Role of IL-18 on the activation of $V\delta_2^+$ T cells – For the development of novel cancer immunotherapy –

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Abstract

IL(interleukin)-18 is known to induce proliferation of $V\delta_2^+$ 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 $V\delta_2^+$ T cells to evaluate the importance of IL-18 for developing $V\delta_2^+$ T cells-based adoptive immunotherapy. Peripheral blood mononuclear cells (PBMCs) were purified from heparinized blood of healthy donors and cancer patients. $CD56^- V\delta_2^+$ T cells isolated from healthy subjects were cultured with IL-2 and IL-18 or with IL-2 alone. The number of $CD56^- V\delta_2^+$ cells increased compared with those with IL-2 alone. However, this increase was not statistically significant. $CD56^- V\delta_2^+$ 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 $CD56^+ V\delta_2^+$ T cells. IL-18 promotes the proliferation of $V\delta_2^+$ 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 $\gamma\delta$ T cells expressing $V\gamma_9V\delta_2$ (also known as $V\gamma_2V\delta_2$) recognize nonpeptide antigens derived from microbial pathogens^{1,2)}. These $V\delta_2^+$ T cells are also activated in an major histocompatibility complex-nonrestricted NK (natural killer) receptor-mediated manner¹⁾. $V\delta_2^+$ T cells show natural cytolytic activity against tumor cells *in vitro*³⁾. $V\delta_2^+$ 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 $CD56^5)$.

$V\delta_2^+$ T cells have attracted attention as effector cells in immunotherapy in recent years⁶⁾. Peripheral blood $V\delta_2^+$ 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 $V\delta_2^+$ T cells or those expanded by a combination of IPP and IL(interleukin)-2 express CD56 on their surface, and IPP-activated $CD56^+ V\delta_2^+$ T cells are reported to be more potent antitumor effectors than their $CD56^-$ counterparts¹⁰⁾.

IL-18 is an activator of T helper 1 cells leading to the production of gamma interferon (IFN- γ) and the proliferation of lymphocytes¹¹⁾, and IL-18 is classified as one of the members of the IL-1 cytokine superfamily, which are regulators of innate and acquired immune responses¹²⁾. $V\delta_2^+$ 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 $V\delta_2^+$ T cells. IL-18 acts directly on $V\delta_2^+$ T cells through its receptors to augment their survival, proliferation, and cytokine production¹³⁾. $CD56^{\text{bright}} CD11c^+$ cells strongly facilitate IL-18-mediated expansion of $V\delta_2^+$ T cells stimulated by 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP). IL-18 appears to

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promote the proliferation of $\gamma\delta$ 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^{bright} CD11c cells using zoledronate through V δ_2 ⁺ T cells receptor-mediated recognition and the results concluded that IL-18 promotes growth of V δ_2 ⁺ T cells.

Here, we cultured CD56⁺-depleted cells and 2M3B1PP was utilized for antigens to exclude the impact of CD56^{bright} 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 V δ_2 ⁺ T cells.

Materials and Methods

Reagents

2M3B1PP, a pyrophosphomonoester, was prepared at our institution as previously described^{2, 14}. 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). LymphoprepTM was obtained from Fresenius Kabi Norge AS for Axis-Shield PoC AS (Oslo, Norway).

Antibodies

Anti-V δ_2 (FITC), anti-CD56 (PE), anti-CD3 (Phycoerythrin-Cy5: PC5), and anti-Mouse IgG (PC7) were obtained from Beckman Coulter (Marseille, France). PE mouse IgG1 κ (isotype control) was obtained from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA).

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 $\gamma\delta$ 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 purified from the heparinized blood of healthy donors and cancer patients. The median patient age was 69.0 years. Cancer patients included six patients with ovarian cancer (four high grade serous carcinoma, one clear cell carcinoma, and one endometrioid and neuroendocrine carcinoma) and four patients with primary peritoneal cancer (four high grade serous carcinoma). The stage 3c cancers were eight and 4b were two patients.

PBMCs were cultured in Alys505 supplemented with 10% pooled AB serum. For the expansion of $\gamma\delta$ T cells, total PBMCs ($1.0\text{--}3.3 \times 10^6/\text{mL}$) were stimulated with 2M3B1PP (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 EasySepTM (Stemcell Technologies, Vancouver, Canada). CD56⁺ and CD56[−] cells were isolated from PBMCs using the EasySepTM Human CD56 positive selection Kit (Stemcell Technologies), and CD56⁺ and CD56[−] V δ_2 ⁺ cells were isolated from cultured V δ_2 ⁺ cells using the EasySepTM Human CD56 positive selection Kit.

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^{15, 16} to detect internal IPP-dependent cytotoxicity. V δ_2 ⁺ 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 ARVOTM 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 V δ_2 ⁺ T cells from CD56⁺ depleted cells by IL-2 and IL-18

CD56⁺ and CD56[−] cells were isolated and cultured with a combination of 2M3B1PP and IL-2 or a combination of 2M3B1PP, IL-2, and IL-18 (Fig. 1A). A higher

expansion of $V\delta_2^+$ 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 expres-

sion of $CD56$ compared with treatment with IL-2 alone (Fig. 1A-c, 1C). Thus, IL-18 was found to promote proliferative $V\delta_2^+$ 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 $V\delta_2^+$ 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 $V\delta_2$ -stained gated $CD3^+$ T cells are shown.

A representative result of five independent experiments is shown.

B. Proliferation of $V\delta_2^+$ T cells (fold). Absolute cells numbers were determined and cell expansion was quantified. Box-plot diagrams from five individuals are shown.

x : Statistics outliers

C. Percentage of $CD56^+ V\delta_2^+$ T cells/ $V\delta_2^+$ T cells (%) expression. Box-plot diagrams from five individuals are shown.

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 cyto-

toxicity 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-

Fig. 2 Higher anti-U937 cytotoxicity of CD56⁺ Vδ₂⁺ T cells cultured with IL-2 and IL-18 compared with those cultured with IL-2 alone.

- CD56⁺ cells were isolated from healthy donor PBMCs. The cells were cultured with IL-2 alone or in combination with IL-18. After 14 days, CD56⁺ and CD56⁺ Vδ₂⁺ cells were isolated from cultured Vδ₂⁺ cells. Dot plots of CD56-stained and Vδ₂-stained gated CD3⁺ T cells are shown. Representative dot plots from three experiments are shown.
- We performed cytotoxicity assays using N-SPC and the U937 cell line. The E: T ratio used was 40:1 (Vδ₂⁺ T cells: 2 × 10⁵ cells, U937: 5 × 10³ cells) in triplicate culture. Representative data from four independent experiments are shown.

Fig. 3 IL-18 had the effect of proliferating Vδ₂⁺ T cells in ovarian cancer patients

PBMCs were purified blood from cancer patients included six ovarian cancer patients and four peritoneal cancer patients. At pre-culture and after 14 days of culture, cells were stained to confirm Vδ₂⁺ T cell count. Absolute cell numbers were determined, and Vδ₂⁺ T cell expansion was quantified. Box-plot diagrams are shown.

sion of $V\delta_2^+$ 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 $V\delta_2^+$ T cells was obtained by a combination of IL-2 and IL-18 than in those by IL-2 alone (Fig. 3).

Discussion

$V\delta_2^+$ 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 $V\delta_2^+$ T cell responses to zoledronate and that IL-2 and CD56^{bright} CD11c⁺ cells play a key role in the IL-18 mediated proliferation of $V\delta_2^+$ T cells¹⁷⁻¹⁹. The mechanism of activation of by IL-18 leading to CD56⁺ $V\delta_2^+$ T cells or CD56⁻ $V\delta_2^+$ T cells from PBMCs has not been studied. In this study, we focused on the CD56 expression of $V\delta_2^+$ T cells to clarify the direct effects of IL-18 to $V\delta_2^+$ T cells. Then we used CD56⁻ depleted cells and 2M3B1PP for antigens to exclude the impact of CD56^{bright} 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 $V\delta_2^+$ cells. Memory CD8⁺ T cells and NK cells are known to express IL-18 receptors (IL-18Rs). A previous study reported that $\gamma\delta$ T cells represent memory cells in circulation and express IL-18 receptors. The study also concluded that IL-18 has a direct action on $\gamma\delta$ T cells through IL-18Rs to activate antiapoptotic signals and expand $\gamma\delta$ T cells¹³.

Then, we investigated the cytotoxicity of CD56⁻ cells. A previous study reported that CD56⁺ $\gamma\delta$ T cells can kill squamous cell carcinoma cells more effectively than CD56⁻ $\gamma\delta$ T cells using standard ⁵¹Cr-release assay¹⁰.

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 $V\delta_2^+$ cells is dependent on IPP.

Previous studies have shown that IL-18 promotes the growth of $V\delta_2^+$ T cells^{13, 17, 20, 21}, enhances the expression of CD56, and enhances cytotoxic activity¹⁰. However, it is unknown whether CD56⁻ $V\delta_2^+$ T cells are activated to express CD56 before culture, or CD56⁺ $V\delta_2^+$ T cells expand more than CD56⁻ $V\delta_2^+$ T cells. We found that CD56⁻ $V\delta_2^+$ T cells can be produced even when CD56⁺ cells are cultured, and CD56⁺ $V\delta_2^+$ 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 $V\delta_2^+$ T cells; thus,

we thought that IL-18 may be effective for CD56⁻ cells.

The cytotoxicity was variable among patients and individuals. CD56⁻ $V\delta_2^+$ T cells cultured with IL-2 and IL-18 showed high cytotoxicity in some cases. CD56⁺ $V\delta_2^+$ T cells cultured from CD56⁺-depleted PBMCs were also not always more cytotoxic than CD56⁻ $V\delta_2^+$ T cells cultured from CD56⁺-depleted PBMCs.

The number of $\gamma\delta$ T cell was shown to decrease in peripheral blood from patients with ovarian cancer²². Obtaining sufficient effector cells to perform adoptive cellular immunotherapy seems not to be easy. In this study, it was found that high amount of $V\delta_2^+$ 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 $V\delta_2^+$ T cells compared with IL-2 alone, three out of 10 patients showed $V\delta_2^+$ 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⁻ $V\delta_2^+$ T cells than CD56⁺ $V\delta_2^+$ 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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