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IL32γ activates natural killer receptor-expressing innate immune cells to produce IFNγ via dendritic cell-derived IL12

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ABSTRACT

The inflammatory cytokine IL32γ acts on dendritic cells (DCs) to produce IL12 and IL6, which are involved in the differentiation of Th1 and Th17 cells. Natural killer (NK) and NKT cells play important roles in IL12-mediated adaptive immune responses, such as antitumor immunity. Herein we demonstrate the effect of IL32γ on the activation of NK and NKT cells. Upon IL32γ stimulation, splenic NK and NKT cells could be activated, and this activation was dependent on both IL12 and DCs, which was confirmed by using IL12p35 knockout and CD11c-diphtheria toxin receptor transgenic mouse models. Furthermore, IL32γ could induce the production of proinflammatory cytokines by NKDCs, a subset of DCs expressing NK cell markers, known to enhance NKT cell function. Unlike conventional DCs, NKDCs produced IFNγ and TNFα rather than IL12 upon stimulation with IL32γ. Taken together, IL32γ will be useful as an adjuvant to boost the cytotoxicities of NK and NKT cells that play critical roles in antitumor immunity.
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1. Introduction

Originally reported as a transcript in IL2 activated NK and T cells (named the NK4 transcript), IL32 is a cytokine promoting the production of a number of proinflammatory cytokines, including TNFα, IL1β, IL18, IL6 and other chemokines in primary monocytic leukocytes and cultured cell lines. Among the six isoforms of IL32, the γ isoform is known to be the most active [1,2]. Recently it was revealed that IL32 is associated with a variety of immune diseases, such as infectious diseases (i.e., influenza A virus and HIV infection) and autoimmune inflammatory diseases (i.e., rheumatoid arthritis, osteoarthritis, and Crohn disease), suggesting that IL32 plays critical roles in the regulation of immune responses [3,4].

Despite the clinical importance of IL32, it is difficult to investigate the immunological functions of IL32 *in vivo* because a mouse homolog of human IL32 and the corresponding murine receptor have thus far not been identified. However, through a transgenic mouse model overexpressing human IL32, the *in vivo* functions of IL32 have been described in autoimmune diseases, such as rheumatoid arthritis (RA). For example, the synovial expression of IL32 is strongly correlated with that of TNFα and IL1β and also with the severity of joint inflammation, implying that IL32 is a strong mediator of inflammatory processes during RA [3,4]. In addition, it was recently shown that human IL32γ induces the maturation of murine DCs to polarize Th1 and Th17 cell differentiation by enhanced IL12 and IL6 production, revealing an IL32-IL12 axis that elicits inflammatory immune responses [5].

Recently, it was also demonstrated that natural killer (NK) cell cytotoxicity was decreased when cells were transfected with small interfering (si) RNA against IL32, suggesting that IL32 can enhance the cytotoxic effect of NK cells. In addition, overexpression of IL32α increased NK cell-mediated killing through the up-regulation of Fas and UL16 binding protein 2 (ULBP2) expression in human chronic myeloid leukemia cells [6]. In addition to NK cells, a subset of T cells expressing both TCR and NK cell markers, so-called NKT cells, are involved in antitumor immune responses. Among heterogeneous NKT cell populations, invariant NKT (iNKT) cells are a well-characterized NKT subset that express an invariant TCR encoded by both the Vα14-Jα281 gene segment and the Vβ8, 7, or 2 gene

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segments. These iNKT cells recognize a glycolipid antigen, such as α-GalCer, in MHC I-like CD1d-dependent manner. Due to their rapid production of cytokines, such as IL4 and IFNγ, iNKT cells play a critical role in regulation of adaptive immune responses [7].

DCs are known to be the most potent antigen presenting cells. In the mouse spleen, three DC subsets have been described: $CD11b^{+}B220^{-}$ myeloid DCs, $CD11b^{-}B220^{-}$ DCs, and $CD11b^{-}B220^{+}$ plasmacytoid DCs. Previous studies revealed the distinct differences in immune regulation among these DC subsets [8]. In addition to the subsets described above, a novel population of DCs co-expressing the NK cell marker NK1.1/DX5 and the DC marker CD11c have been identified in humans and rodents [9]. These so-called natural killer dendritic cells (NKDCs) play a significant role in immunity because of their dual antigen presenting and lytic functions. Upon stimulation, NKDCs can secrete both IL12 and IFNγ and enhance their IFNγ production via an autocrine IL12 loop [10].

Although NK and NKT cells can be considered to be cellular targets for IL32γ due to their functions as key immune regulators, it remains unclear whether *in vitro* treatment of human IL32γ (hIL32γ) can activate NK and NKT cells. In this study, we investigated the *in vitro* effects of hIL32γ on NK and NKT cells. Our results demonstrated that hIL32γ can activate murine NK and NKT cells through IL12, TNFα, and IFNγ produced by DCs, including NKDCs. Because it has been well recognized that NKT cells are involved in a variety of immune responses, such as antitumor immunity, IL32γ can be a useful adjuvant to boost antitumor immune responses elicited by NKT cells.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) wild type (WT) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Korea). CD1d knockout (KO) mice were described previously [11,12]. IL12p35 KO mice were kindly provided by Dr. R. Locksley (University of California at San Francisco, CA, USA). CD11c-specific diphtheria toxin receptor (CD11c-DTR) transgenic (Tg) mice were obtained from Dr. E. Choi (Seoul National University, Seoul, Korea). All mice were in a B6 genetic background, maintained at Sejong University, and used at 6–12 weeks of age. The animal experiments were approved by the Institutional Animal Care and Use Committee at Sejong University (SJ-20130801).

2.2. Reagents

Diphtheria toxin and LPS derived from E. coli (serotype 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS was used at a final concentration of 1 μg/ml. Recombinant human IL32γ (hIL32γ) was obtained from Dr. Soohyun Kim (Konkuk University, Korea). For *in vitro* stimulation, hIL32γ was used at the range of concentration between 500 ng/ml and 2 μg/ml.

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

A single-cell suspension of splenocytes was prepared and resuspended in RPMI complete medium consisting of 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. $CD11c^{+}$ splenic DCs were enriched using CD11c MACS beads (Miltenyi Biotec, Germany), and the DC population was >97% pure after MACS.

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, CA, USA) were used: fluorescence-conjugated anti-CD11c (clone HL3), anti-CD69 (H1.2.F3), anti-CD3e (clone 145-2C11), anti-NK1.1 (clone PK-136), anti-IL12p40 (clone C15.6), anti-TNFα (clone MP6-XT22), and IgG1, κ as the isotype control (clone R3-34). Fluorescence-conjugated anti-IFNγ (clone XMG1.2) was purchased from eBioscience (San Diego, CA, USA). All flow cytometric data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Intracellular cytokine staining

Splenocytes or purified $CD11c^{+}$ cells were stimulated with LPS (1 μg/ml), hIL32γ (0.5 μg/ml, 1 μg/ml, or 2 μg/ml) or vehicle for 16 h *in vitro*. To prevent extracellular secretion of cytokines, brefeldin A (10 μg/ml) was added for the last 2 h of incubation. The cells were 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-IL12p40, anti-IFNγ, anti-TNFα, or appropriate isotype controls for an additional 30 min at 4 °C.

2.6. Statistical analysis

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

3. Results

3.1. IL32γ activates murine NK and NKT cells

IL32γ is known to increase the production of IL12 and IL6 by DCs [5]. However, the effects of this cytokine on the functions of other innate immune cells, such as NK and NKT cells, have not been studied. There are two main types of NKT cells: invariant (CD1d-dependent) and CD1d-independent NKT cells. Although $NK1.1^{+}$ T cells consist mainly of the iNKT population, our data indicated that a fair number of $NK1.1^{+}$ T cells still developed in CD1d-deficient mice (Fig. 1A). To examine the stimulatory effect of IL32γ on NKT cells, splenocytes from either WT or CD1d KO mice were stimulated with IL32γ *in vitro*, and the subsequent expression of the activation marker CD69 was assessed. We found that CD69 expression increased upon IL32γ stimulation in total NKT cells, CD1d-independent NKT cells, and CD1d-dependent iNKT cells (α-GC/CD1d $dimer^{+}$ $CD3^{+}$) in a dose-dependent manner (Fig. 1B). In addition to CD69, we investigated whether NKT cells produce IFNγ and TNFα upon *in vitro* IL32γ stimulation because NKT cells are one of the early producers of IFNγ and TNFα [7]. As expected, production of IFNγ and TNFα was enhanced in all of the NKT cells regardless of their CD1d-dependency (Fig. 1B). However, IL32γ had little effect on IL4 production by iNKT cells (data not shown). Because we have shown that *in vivo* α-GC-activated iNKT cells increase IFNγ production by NK cells [13], we next examined whether NK cells could be activated by IL32γ and whether this activation could be affected by iNKT cells. To test this hypothesis, total splenocytes from WT and CD1d KO mice were stimulated with IL32γ *in vitro*, and the resultant NK cell production of IFNγ and TNFα was assessed. We found that cytokine production by WT NK cells upon IL32γ stimulation was increased but that by CD1d KO mice was

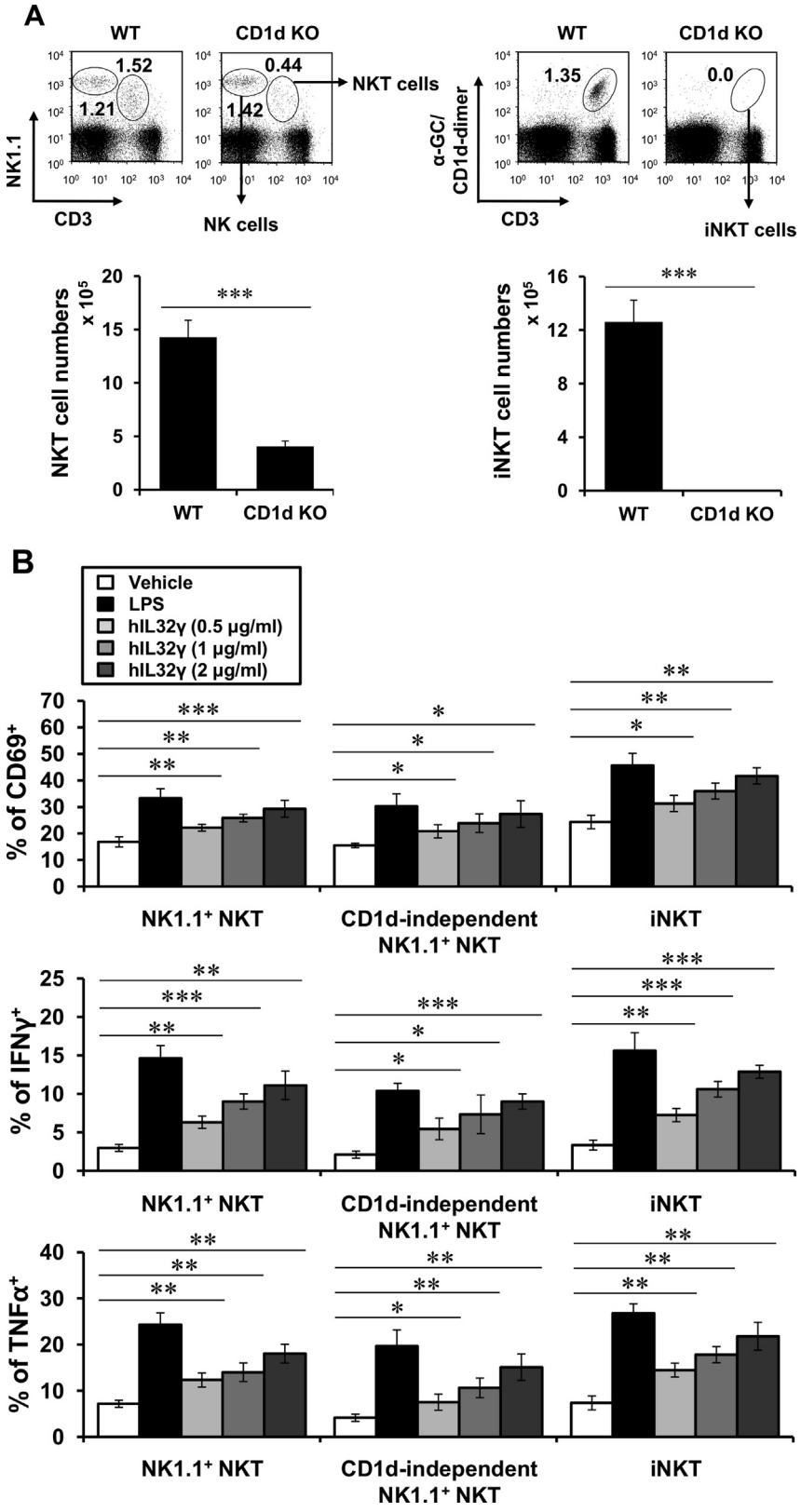

Fig. 1. IL32γ activates murine NK and NKT cells. (A) The percentage of NK1.1$^+$ NKT cells (NK1.1$^+$CD3$^+$) and iNKT cells (α-GC/CD1d dimer$^+$CD3$^+$) among the total splenocytes from WT and CD1d KO mice was plotted (upper panel). Absolute cell numbers of NK1.1$^+$ NKT cells and iNKT cells were assessed by flow cytometry (lower panel). The mean values ± SD (n = 3, ***P < 0.001) are shown. (B) Splenocytes were isolated from WT and CD1d KO mice and cultured in the absence or presence of LPS (1 μg/ml)/IL32γ (0.5, 1, or 2 μg/ml) for 16 h. Both cell surface CD69 expression and intracellular IFNγ and TNFα production were analyzed in NK1.1$^+$ NKT cells (NK1.1$^+$CD3$^+$), CD1d-independent NK1.1$^+$ NKT cells, and iNKT cells (α-GC/CD1d dimer$^+$CD3$^+$). The mean values ± SD are shown (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001). (C) Intracellular production of IFNγ and TNFα was analyzed in NK cells (NK1.1$^+$CD3$^-$). The mean values ± SD are shown (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

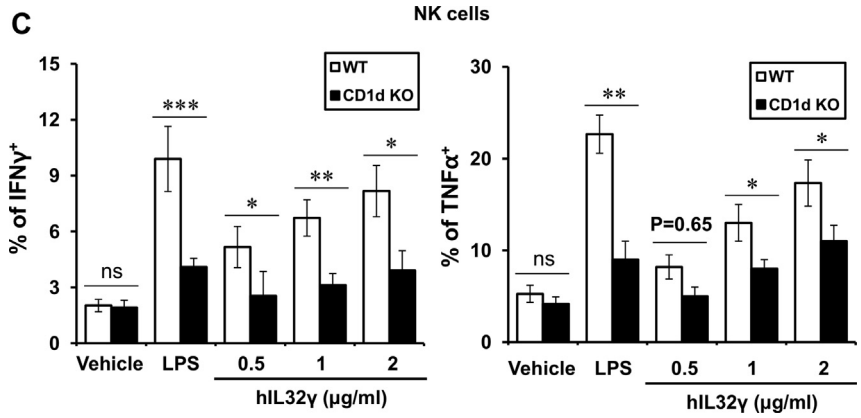

Fig. 1. (*continued*).

significantly decreased (Fig. 1C), indicating that optimal NK cell activation occurred in the presence of IL32γ requires iNKT cells.

3.2. IL32γ-induced NK and NKT cell activation is dependent on IL12

IL12 has been reported to be crucial for the activation of NK and NKT cells [14,15]. Thus, we hypothesized that NK and NKT cell activation by IL32γ is mediated by IL12. To test this, splenocytes from either WT or IL12p35 KO mice were stimulated with IL32γ for 16 h and the subsequent activation status of NK and NKT cells was examined by flow cytometry. Although the numbers of NK and NKT cells were not significantly different between WT and IL12p35 KO mice (Fig. 2A), the extent of activation (CD69 expression and IFNγ production) was dramatically reduced in NK and NKT cells from IL12p35 KO mice compared to those from WT mice (Fig. 2B and C). Taken together, our results clearly demonstrated that IL12 plays a critical role in IL32γ-mediated NK and NKT cell activation.

3.3. IL32γ-induced NK and NKT cell activation is mainly dependent on CD11c$^+$ DCs

The communication between innate immune cells is important in the elicitation of appropriate adaptive immune responses. Previously, it was demonstrated that DCs initiate adaptive immune responses through cross-talk with other innate immune cells, such as NK and NKT cells [16,17]. Because IL12 was found to be indispensable for the IL32γ-induced activation of NK and NKT cells, we next examined whether the activation of NK and NKT cells with IL32γ is dependent on the presence of DCs, which are potent producers of IL12. To test this, we took advantage of the CD11c-DTR Tg mice in which CD11c$^+$ DCs can be depleted by a single i.p. injection of diphtheria toxin (DT). Injection of DT effectively induced the depletion of splenic DCs (CD11c$^+$/GFP(CD11c)$^+$) (Fig. 3A). Total splenocytes from either PBS- or DT-treated mice were prepared and subsequently stimulated with IL32γ. Sixteen hours later, the activation status of NK and NKT cells was examined by flow cytometry. We found that both CD69 expression and IFNγ production in NK and NKT cells from DT-treated mice were significantly lower than those of PBS-treated controls (Fig. 3B and C). These results indicate that IL32γ triggers CD11c$^+$ DC-dependent activation of NK and NKT cells.

3.4. Among IL32γ-stimulated bulk DC populations, NK1.1$^-$ DCs and NK1.1$^+$ DCs are major source of IL12 and IFNγ, respectively

Given that DCs consist of heterogeneous populations with distinct phenotypes, we hypothesized that IL32g could activate DCs other than conventional DCs (i.e., NK1.1$^+$ DCs known to secrete IFNγ upon stimulation). Thus, we first examined what types of cytokines were produced following IL32g stimulation of NK1.1$^+$ DCs. To test this, purified splenic DCs isolated by CD11c-MACS were stimulated with IL32γ *in vitro* and the subsequent production of 3 cytokines (IL12, IFNγ, and TNFα) was assessed in gated CD11c$^+$ DC subpopulations, including NK1.1$^+$ NKDCs and NK1.1$^-$ conventional DCs (cDCs). MACS-purified CD11c$^+$ total DCs contains NKDCs expressing high levels of CD11c and low levels of B220 which are clearly distinguishable from interferon α-producing IKDCs (CD11c^{low}B220$^+$NK1.1$^+$) [18] (data not shown). We found that the production of all three cytokines was increased to some extent in NKDCs following IL32γ treatment. However, compared to cDCs, NKDCs produced 10 times more IFNγ but approximately 4 times less IL12, which suggests that IL32g differentially acts on DC subsets to induce varied cytokine expression (Fig. 4A and B). In addition, IL32γ-mediated TNFα production in NKDCs was comparable with that observed in cDCs (Fig. 4C). These findings suggest that NKDCs as well as cDCs play separate parts in IL32γ-induced immune cascades.

4. Discussion

Here we demonstrated that IL32γ treatment led to the activation of splenic NK and NKT cells, resulting in IFNγ production. Employing IL12p35 KO and CD11c-DTR Tg mice, we also clearly showed that IL32γ-mediated NK and NKT cell activation considerably depends on both IL12 and DCs.

Emerging evidence has shown that expression of the pro-inflammatory cytokine IL32 is increased in a variety of diseases, including chronic obstructive pulmonary disease, inflammatory bowel disease, allergic rhinitis [3,4], psoriasis [19] and myasthenia gravis [20]. In particular, the synovial expression of IL32γ is strongly correlated with that of TNFα and IL1β and also with the severity of joint inflammation, implying that IL32γ is a strong mediator of inflammatory processes during RA. Moreover, IL32γ exerts direct effects on joint inflammation in mice [3]. Recently, Park et al. reported that IL12p35 promotes antibody-induced joint inflammation by activating NKT cells that are known to respond to even low levels of IL12 stimulation due to their high expression of the IL12 receptor [14], indicating that NKT cells can negatively regulate joint inflammation. Because our present study showed that IL32γ can activate NKT cells in an IL12-dependent manner, it could be hypothesized that the increased expression of synovial IL32γ during joint inflammation might reinforce inflammatory responses in the affected tissue via NKT cell activation. Taken together, our results may indicate that IL12-dependent NKT

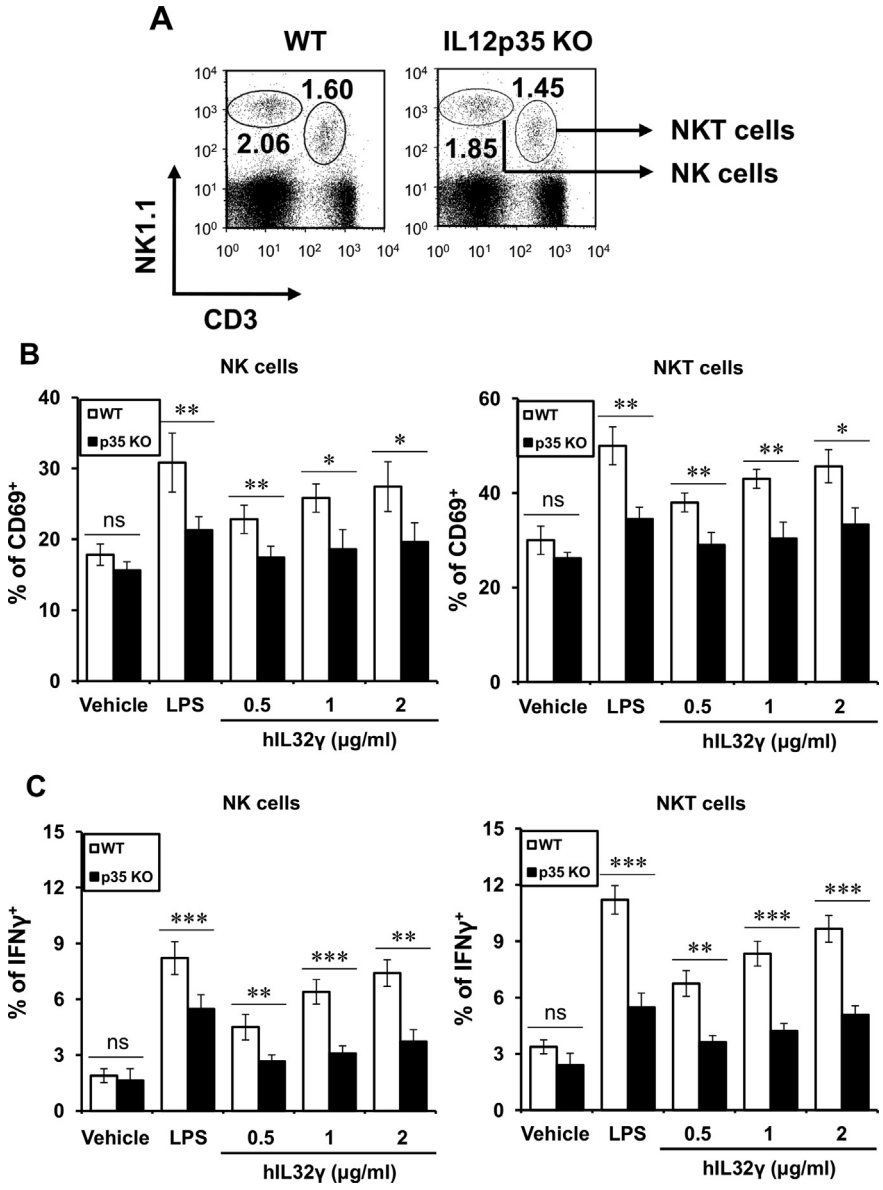

Fig. 2. IL32γ-induced NK and NKT cell activation is dependent on IL12. (A) The percentages of NK cells ($NK1.1^+CD3^-$) and NKT cells ($NK1.1^+CD3^+$) among the total splenocytes from WT and IL12p35 KO mice were plotted. (B–C) Splenocytes were isolated from WT and IL12p35 KO mice and subsequently cultured in the absence or presence of LPS (1 μg/ml)/ IL32γ (0.5, 1, or 2 μg/ml) for 16 h. Both cell surface CD69 expression and intracellular IFNγ production were analyzed in NK cells ($NK1.1^+CD3^-$) and NKT cells ($NK1.1^+CD3^+$). The mean values ± SD are shown (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

activation by IL32γ is an important factor for IL32γ-driven joint inflammation.

In contrast to the harmful effects of NKT cells on joint inflammation, NKT cells as well as NK receptor-expressing innate immune cells, such as NK cells and NKDCs, play important roles in antitumor immunity. These populations are equipped with a variety of cytotoxic effector molecules, such as TNFα, to kill tumor cells. Furthermore, one recent study provided evidence that TNFα is critical for spontaneous antitumor responses [21]. Because our results demonstrated that IL32γ up-regulated TNFα production from the aforementioned NKR-expressing immune cells with tumor cytolytic function, *in vivo* treatment with IL32γ will provide excellent immune regulation against cancer by boosting antitumor immune responses. Moreover, because our previous study demonstrated that the interaction between NKDCs and NKT cells was required for optimal antitumor immunity [13], IL32γ may exhibit synergistic antitumor effects through collaboration with NKR-expressing immune cells. In particular, we previously reported that NKDCs expressed a higher level of the IL12 receptor than did cDCs, whereas NKDCs expressed a lower level of the IFNγ receptor than did cDCs [22]. Among the total DCs, NKDCs were the major producer of IFNγ in response to IL32γ, whereas IL12 was mainly produced by cDCs upon IL32γ stimulation. These results imply that IL12 produced by cDCs following IL32γ stimulation subsequently acts on NKDCs to release IFNγ, and this interaction between NKDCs and cDCs could be instrumental for effective anti-tumor immunity.

It was previously demonstrated that IFNγ could induce IL32 in epithelia cells as well as in PBMCs [23] and that endogenous IFNγ was required for the optimal IL32 production induced by Mycobacterium tuberculosis [24]. Because we observed that IL32γ can stimulate IFNγ production by NK and NKT cells, it can be speculated that IL32γ-induced IFNγ from these cells in turn acts in a positive feedback loop, boosting the activation of PBMCs to increase IL32g production. If this is the case, IL32γ can be applicable for clinical

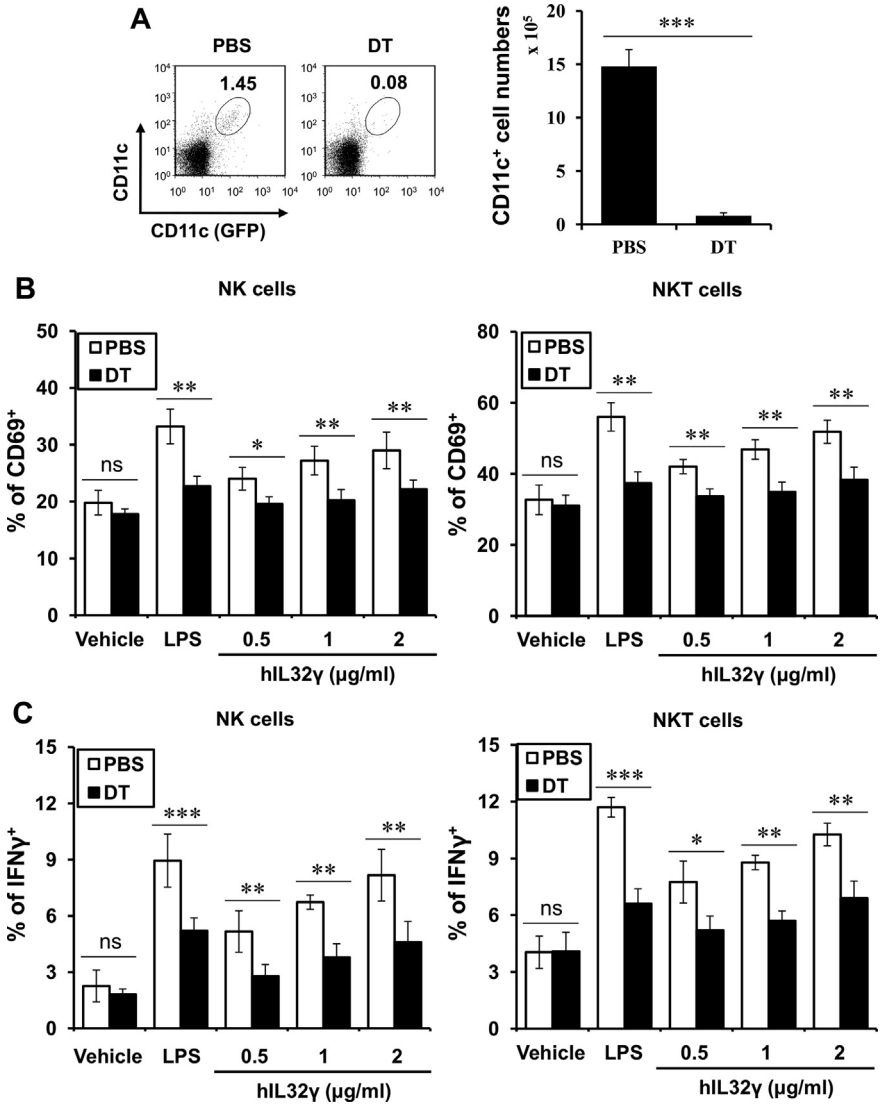

Fig. 3. IL32γ-induced NK and NKT cell activation is mainly dependent on CD11c$^+$ DCs. (A–C) CD11c-DTR Tg recipient mice were i.p. injected with DT (120 ng/mouse) to remove the CD11c$^+$ DC population. (A) The percentages of CD11c$^+$ cells (CD11c$^+$/GFP(CD11c)$^+$) among the total splenocytes from either PBS- or DT-injected mice were plotted (left panel). The absolute cell number of CD11c$^+$ cells was assessed by flow cytometry (right panel). The mean values ± SD (n = 3, ***P < 0.001) are shown. (B–C) Splenocytes were isolated from either PBS- or DT-injected mice and were subsequently cultured in the absence or presence of LPS (1 μg/ml)/IL32γ (0.5, 1, or 2 μg/ml) for 16 h. Both cell surface CD69 expression and intracellular IFNγ production were analyzed in NK cells (NK1.1$^+$CD3$^-$) and NKT cells (NK1.1$^+$CD3$^+$). The mean values ± SD are shown (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

antitumor immunotherapy. Thus, our results strongly suggest that IL32γ can be an attractive candidate as a tumor vaccine adjuvant. The following scenario might be possible for IL32γ to elicit antitumor responses: IL32γ induces the maturation of cDCs leading to IL12 production and IL32γ-induced IL12 subsequently activates NKT and NK cells to express cytotoxic cytokines, such as TNFα, to elicit enhanced antitumor effects. Furthermore, NKDCs have increased tumor cytolytic capacities as a result of their cytokine-mediated interaction with cDCs.

On the other hand, our results cannot exclude the possibility that IL32γ-induced IFNγ might render cells more responsive to exogenous IL32γ or endogenously produced IL32γ, although it is not yet clear whether IFNγ can induce IL32g receptor expression. Although a receptor for IL32g is currently unknown, several studies have demonstrated that a variety of immune cells can respond to IL32γ, such as endothelial cells [25] and eosinophils [26], indicating its broad range of target cells. Recently, a specific IL32-binding protein known as PR3 was found to localize to the cell surface and the cytoplasm of eosinophils, which suggests that the eosinophil IL32γ-PR3 signaling pathway might be involved in the induction of allergic inflammation-related immune responses.

In addition to the effect of IL32γ on eosinophils, it was recently reported that IL32γ may influence macrophage differentiation. Macrophages can be classified into two distinct subsets as follows: M1 macrophages differentiated in an IFNγ-dominant environment display proinflammatory characteristics (i.e., production of IL12), while M2 macrophages are differentiated under IL4 conditions to regulate inflammatory immune responses. A recent study demonstrated that IL32γ can induce proinflammatory M1-like macrophages but can also preferentially accelerate M2 polarization in the presence of M-CSF [27]. Thus, it may be possible that macrophages activated by IL32γ also induce the activation of NK and NKT cells, most likely via IL12 production. Considering that our DC-depletion experiments using CD11c-DTR Tg mice dramatically reduced the level of NK and NKT cell activation induced by IL32γ, macrophages might be much less influential on IL32γ-mediated activation of NK and NKT cells than DCs.

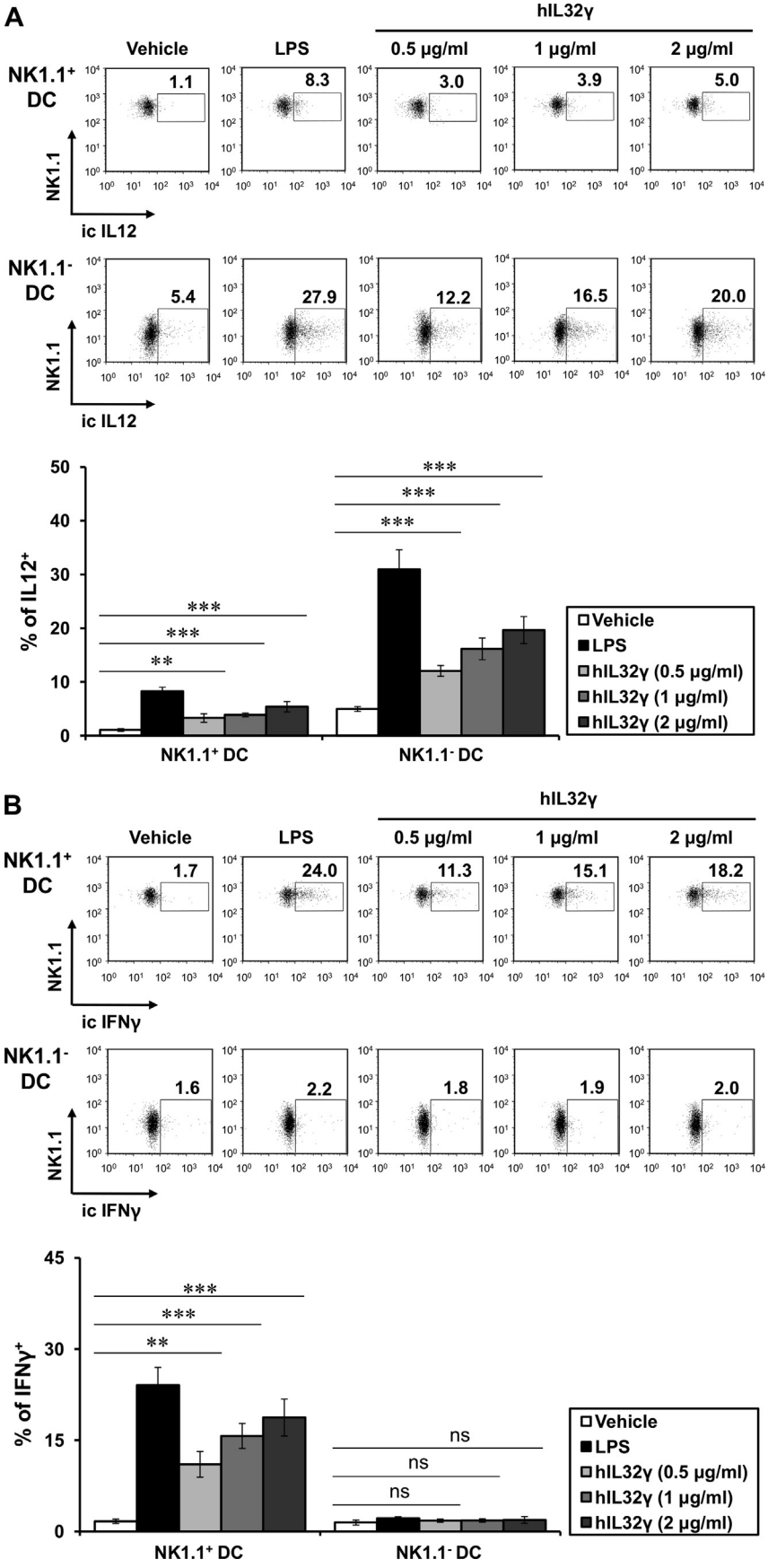

Fig. 4. Among IL32γ-stimulated bulk DC populations, NK1.1$^-$ DCs and NK1.1$^+$ DCs are major source of IL12 and IFNγ, respectively. (A–C) CD11c$^+$ splenic DCs from WT mice were enriched and subsequently cultured in the absence or presence of LPS (1 μg/ml)/IL32γ (0.5, 1, or 2 μg/ml) for 16 h. (A) Intracellular IL12, (B) intracellular IFNγ, and (C) intracellular TNFα production by both NK1.1$^+$ DCs (NK1.1$^+$CD11c$^+$) and NK1.1$^-$ DCs (NK1.1$^-$CD11c$^+$) were evaluated by flow cytometry. *Top*, the representative FACS plot; *bottom*, summary figures. Mean values ± SD are presented (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

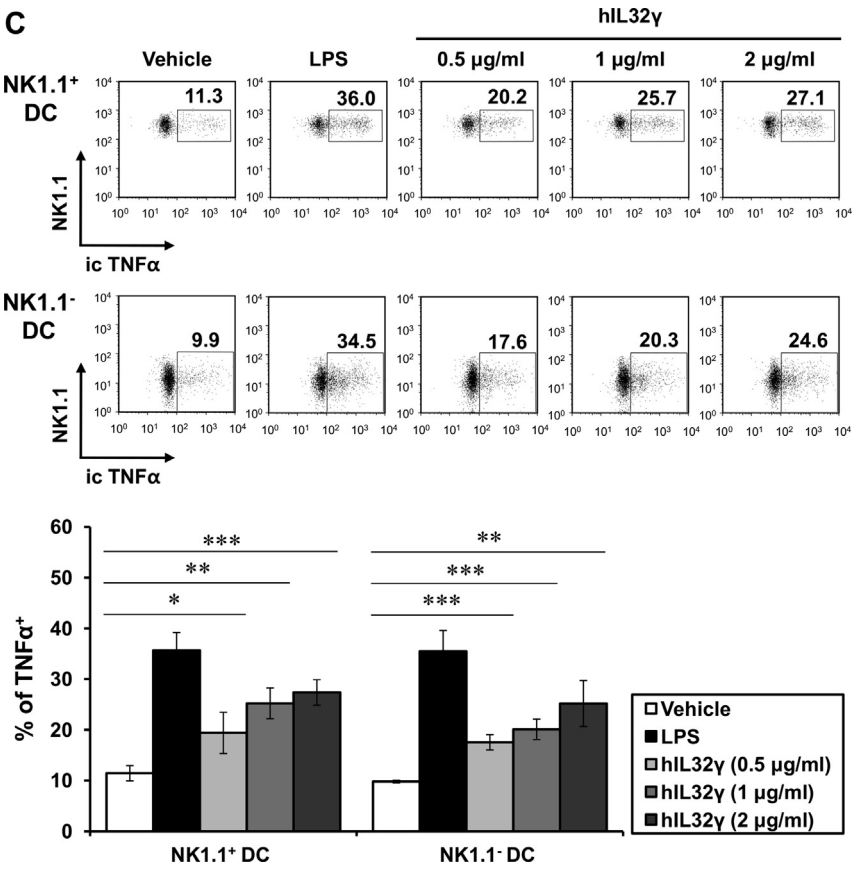

Fig. 4. (*continued*).

In conclusion, we demonstrated that IL32γ can activate NKR-expressing innate immune cells to produce IFNγ via IL12-dependent DCs. Because emerging evidence shows that IL32γ can influence a variety of immune cells, including DCs, macrophages, and eosinophils, it will be of great interest to further investigate IL32g-induced immune responses in many different immune disease models, including infection, cancer and allergy.

Conflict of interest

None.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.174.

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