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Original Article

Thymic stromal lymphopoietin production in DN32.D3 invariant natural killer T (iNKT) cell line and primary mouse liver iNKT cells

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ABSTRACT

Background: Invariant natural killer T (iNKT) cells are known as the fast responder in allergic inflammation and the source of interleukin (IL)-4, IL-13, and interferon-gamma. Absence of iNKT cells down-regulated thymic stromal lymphopoietin (TSLP) production at the early stage of type 2 immune responses in the airway. However, it has not been reported whether iNKT cells are able to produce TSLP via stimulation of T-cell receptor (TCR).
Objective: We aimed to evaluate TSLP production from iNKT cells by TCR specific stimulations with anti-CD3/CD28 antibodies and α-galactoceramide (α-GalCer).
Methods: DN32.D3 iNKT cell line was stimulated with anti-CD3/CD28 antibodies, and TSLP production was measured in culture supernatants. Next, to confirm the TSLP production in primary mouse iNKT cells, the cells were sorted using α-GalCer-CD1d tetramer from mouse liver, and stimulated with anti-CD3/CD28 antibodies and α-GalCer. Then, cytokine productions were evaluated by enzyme-linked immunosorbent assay and quantitative polymerase chain reaction.
Results: TCR specific stimulation in DN32.D3 cells induced TSLP production as well as signature cytokines of iNKT cells. On the other hand, isolated primary mouse iNKT cells from liver did not show any induction of TSLP by TCR specific stimulations including anti-CD3/CD28 antibodies and α-GalCer, on the contrary to other cytokines.
Conclusion: This study suggested the possibility of TSLP production in iNKT cells, especially from DN32.D3 although primary mouse liver iNKT cells showed a different result.

Keywords: Natural killer T cells; Thymic stromal lymphopoietin; T-cell receptors

INTRODUCTION

Among diverse cells involved in type 2 inflammation of the airway such as asthma, natural killer T (NKT) cells are regarded as one of the important players, because they are related with the development and phenotype of type 2 inflammation. In asthmatics, high

Conflict of Interest
The authors have no financial conflicts of interest.

Author Contributions
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levels of pulmonary NKT cells having invariant T-cell receptor (TCR) were observed in bronchoalveolar lavage fluids [1]. In an animal model, the absence of NKT cells failed to induce type 2 inflammation phenotype [2]. Moreover, they are important source of type 2 cytokines such as interleukin (IL)-4 and IL-13 in the beginning of development of type 2 inflammation [3] or in the challenge period via coordination with macrophages or other immune cells [4, 5].

There are 3 types in NKT cells: type 1 to 3. Especially, type 1 NKT cells are also called as invariant NKT (iNKT) cells. iNKT cells are known as rapid responders to lipid antigen and producers of IL-4 and IL-13 via lipid antigen loaded CD1d and invariant TCR recognition [6-8]. Other stimulators including cytokines such as IL-33 and TNF-α can also induce type 2 cytokines by the activation of iNKT cells [8, 9]. Because of these characters, iNKT cells show a considerable involvement in type 2 airway inflammation [3]. To investigate the role of mouse iNKT cells, an immortalized cell line, DN32.D3, was established using hybridoma system [10] and has been used in previous studies [11, 12].

In addition to iNKT cells in innate immune system, there is an alternative source of type 2 cytokines from innate lymphoid cells (ILCs). They can be activated by thymic stromal lymphopoietin (TSLP) secreted from diverse immune and structural cells such as epithelial cells in airways [13, 14], and under the stimulation of allergen/protease or viral components [9, 15, 16]. Interestingly TSLP can also induce IL-4 and IL-13 via activating iNKT cells without any interferon-gamma (IFN-γ) production [17]. In this context, TSLP can play a major role in the induction of type 2 cytokines in innate immune system via ILCs and iNKT cells.

Our previous study showed that the absence of iNKT cells down-regulated TSLP level at the early stage of type 2 immune responses in the airway [18]. However, it has not been reported whether iNKT cells are able to produce TSLP via activation of TCR. In this study, we investigated the TSLP production from iNKT cells using cell-specific stimulation.

MATERIALS AND METHODS

Cell culture and stimulation

DN32.D3 iNKT cells were cultured in DMEM (Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS) (Biowest LLC, Riverside, MO, USA) and 1× antibiotics (Penicillin and streptomycin, Welgene) as described in the previous study [3]. To stimulate DN32.D3 cells, overnight starved cells were cultured in 24 well-culture plate (Corning Incorp., Corning, NY, USA) precoated with anti-mouse CD3/CD28 antibodies (Biolegend, San Diego, CA, CA) at a concentration of 1.0×10^6 cells/mL in 500 μL of serum-free medium.

Primary iNKT cells were isolated from mouse liver mononuclear cells by enrichment using MACs separator (Miltenyi Biotec Inc., Sunnyvale, CA, USA; purity > 85%) with allophycocyanin (APC)-conjugated α-galactoceramide (α-GalCer)/CD1d tetramers (National Institutes of Health, Bethesda, MD, USA) and anti-APC magnetic beads. Isolated iNKT cells were stimulated in 96 well-culture plate (Corning Incorp.) with anti-mouse CD3/CD28 antibodies at a concentration of 2.0×10^5 cells in 200 μL RPMI 1640 medium (10% FBS, 1× antibiotics, Welgene). For stimulation with α-GalCer, isolated iNKT cells stimulated with 200 ng/mL of α-GalCer in the same condition as anti-CD3/CD28 stimulation.

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Cytokine evaluation from culture supernatant

To evaluate cytokine levels from cell culture supernatants, mouse IL-4, IL-13, IFN-γ, and TSLP enzyme-linked immunosorbent assay (ELISA) Duoset (R&D Systems, Minneapolis, MN, USA) were used following manufacturer's protocol.

Evaluation of RNA expression from iNKT cells

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guideline. Next, cDNA was synthesized from RNA using the Maloney murine leukemia virus reverse transcriptase Taq polymerase (M1705, Promega, Madison, WI, USA). For quantitative reverse-transcription polymerase chain reaction (PCR), SensiFAST SYBR Lo-ROX buffer (Bioline, Memphis, TN, USA) and gene-specific PCR products were quantified using an Applied Biosystems 7500 Sequence Detection System. The primer sequences were as follows; Ifng forward: cggcacagtcattgaaagccta, reverse: gttgctgatggcctgattgtc; β-actin forward: tgctaggagccagagcagta, reverse: agtgtgacgttgacatccgt; TSLP forward: cggatggggctaacttaca, reverse: tcctcgatttgctcgaactt.

Statistics

For statistical analysis, GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) was used for the preparation of the graphs, and data were presented as mean ± standard errors of the mean. Statistical significance was elucidated using 1-way analysis of variance test or Student *t* test with *post hoc* comparison (Kruskal-Wallis or Mann-whitney *U* test). A *p* value less than 0.05 was regarded as statistically significant.

RESULTS

iNKT cell line was able to produce TSLP

First, to confirm the possibility that TSLP induction in iNKT cell line, DN32.D3 cells were stimulated with anti-CD3/CD28 antibodies, and the supernatants were evaluated by ELISA at different time points. The production of IL-4, IL-13, and IFN-γ were elevated by stimulation from 4 to 16 hours. Interestingly, TSLP production was also highly induced from 4 hours after stimulation (**Fig. 1**).

Primary mouse liver iNKT cells were not able to produce TSLP by stimulation via TCR

Next, to evaluate the TSLP production in primary iNKT cells, the cells were isolated from mouse liver (**Fig. 2A**). Stimulation of the isolated iNKT cells with anti-CD3/CD28 antibodies showed up-regulation of IL-4, IL-13, and IFN-γ cytokines in the culture supernatants. However, unlike the result of DN32.D3 cells, TSLP was not detected (**Fig. 2B**). We also evaluated the mRNA expression level from cell lysate with IFN-γ and TSLP specific primers, which showed the up-regulated expression of IFN-γ mRNA but not TSLP mRNA (**Fig. 2C**).

Primary mouse liver iNKT cells were not able to produce TSLP by α-GalCer

It is known that iNKT cells also respond to lipid antigen, α-GalCer. Thus, we stimulated the isolated primary iNKT cells using α-GalCer to check the response according to different stimulators. However, we did not find any difference from the results with anti-CD3/CD28 antibodies (**Fig. 3**).

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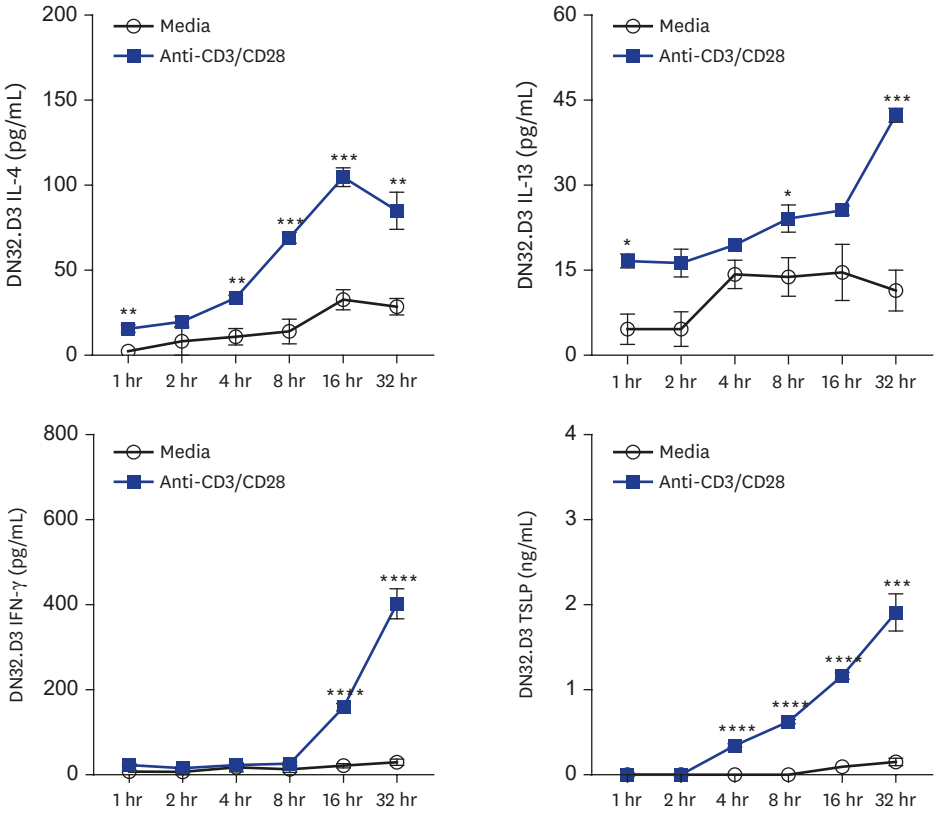

Fig. 1. Cytokine induction profile in each time point stimulation with anti-CD3/CD28 antibodies in DN32.D3 cells. The experiment was repeated at least 2 times. In all experiments, comparisons are relative to media control group at each time point. IL, interleukin; IFN, interferon; TSLP, thymic stromal lymphopoietin. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

DISCUSSION

In this study, we activated iNKT cells with TCR specific stimulations to confirm whether TSLP induction from iNKT cells was possible. TSLP was induced in DN32.D3 iNKT cells by anti-CD3/CD28 stimulation as well as IL-4, IL-13, and IFN-γ. In primary iNKT cells from mouse liver, we could not detect TSLP by TCR specific stimulations with anti-CD3/CD28 and α-GalCer although the other 3 cytokines were detected. This study showed that DN32.D3 and primary mouse liver iNKT cells showed a different response in TSLP production.

Based on different results from DN32.D3 and primary mouse liver iNKT cells, we checked whether this could be caused by the character of hybridoma. DN32.D3 iNKT cell line was established from the hybridization of thymus derived mouse iNKT cells and BW5147, TCR-/- thymoma cells [10]. According to the previous study, some solid tumors including breast tumor could be the direct producer of TSLP [19]. BW5147 is derived from the mouse thymus lymphoma in AKR/J mouse [20]. It has been reported that TSLP production in disease-related thymoma was down-regulated than normal subject [19]. Even though the TSLP could be induced by some cancer cells, undifferentiated or fully differentiated T cell is not known as the source of TSLP [14]. In this context, we could postulate that the character of BW5147 was not the cause of TSLP production in DN32.D3 cells.

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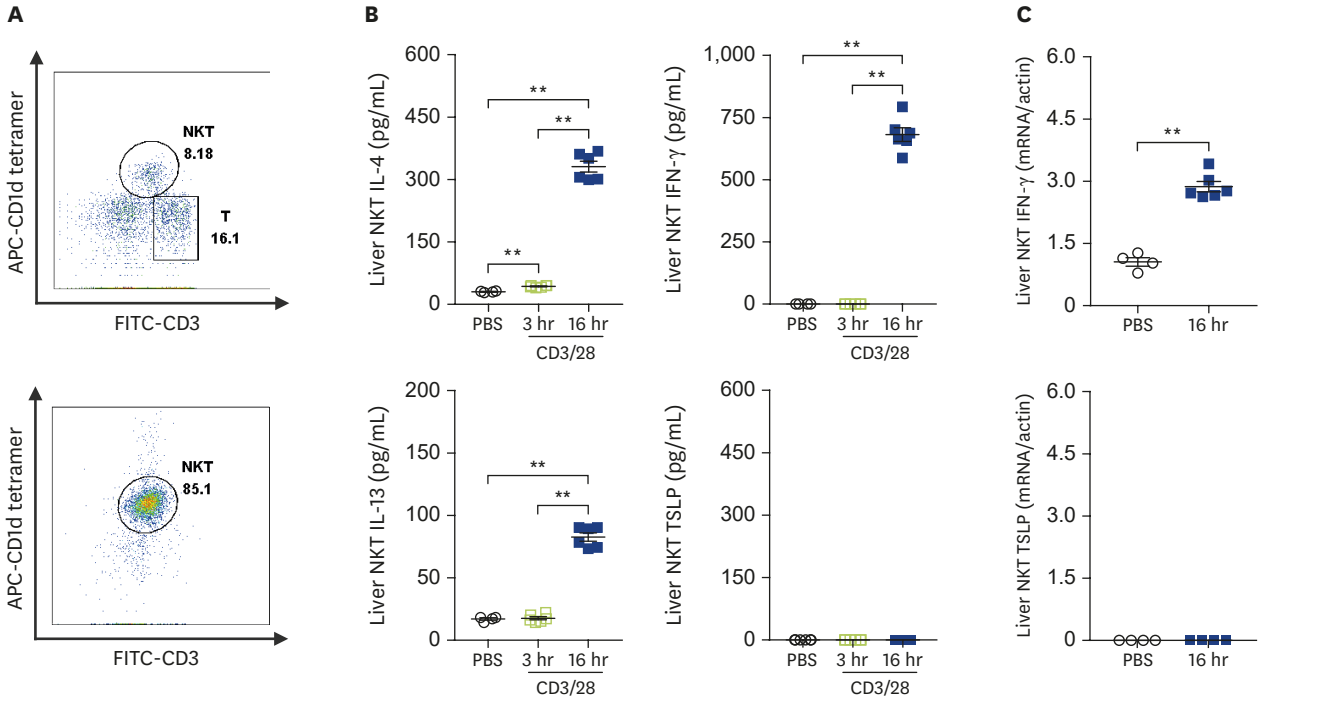

Fig. 2. Cytokine production in primary mouse liver invariant natural killer T (iNKT) cells with anti-CD3/CD28 stimulation. (A) The purity of isolated iNKT cells before (upper) and after (lower) MACs sorting. (B) Cytokine production in culture supernatant. (C) Change of mRNA levels by anti-CD3/CD28 stimulation. The experiment was repeated at least 2 times. In all experiments, comparisons are relative to media control group at each time point. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; IL, interleukin; IFN, interferon; TSLP, thymic stromal lymphopoietin. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

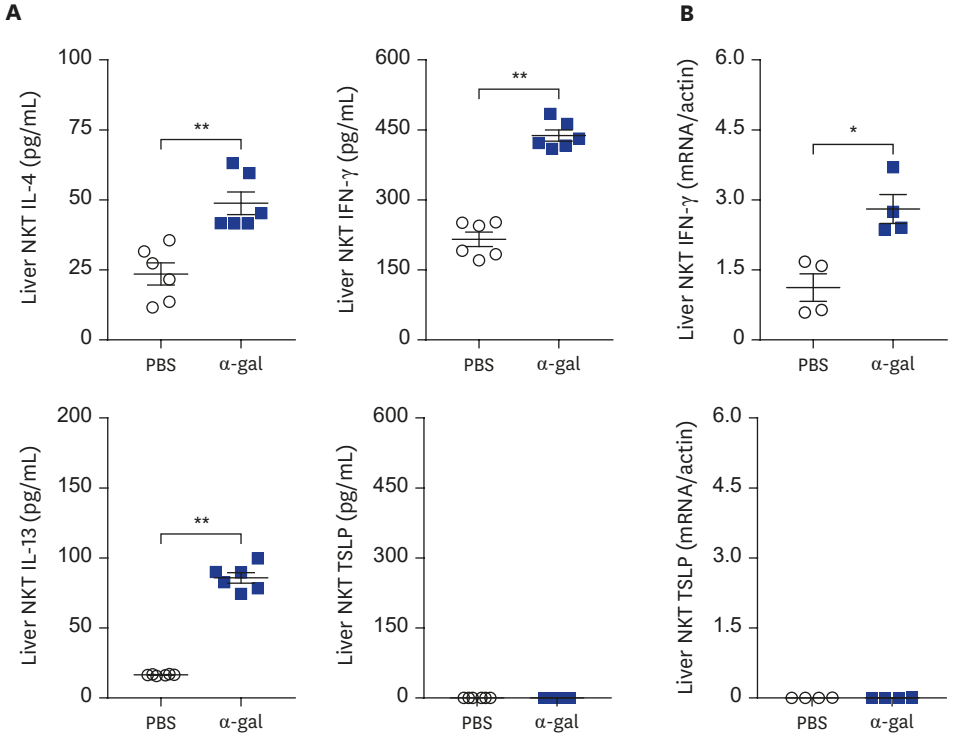

Fig. 3. Cytokine production in primary mouse liver invariant natural killer T (NKT) cells with α-galactoceramide (α-GalCer) stimulation. (A) Cytokine production in culture supernatant and (B) Change of mRNA levels by α-GalCer stimulation. The experiment was repeated at least 2 times. In all experiments, comparisons are relative to media control group at each time point. PBS, phosphate-buffered saline; IL, interleukin; IFN, interferon; TSLP, thymic stromal lymphopoietin. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

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Next, based on cell line experiments, we attempted to evaluate the changes of protein and mRNA production at 4 and 16 hours after stimulation to confirm the change of TSLP production in the primary iNKT cells. Previously the cells showed enormous cytokine production within 24 hours after anti-CD3 stimulation [21]. We could observe the induction of IL-4, IL-13, and IFN-γ at 16 hours after both stimulations as previously studied. However, TSLP protein and mRNA were not detected. It could be possible that our detection time was short. However, even if TSLP appears in much later time, mRNA should have been increased at 16 hours since iNKT cells show rapid responses to stimulation [21]. Based on above clues, we could postulate that the evaluation time points were appropriate for the detection of TSLP even in the primary cells.

In this study, to stimulate primary iNKT cells, we isolated them from mouse liver using α-GalCer tetramer. DN32.D3 cells were derived from thymic iNKT cells. This difference of cell origin could be another variable for TSLP production in iNKT cells. There could be different responses including production of IL-4 and IFN-γ, degree of activation, and cytokine profile according to the diverse origins of iNKT cells [21, 22].

To stimulate iNKT cells, we treated α-GalCer which is a well-known specific lipid antigen of iNKT cells. In addition to this, since they have TCR and linked signal transducer CD3 and CD28 as T cells, we used anti-CD3/CD28 antibodies to cover a wide range of TCR specific stimulations. However, we could not find any alteration of TSLP production with TCR specific stimulations in primary mouse liver iNKT cells. Interestingly, there are alternative activating pathways of iNKT cells secreting IL-4, IL-13, and IFN-γ by cytokines such as IL-12, IL-18, IL-33, TNF-α, and bacteria-derived lipid components [8, 9, 23]. Considering this, there is a possibility that TSLP might be induced by alternative pathways more than direct TCR activations, and this have to be confirmed in further studies.

In conclusion, this study suggested the possibility of TSLP production in iNKT cells, especially from DN32.D3 iNKT cells although primary mouse liver iNKT cells showed a different result.

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