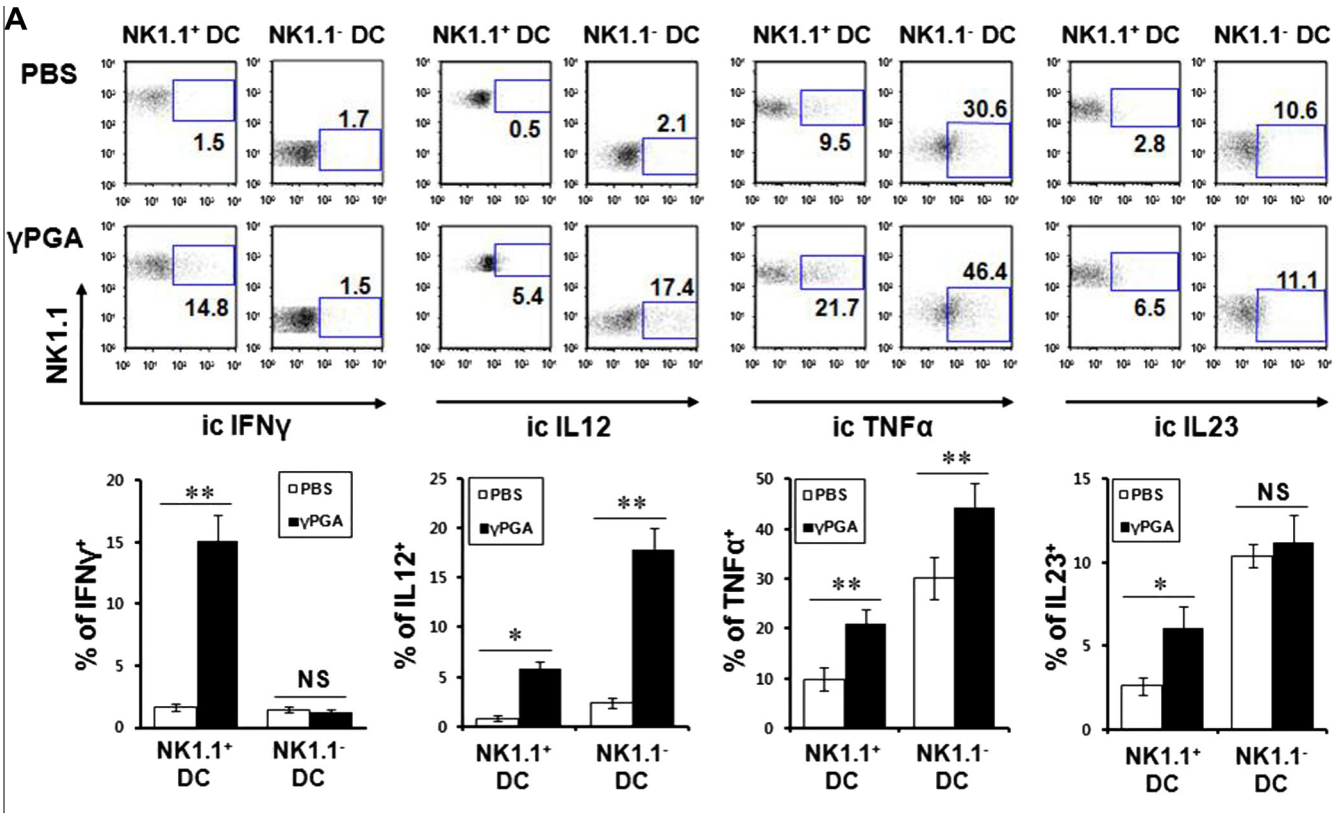


Fig. 2. IFNγ production of NKDCs by γPGA stimulation. (A and B) DCs from B6 mice were cultured in the presence of γPGA, LPS, or vehicle control for 16 h. (A) IFNγ secretion by total DCs after γPGA (0.33, 1 or, 3 mg/ml) or LPS (1 μg/ml) stimulation was assessed by ELISA ($n = 3$, $^*P < 0.05$). (B) Intracellular IFNγ production was analyzed in NK1.1$^+$ CD11c$^+$ (NK1.1$^+$ DC) and NK1.1$^-$ CD11c$^+$ cells (NK1.1$^-$ DC). Representative results from four independent experiments are shown. (C) Total splenocytes were isolated from yet40 and yeti mice. After γPGA stimulation for 16 h, cytokine production in NK1.1$^+$ and conventional DCs (NK1.1$^-$ CD11c$^+$) was assessed using flow cytometry. Representative results from two independent experiments are shown.



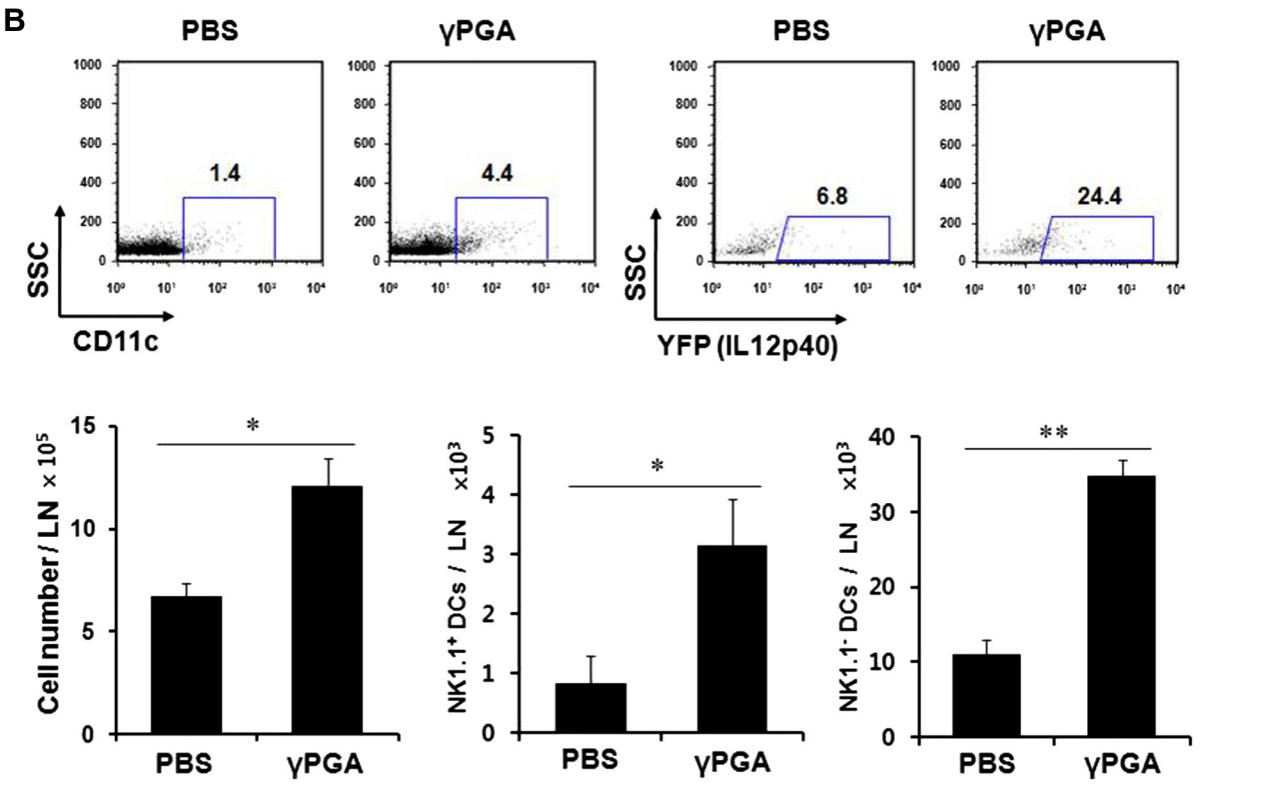


Fig. 3. Increase of NKDCs in the draining lymph nodes by *in vivo* administration of γPGA. (A) B6 mice were injected i.p. with γPGA (2 mg) or PBS. After 16 h, splenic $CD11c^+$ cells were isolated. Intracellular IFNγ, IL12p40, TNFα, and IL23p19 production was assessed by flow cytometry in $NK1.1^+$ ($NK1.1^+$ $CD11c^+$) and $NK1.1^-$ DCs ($NK1.1^-$ $CD11c^+$). Means ± SD are shown in the graphs below ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$). (B) $NK1.1^+$ DCs in the popliteal LN were analyzed 48 h after γPGA (10 μg/mouse) footpad injection in yet40 mice. IL12 expression was analyzed in $CD11c^+$ DCs by YFP expression. Absolute numbers of total lymphocytes, $NK1.1^+$ ($NK1.1^+$ $CD11c^+$) and $NK1.1^-$ DCs ($NK1.1^-$ $CD11c^+$) per popliteal LN were determined and are observed in the lower panels. Means ± SD are shown ($n = 3$).

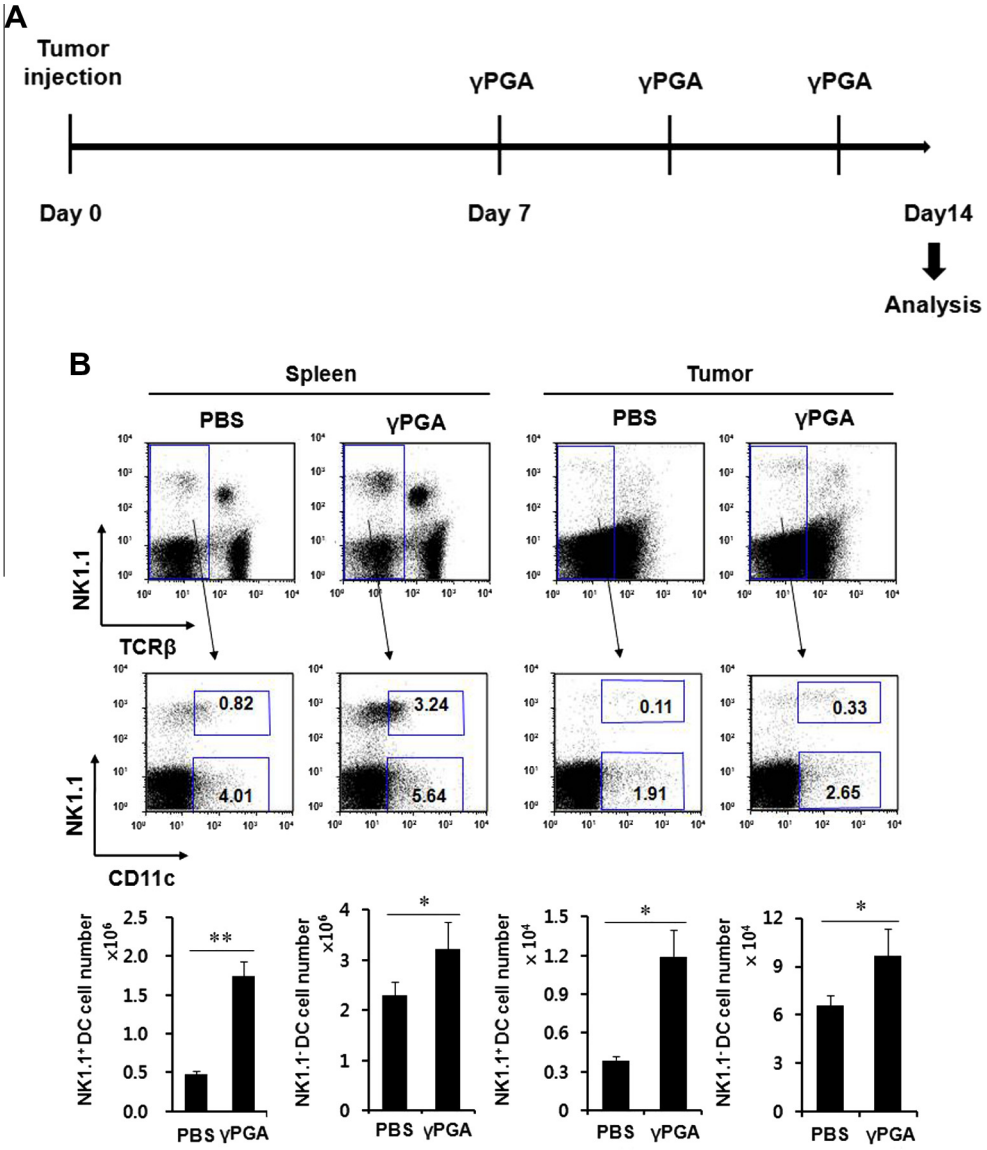


Fig. 4. Increase of NKDC numbers at the tumor sites and cytotoxicity upon stimulation with γPGA. (A) γPGA (2 mg/injection) was i.p. injected into B6 three times a week from day 7 post EL4 tumor cell injection. Splenocytes and tumor-infiltrating lymphocytes were analyzed at day 14, as observed in B. (B) NK1.1$^-$ and NK1.1$^+$ DC numbers were calculated by gating NK1.1$^-$ TCRβ$^-$ CD11c$^+$ and NK1.1$^+$ TCRβ$^-$ CD11c$^+$, as observed in the upper panels. The values on the dot plots show the percentages of the gated cell populations. Means ± SD are shown ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$). (C) Splenic DCs were isolated from B6 mice 16 h after i.p. injection of γPGA (2 mg) or PBS. NK1.1$^+$ and NK1.1$^-$ DCs were analyzed for perforin, FasL, and TRAIL expression. Means ± SD are shown ($n = 3$). (D) CD11c$^+$ DCs were stimulated with γPGA (1 mg/ml) or CpG (5 μg/ml) for 16 h and then co-cultured with CFSE-labeled EL4, RMA, or RMA-S cells at the indicated ratios. After 10 h of co-culture, cytotoxicity was measured using the CFSE/7-AAD cytotoxicity assay. Means ± SD are shown ($n = 2–3$). (E) γPGA (2 mg) or PBS was i.p. injected into B6 mice. 16 h later, total or NK1.1$^+$ DC-depleted DCs isolated from these mice were used as effector cells for a cytotoxicity assay. CFSE-labeled YAC-1 cells were co-cultured with effector cells at the indicated E:T ratios for 10 h. Cytotoxicity at an E:T ratio of 27:1 is displayed in the right panel. Means ± SD are shown ($n = 3$).

and γPGA stimulation. This result suggests that, unlike NK cells, NKDCs might have alternative pathways to kill target cells.

To confirm the enhanced cytotoxicity of NKDCs by γPGA, total or NKDC-depleted DCs were isolated from γPGA-treated mice and incubated with CFSE-labeled YAC-1 target cells. As expected, γPGA-stimulated total DCs killed YAC-1 tumor cells more efficiently than NKDC-depleted DCs by approximately 200% (Fig. 4E). Taken together, NKDCs activated by low-molecular-weight γPGA have up-regulated cytotoxic molecules, resulting in efficient killing of tumor cells.

4. Discussion

In this study, we demonstrate that NKDCs are stimulated by low-molecular-mass (50 kDa) γPGA to produce cytokines and eliminate tumor cells. In brief, treatment with γPGA up-regulated the expression of the activation marker, MHC, co-stimulatory molecules, pro-inflammatory cytokines, cytolytic molecules, and death ligands in NKDCs. More importantly, natural cytotoxicity of NKDCs against various tumor cells increased *in vitro* and *in vivo* upon γPGA stimulation through increased perforin, TRAIL, and FasL expression. Enhanced killing activity of NKDC by γPGA might lead to rapid elimination of tumor cells and the consequent presentation of tumor antigens to prime antigen-specific T cells [17]. Notably, unstimulated NKDCs already expressed high levels of TRAIL, when compared to conventional DCs. Previous reports have shown that rejection of melanoma by NKDCs is dependent on a TRAIL-mediated mechanism [16] and that human CD11c$^+$ blood DCs express TRAIL after stimulation with IFNγ or IFNα and acquire cytotoxicity against

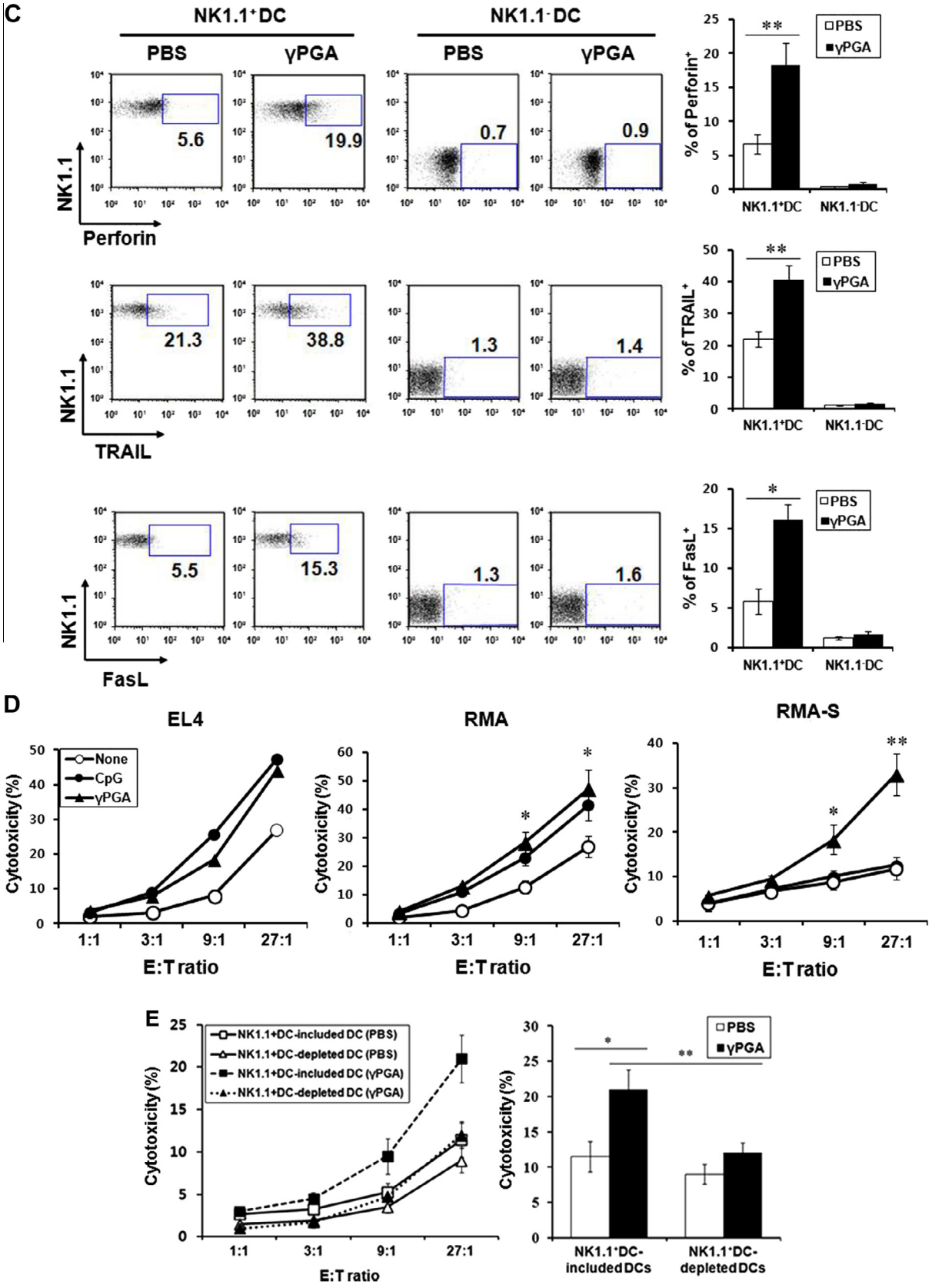


Fig. 4 (*continued*)

TRAIL-sensitive tumor cells [18]. The results have also shown the accumulation of NKDCs in response to γPGA *in situ* and in the DLN and at tumor sites, which could help in effective killing. However, it is not clear whether the accumulation of DCs in the DLN by γPGA is a result of active cell proliferation or increased trafficking.

Our data suggest possible crosstalk between NKDCs and conventional DCs in γPGA-mediated anti-tumor immunity. IFNγ secretion by NKDCs depends on IL12 [14]. NKDCs were the major producer of IFNγ in this study and in previous reports [13]. In contrast, IL12 was mainly produced by conventional DCs upon γPGA stimulation. NKDCs expressed a higher level of IL12Rβ1 receptor than conventional DCs (MFI, 32.1 ± 2.6 vs 6.9 ± 1.1; $P < 0.01$) (data not shown). Furthermore, the expression level of the IFNγR1 receptor on NKDCs was lower than that of conventional DCs (MFI, 76.3 ± 6.3 vs 117.3 ± 7.6; $P < 0.01$) (data not shown). These results imply that γPGA induces conventional DC maturation and IL12 production and that IL12 produced by conventional DCs activates NKDCs to release IFNγ. Together with their antigen-presenting capacity and cytotoxicity, the interaction between NKDCs and DCs via cytokines could be instrumental for effective anti-tumor immunity, which requires complex innate and adaptive immune responses.

The known cellular receptor of γPGA is TLR4, but the low expression of TLR4 in NKDCs and the involvement of MyD88 in the stimulatory effect of γPGA (data not shown) imply that other TLRs might be involved in the recognition of γPGA. Alternatively, NKDCs might be sensitive enough to be stimulated via the TLR4 signaling pathway, even with their relatively lower expression of TLR4 when compared to that of conventional DCs. It is also possible for γPGA to stimulate NKDCs directly and indirectly through TLR4.

These results suggest that low-molecular-weight γPGA could be useful as an adjuvant for cancer vaccines and other chemo- and immunotherapeutics. First, γPGA is non-toxic and biodegradable, so it would be relatively safe. Second, γPGA enhances NKDC-mediated anti-tumor immunity. Finally, γPGA augments pro-inflammatory cytokine production and antigen presentation of NKDCs, consequently boosting the innate and adaptive immune responses against tumors. Notably, the induction of NK cell-mediated anti-tumor immunity was only found with high-molecular-mass γPGA [3]. Further investigation that compares the effect of γPGA with high and low molecular masses on an array of immune cell types is required.

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