Role of IL-18 on the activation of Vδ2+ T cells
– For the development of novel cancer immunotherapy –

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Abstract
IL-(interleukin)-18 is known to induce proliferation of Vδ2+ T cells and expression of CD56, resulting in enhancement of cytotoxic activity. We focused on the expression of CD56 and the direct action of IL-18 on Vδ2+ T cells to evaluate the importance of IL-18 for developing Vδ2+ T cells-based adoptive immunotherapy. Peripheral blood mononuclear cells (PBMCs) were purified from heparinized blood of healthy donors and cancer patients. CD56+ Vδ2+ T cells isolated from healthy subjects were cultured with IL-2 and IL-18 or with IL-2 alone. The number of CD56+ Vδ2+ cells increased compared with those with IL-2 alone. However, this increase was not statistically significant. CD56+ Vδ2+ T cells derived from CD56-depleted PBMCs were stimulated by phosphoantigens and cultured with IL-2 and IL-18, and showed significantly stronger cytotoxic activity than resultant CD56+ Vδ2+ T cells. IL-18 promotes the proliferation of Vδ2+ T cells from PBMCs of ovarian cancer patients. Thus, IL-18 is worth to be considered as a strong candidate in the development of effective cancer immunotherapy.

Keywords: CD56, Cytotoxicity, Gamma Delta T Cells, Interleukin-18, Ovarian cancer

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Introduction
Human γδ T cells expressing VγVδ2 (also known as Vγ2,Vδ2) recognize nonpeptide antigens derived from microbial pathogens1,2. These Vδ2+ T cells are also activated in an major histocompatibility complex-nonrestricted NK (natural killer) receptor-mediated manner3. Vδ2+ T cells show natural cytolytic activity against tumor cells in vitro3. Vδ2+ T cells can respond to tumor cells by detecting of phosphoantigens in target cells4.

CD56, also known as neural cell adhesion molecule, is a calcium-independent adhesion molecule, originally discovered in the nervous system. CD56 is a marker of NK cells and effector function of cytotoxic T lymphocytes (CTL) correlates with surface expression of CD565.

Vδ2+ T cells have attracted attention as effector cells in immunotherapy in recent years6-9. Peripheral blood Vδ2+ T cells were stimulated by isopentenyl pyrophosphate (IPP) or nitrogen containing bisphosphonates (N-BPs), which inhibit farnesyl pyrophosphate synthetase, resulting in intracellular accumulation of IPP in monocyte lineage cells7-9.

The 30%-70% of fresh Vδ2+ T cells or those expanded by a combination of IPP and IL-(interleukin)-2 express CD56 on their surface, and IPP-activated CD56+ Vδ2+ T cells are reported to be more potent antitumor effectors than their CD56-counterparts9.

IL-18 is an activator of T helper 1 cells leading to the production of gamma interferon (IFN-γ) and the proliferation of lymphocytes10, and IL-18 is classified as one of the members of the IL-1 cytokine superfamily, which are regulators of innate and acquired immune responses11. Vδ2+ T cells express cytotoxicity-related markers such as NKG2D, perforin, and CD56 which is related to the cytolytic activity of lymphocytes.

No clear differences in the expression of these surface markers have been reported on the specific action of IL-18 on the expansion of Vδ2+ T cells. IL-18 acts directly on Vδ2+ T cells through its receptors to augment their survival, proliferation, and cytokine production12. CD56bright CD11c+ cells strongly facilitate IL-18-mediated expansion of Vδ2+ T cells stimulated by 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP). IL-18 appears to
promote the proliferation of γδ T cells in multiple ways including forming growth centers, rendering signals through co-stimulatory molecules, and directly activating survival signals. These studies employed an activation of CD56<sup>bright</sup> CD11c cells using zolendronate through Vδ<sub>2</sub><sup>+</sup> T cells receptor-mediated recognition and the results concluded that IL-18 promotes growth of Vδ<sub>2</sub><sup>+</sup> T cells.

Here, we cultured CD56<sup>+</sup>-depleted cells and 2M3BIPP was utilized for antigens to exclude the impact of CD56<sup>bright</sup> CD11c cells and the endogenous production of IL-18 by monocyte lineage cells in order to clarify the direct function of exogenous IL-18 to Vδ<sub>2</sub><sup>+</sup> T cells.

**Materials and Methods**

**Reagents**

2M3BIPP, a pyrophosphonomonooester, was prepared at our institution as previously described<sup>2,10</sup>. Recombinant human interleukin-2 (rIL-2, Proleukin) was obtained from Novartis (Basel, Switzerland). Human rIL-18 was obtained from Medical & Biological Laboratories (Nagoya, Japan). ALyS505 NO medium (Iscove’s MEM-based serum-free medium) was obtained from Cell Science & Technology institute (Sendai, Miyagi, Japan). Lymphoprep<sup>TM</sup> was obtained from Fresenius Kabi Norge AS for Axis-Shield PoC AS (Oslo, Norway).

**Antibodies**

Anti-Vδ<sub>2</sub> (FITC), anti-CD56 (PE), anti-CD3 (Phycoerythrin-Cy5: PC5), and anti-Mouse IgG (PC7) were obtained from Beckman Coulter (Marseille, France). PE mouse IgG1κ (isotype control) was obtained from Becton Dickinson Biosciences. Mouse IgGs was used as isotype controls.

**Culture of U937 cells**

U937, a human histiocytic lymphoma cell line was cultured in complete RPMI 1640 supplemented with 10% FBS.

**Cell culture, stimulation, and γδ T cell expansion**

All donors gave informed consent in accordance with the Declaration of Helsinki for the use of their blood for research purposes and this study was approved from the Ethics Committee of the Tokyo Women’s Medical University (Approval number: 3126).

PBMCs were cultured in Alys505 supplemented with 10% pooled AB serum. For the expansion of γδ T cells, total PBMCs (1.0–3.3 × 10<sup>7</sup>/mL) were stimulated with 2M3BIPP (100 μM). On the next day, 10% human type AB serum was added with 200 U/mL of rIL-2 alone or in combination with rIL-18 (100 ng/mL). IL-2 and IL-18 were added every 2–3 days, and the cells were incubated for a total of 14 days. After incubation for assigned days, PBMCs were stained with specific antibodies and analyzed using flow cytometry (FACS Calibur, BD Biosciences). Mouse IgGs was used as isotype controls.

**Magnetic cell separation**

Cell isolation or depletion was performed using EasySep™ (Stemcell Technologies, Vancouver, Canada). CD56<sup>+</sup> and CD56<sup>−</sup> cells were isolated from PBMCs using the EasySep™ Human CD56 positive selection Kit (Stemcell Technologies). All procedures were performed following the manufacturer’s instructions. Cell purity was evaluated using flow cytometry (FACS Calibur, BD Biosciences).

**Cytotoxicity assay**

We performed cytotoxicity assay using the N-SPC Non-Radioactive Cellular Cytotoxicity Assay Kit (Technosuzuta, Nagasaki, Japan). U937 cells were used as a target. Tetrakis-pivaloyloxymethyl 2-(thiazole-2-ylamino) ethylidene-1, 1-bisphosphonate (PTA) (active bisphosphonate prodrug, Technosuzuta) was used at a concentration of 100 nM<sup>15,16</sup> to detect internal IPP-dependent cytotoxicity. Vδ<sub>2</sub><sup>+</sup> cells (at 14 days of culture as described above) were used as effector cells. The cytotoxicity assay was performed at E:T ratios ranging from 40:1–0.625:1 in triplicate culture. Time-resolved fluorescence was measured using an ARVO multiplate reader (2030 ARVO™ X, Perkin Elmer, Waltham, MA, USA). All procedures were performed following the manufacturer’s instruction.

**Statistical analysis**

Data were analyzed using 2-way tests, Wilcoxon signed-rank test, and Student’s t test depending on the data. A p-value of <0.05 was considered as significant. We used the software SAS version 9.4 (SAS Institute, NC, USA) with GLM Procedure or JMP version 13.0.

**Results**

**Proliferation of Vδ<sub>2</sub><sup>+</sup> T cells from CD56<sup>−</sup> depleted cells by IL-2 and IL-18**

CD56<sup>+</sup> and CD56<sup>−</sup> cells were isolated and cultured with a combination of 2M3BIPP and IL-2 or a combination of 2M3BIPP, IL-2, and IL-18 (Fig. 1A). A higher
expansion of Vδ2 T cells was obtained by a combination of IL-2 and IL-18 compared with IL-2 alone (Fig. 1B-e, f). However, this increase was not statistically significant. CD56+ cells cultured with IL-2 and IL-18, and with IL-2 alone, and there was no difference (Fig. 1B-c, d). Treatment of purified CD56+ cells with the combination of IL-2 and IL-18 (Fig. 1A-d) promoted higher expression of CD56 compared with treatment with IL-2 alone (Fig. 1A-c, 1C). Thus, IL-18 was found to promote proliferative Vδ2 T cells from CD56-depleted cells (Fig. 1B). However, this increase in expression was not significant. Further, box-and-whisker plot showed differences in expansion rates among individuals.

Fig. 1 IL-18 promotes proliferative Vδ2+ T cells from purified CD56- cells.
A. CD56+ and CD56- cells were isolated from PBMCs of healthy donor. The cells were cultured with IL-2 alone or in combination with IL-18. Dot plots of CD56-stained and Vδ2-stained gated CD3+ T cells are shown. A representative result of five independent experiments is shown.
B. Proliferation of Vδ2+ T cell (fold). Absolute cells numbers were determined and cell expansion was quantified. Box-plot diagrams from five individuals are shown.
C. Percentage of CD56+ Vδ2+ T cell / Vδ2+ T cell (%). Box-plot diagrams from five individuals are shown.

\[ \text{Role of IL-18 in Vδ2 T Cell Activation} \]
Cytotoxicity of CD56⁻ Vδ²⁺ T cells cultured with IL-2 and IL-18 and with IL-2 alone against U937 cells

Vδ²⁺ CD56⁻ cells were cultured with IL-2 and IL-18 and with IL-2 alone for 14 days. The cells cultured with IL-2 alone (Fig. 2A-a), those with IL-2 and IL-18 (Fig. 2A-b), CD56⁺ cells of those with IL-2 and IL-18 (Fig. 2A-c), and CD56⁻ cells of those with IL-2 and IL-18 (Fig. 2A-d) were compared on cytotoxicity against U937.

The cytotoxicity were increased in three of the four cases and these results showed significant higher cytotoxicity of the CD56⁻ Vδ²⁺ T cells cultured with IL-2 and IL-18 (Fig. 2A-d) compared with those cultured with IL-2 alone (Fig. 2A-c) against U937 cells (Fig. 2B). No difference in cytotoxicity was found using CD56⁻ cells cultured with IL-2 alone (Fig. 2A-a) or those cultured with IL-2 and IL-18 (Fig. 2A-b).

Proliferation of Vδ²⁺ T cells obtained from patients with ovarian cancer by IL-18

We investigated the usefulness of IL-18 in the expan-
sion of Vδ2+ T cells from PBMCs of cancer patients. PBMCs from 6 patients ovarian cancer and 4 with peritoneal cancer were cultured with IL-2 and IL-18, and with IL-2 alone. A significantly higher proliferation of Vδ2+ T cells was obtained by a combination of IL-2 and IL-18 than in those by IL-2 alone (Fig. 3).

Discussion

Vδ2+ T cells are considered to play an important role as immune-surveillance against tumors and infections. Okamura et al. reported that IL-18 markedly amplified Vδ2+ T cell responses to zoleodarate and that IL-2 and CD56bright CD11c+ cells play a key role in the IL-18 mediated proliferation of Vδ2+ T cells17-19. The mechanism of activation of by IL-18 leading to CD56+ Vδ2+ T cells or CD56– Vδ2+ T cells from PBMCs has not been studied. In this study, we focused on the CD56 expression of Vδ2+ T cells to clarify the direct effects of IL-18 to Vδ2+ T cells. Then we used CD56+ depleted cells and 2M3B1PP for antigens to exclude the impact of CD56bright CD11c cells and the endogenous IL-18 secreted by monocyte lineage cells.

These results suggests that the action of IL-18 might be greater on CD56+ cells than on CD56– cells. In other words, it was suggested that IL-18 had a direct action on Vδ2+ cells. Memory CD8+ T cells and NK cells are known to express IL-18 receptors (IL-18Rs). A previous study reported that γδ T cells represent memory cells in circulation and express IL-18 receptors. The study also concluded that IL-18 has a direct action on γδ T cells through IL-18Rs to activate antiapoptotic signals and expand γδ T cells35.

Then, we investigated the cytotoxicity of CD56+ cells. A previous study reported that CD56+ γδ T cells can kill squamous cell carcinoma cells more effectively than CD56– γδ T cells using standard 51Cr-release assay36. U937, a human histiocytic lymphoma cell line was used to assay cytotoxicity. Because U937 cells express MIC A/B, which is a ligand for NKG2D, U937 cells are targets for NK cells that possess NKG2D. We used PTA, an inhibitor of farnesyl pyrophosphate synthase to maintain intracellular IPP accumulation, because U937-directed cytotoxicity of Vδ2+ cells is dependent on IPP.

Previous studies have shown that IL-18 promotes the growth of Vδ2+ T cells13, 17, 20, 21, enhances the expression of CD56, and enhances cytotoxic activity40. However, it is unknown whether CD56+ Vδ2+ T cells are activated to express CD56 before culture, or CD56– Vδ2+ T cells expand more than CD56+ Vδ2+ T cells. We found that CD56– Vδ2+ T cells can be produced even when CD56+ cells are cultured, and CD56+ Vδ2+ T cells can be similarly produced by culture of CD56– cells (Fig. 1A). It was found that CD56– depleted cells cultured with IL-2 and IL-18 (Fig. 1A-f) showed expansion of Vδ2+ T cells; thus, we thought that IL-18 may be effective for CD56– cells. The cytotoxicity was variable among patients and individuals. CD56– Vδ2+ T cells cultured with IL-2 and IL-18 showed high cytotoxicity in some cases. CD56+ Vδ2+ T cells cultured from CD56–depleted PBMCs were also not always more cytotoxic than CD56– Vδ2+ T cells cultured from CD56–depleted PBMCs.

The number of γδ T cell was shown to decrease in peripheral blood from patients with ovarian cancer20. Obtaining sufficient effector cells to perform adoptive cellular immunotherapy seems not to be easy. In this study, it was found that high amount of Vδ2+ T cells were proliferated from PBMCs of patients with ovarian cancer and primary peritoneal cancer using IL-18 added to IL-2, and it is suggesting the usefulness of IL-18. Although culture with a combination of IL-2 and IL-18 resulted in significant higher proliferation of Vδ2+ T cells compared with IL-2 alone, three out of 10 patients showed Vδ2+ T cells increased only 8–36 times even when IL-18 was added. As described above, there is room for improvement of the culture system for such patients.

This study is an exploratory study and the number of specimens was small, therefore there is the limitation of the research.

In summary, our results show that IL-18 proliferate more CD56– Vδ2+ T cells than CD56+ Vδ2+ T cells and has an effect of promoting their higher cytotoxicity. IL-18 can therefore be considered an important cytokine in the development of cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

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