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Possible Application of Ascites-infiltrating Gamma-delta T Cells for Adoptive Immunotherapy

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Abstract. *Background/Aim: Malignant ascites contain many tumour-infiltrating lymphocytes. γδ T cells with antitumour activity have attracted attention as effector cells in cancer immunotherapy. $V\delta_2^+$ T cells were cultured from peripheral blood mononuclear cells (PBMCs) and ascites-infiltrating lymphocytes (AILs) to compare the differences in response to 2-methyl-3-butenyl-1-pyrophosphate (2M3B1-PP) and zoledronate (Zol) as antigens in vitro. Materials and Methods: To expand $V\delta_2^+$ T cells from PBMCs and AILs from 29 patients with cancer, these cells were cultured and subjected to analysis. Results: The proliferation rate of $V\delta_2^+$ T cells was higher in both PBMCs and AILs when cultured with Zol than with 2M3B1-PP. Although $V\delta_2^+$ T cells show a higher rate of expansion in AILs compared to PBMCs, the number of mixed tumour cells in ascites was decreased when cultured with Zol. Conclusion: $V\delta_2^+$ T cells in AILs are cytotoxic to tumour cells in ascites and may be considered in adoptive immunotherapy.*

The usefulness of immunotherapy using tumour-specific tumour-infiltrating lymphocytes (TIL) has been reported (1), but it is difficult to obtain sufficient TIL for therapeutic purposes in solid cancers. Therefore, their use under these circumstances is not yet widespread. However, ascites contains abundant TIL, called ascites-infiltrating lymphocytes (AILs), that are easier to obtain than TILs. A previous report on patients with ovarian cancer and malignant ascites who received treatment with AILs and/or chemotherapy showed that the combination therapy extended time to progression compared to chemotherapy alone (2).

Ovarian cancer is often diagnosed at an advanced stage, and cases of decreased quality of life due to malignant ascites accumulation are common; therefore, ascites control is important. Although puncture drainage of ascites is effective in alleviating symptoms, the nutritional status declines due to a loss of protein. The ascites get accumulated within a few days, and frequent puncturing is necessary (3).

In addition, human γδ T cells expressing $V\gamma_9V\delta_2$ (also known as Vγ2Vδ2) recognize nonpeptide antigens derived from pyrophosphomonoester compounds (4, 5). $V\delta_2^+$ T cells, which possess antitumour activity, are attracting attention as effector cells in cancer immunotherapy; various clinical examinations have been performed, and their usefulness has been reported (6). $V\delta_2^+$ T cells respond to the presence of small isoprenoid metabolites, such as self-isopentenyl pyrophosphonate (IPP) with butyrophilin 3A1(BFN3A1) conformation on normal and tumor cells (7). Nitrogen-containing bisphosphonates (N-BPs) inhibit farnesyl pyrophosphate synthetase in normal and tumor cells, thereby resulting in intracellular accumulation of IPP, and subsequently, $V\delta_2^+$ T cells respond.

The present study focuses on $V\delta_2^+$ T cells in (AILs) for possible clinical application.

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Materials and Methods

Reagents. 2-Methyl-3-butenyl-1-pyrophosphate (2M3B1-PP), a pyrophosphomonoester that acts on $V\delta_2^+$ T cells with an effect 100 times stronger than IPP, was prepared at our institution as described previously (5, 8). Recombinant human interleukin-2 (Proleukin) and Zoledronate (Zol) were obtained from Novartis (Basel, Switzerland). ALyS505 NO medium (Iscove's MEM-based serum-free medium) was obtained from the Cell Science & Technology Institute (Sendai, Miyagi, Japan).

Antibodies. Anti-Vδ_2 (FITC), anti-CD56 (PE), and anti-CD3 (Phycoerythrin-Cy5) were obtained from Beckman Coulter (Marseille, France). PE mouse IgG1ϰ (isotype control) was obtained from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA).

Cell culture, stimulation, and Vδ_2^+ T cell expansion. All donors gave informed consent in accordance with the Declaration of Helsinki for the use of their blood and ascites for research purposes. Approval was obtained from the Review Board of the Tokyo Women's Medical University of Innsbruck (Approval number: 3126). Peripheral blood was obtained from heparinized blood samples, and patient ascites were obtained by peritoneal puncture or from intraoperative ascites. Peripheral blood was diluted with PBS and layered on Lymphoprep. PBMCs were isolated by centrifugation (1,500 rpm, 35 min) and washed three times.

The cellular component of ascites was isolated by centrifugation (2,000 rpm, 10 min). Cellular component was diluted with PBS and layered on Lymphoprep. AILs/cancer cell mixtures (CCs) were purified from ascites by centrifugation (1,500 rpm, 35 min).

In total, 23 patients with ovarian cancer; one patient with fallopian tube cancer; and six patients with primary peritoneal cancer who were at stages 1 c, 2 c, 3 c, and 4 b were included in the present study (Table I).

PBMCs and AILs/CCs were cultured in Alys505 medium and incubated at 37˚C/5% CO_2 atmosphere.

To expand Vδ_2^+ T cells, total PBMCs or AILs (1-1.3×10^6/ml) were stimulated with 2M3B1-PP (100 μM) or Zol (5 μM). The next day, 10% human-pooled type AB serum was added along with 200 U/ml of rhIL-2. rhIL-2 was added every 2-3 days, and the cells were cultured for a total of 14 days. These cultured cells were stained with anti-Vδ_2 (FITC), anti-CD56 (PE), and anti-CD3 (Phycoerythrin-Cy5) for 20 min on ice, as described previously (9). These cells were subjected to flow cytometry (FACS Calibur, Becton Dickinson Biosciences).

Mouse IgGs were used as isotype controls.

Statistical analysis. Data were analysed using a Wilcoxon signed-rank test. A *p*-value <0.05 was considered significant. JMP version 13.0 software was used for statistical analysis.

Table I. *Characteristics of patients*.

Age	Years
Median	65
Min–Max	38-80
Patients	Number
Total	29
Diagnosis	
Ovarian cancer	22
Fallopian tubal cancer	1
Primary peritoneal cancer	6
Stage	
1c	3
2c	2
3c	17
4b	7
Pathology	
High-grade serous carcinoma	20
Low-grade serous carcinoma	1
Mucinous carcinoma	1
Endometrioid carcinoma	2
Clear carcinoma	5
Endometrioid and neuroendocrine carcinoma	1
Sample collection	
Both blood and ascites	14
Blood only	13
Ascites only	2
Blood stimulated with 2M3B1-PP	27
Blood stimulated with Zol	19
Ascites stimulated with 2M3B1-PP	16
Ascites stimulated with Zol	14

Results

Twenty-two patients with ovarian cancer, one patient with fallopian tube cancer, and six patients with primary peritoneal cancer were examined in the present study (Table I). The median patient age was 65.0 years, and stage 3c cancers were the most common (17 cases).

First of all, PBMCs and AILs were cultured with either 2M3B1-PP or Zol to confirm whether Vδ_2^+ T cells in AILs were able to respond to these reagents and expand.

After the culture, the proportion of Vδ_2^+ T cells in $CD3^+$ cells was calculated. No significant difference was observed, with the median for blood being 1.42% and that for ascites being 1.35% prior to culturing (Figure 1A). The proportion of Vδ_2^+ T cells after the culture with Zol increased significantly in peripheral blood (92.7%; Figure 1B), and there was an accompanying significant increase in the proliferation rate (204.9-fold; Figure 1C) compared with the culture with 2M3B1-PP (39.1% and 58.8-fold).

The proportions of Vδ_2^+ T cells in PBMCs and AILs were significantly higher in the presence of either 2M3B1-PP or Zol, whereas in AILs, the ratio tended to be higher with Zol than that with 2M3B1-PP, although this difference was not significant (Wilcoxon signed-rank test, *p*=0.0645; Figure 1B).

In addition, the growth rate of Vδ_2^+ T cells cultured with 2M3B1-PP was significantly greater in PBMCs (58.8 times) than in AILs (1.9 times).

In the ascites, the median growth rate of Vδ_2^+ T cells cultured in Zol was 3.8 times and that of those cultured in 2M3B1-PP was 1.9 times. Zol seemed to be a more potent stimulator for Vδ_2^+ T cells in AILs than 2M3B1-PP, but no significant difference was observed between these reagents. During review of individual cases, 4 of 16 cases (25%) in which AILs were cultured with 2M3B1-PP and 6 out of 14 cases (43%) in which AILs were cultured with Zol showed more than 50 times increased numbers of Vδ_2^+ T cells.

Next, the impact of the two reagents to CCs mixed in ascites during the culture was observed. Representative data are shown in Figure 2. The proportion of Vδ_2^+ T cells in freshly isolated AILs was 0.9% and following culture increased up to 10.8% when stimulated with 2M3B1-PP and

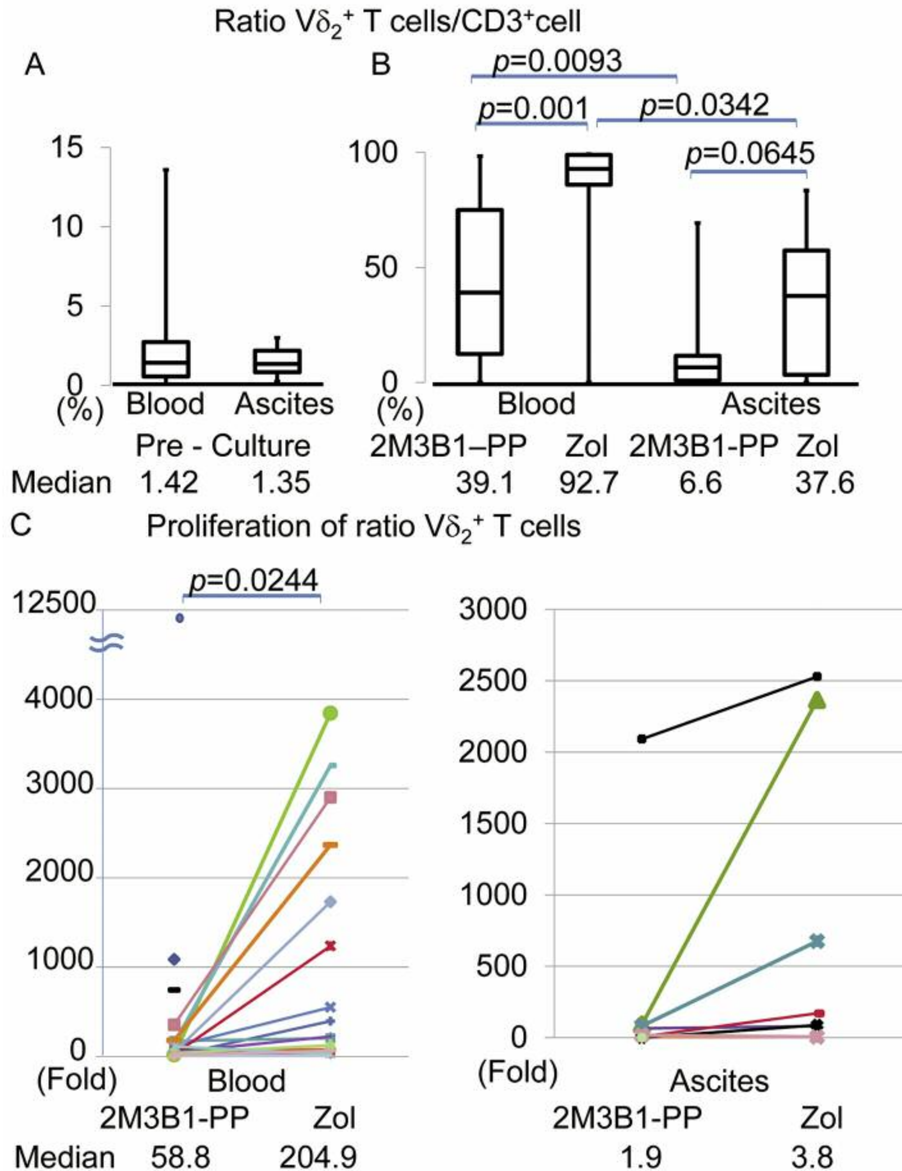

Figure 1. *Ratio Vδ_2^+ T cells/CD3+ cell and proliferation of ratio Vδ_2^+ T cells in peripheral blood and ascites. The proportion of Vδ_2^+ T cells in CD3+ cells in peripheral blood from 20 patients with ovarian cancer, one patient with fallopian tube cancer; and six patients with primary peritoneal cancer and from the ascites of 11 patients with ovarian cancer and five patients with primary peritoneal cancer (A). The proportion of Vδ_2^+ T cells relative to CD3+ cells after culture with each antigen (B). Proliferation of Vδ_2^+ T cells from blood and ascites after 14 days in culture with 2M3B1-PP or Zol (C).*

up to 61.0% when stimulated with Zol (Figure 2A). The number of Vδ_2^+ T cells increased 52-fold when stimulated with 2M3B1-PP and 426- fold when stimulated with Zol. (Figure 2B).

Surprisingly, because Vδ_2^+ T cells in AILs were growing in culture (Figure 2B), the number of mixed CCs decreased and eventually disappeared (Figure 2C). This phenomenon was confirmed since the results showed that mixed CCs disappeared in two of 16 cases (1.5%) whose AILs were stimulated with 2M3B1-PP and in three of 14 cases (21.4%) whose AILs were stimulated with Zol.

Discussion

It has been reported that the ratio of Vδ_2^+ T cells in peripheral blood is 1%-10%. No clear correlation was found between the Vδ_2^+ T cells ratio in peripheral blood and those in ascites in the present study.

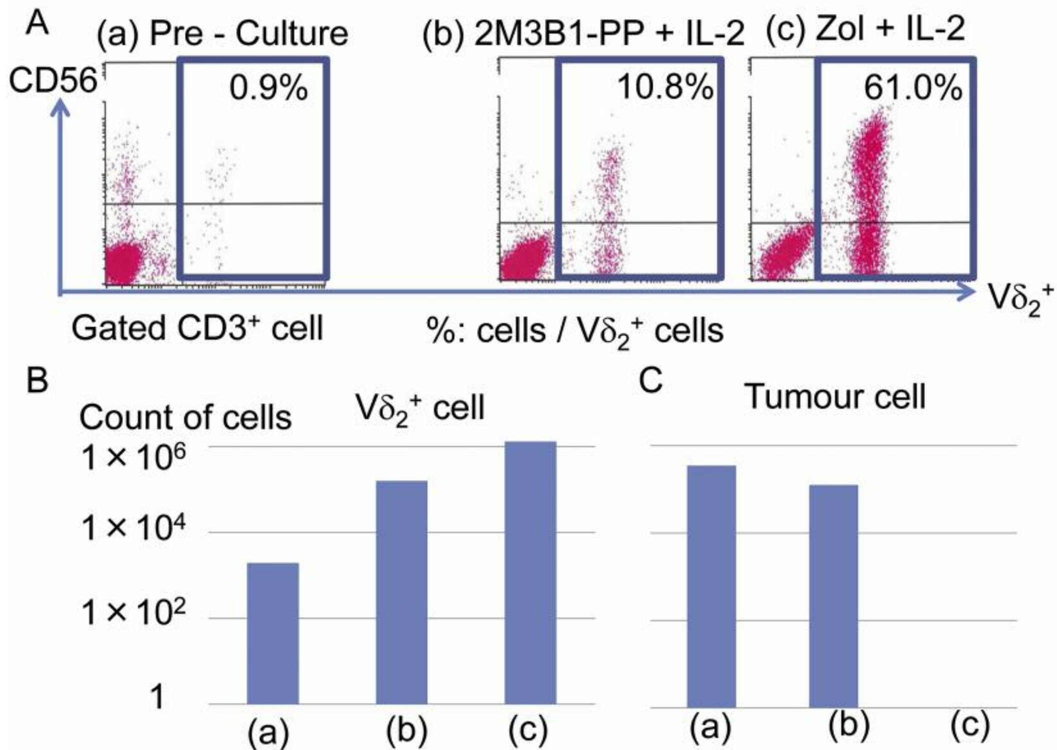

Figure 2. *Representative data from one patient with stage 3c primary peritoneal cancer. Cells were either pre-cultured (a) or cultured in 2M3B1-PP+IL-2 (b) or Zol+ IL-2 (c) for 14 days. PBMCs and tumour cells from ascites were stained with anti-CD3, anti-CD56, and anti-Vδ_2 antibodies and analysed using flow cytometry (A). Dot plots of CD56- and Vδ_2-stained and gated CD3$^+$ T cells are shown. In addition, absolute numbers of V$\delta_2$$^+$ (B) and tumour (C) cells were determined.*

Some studies have investigated the relationship between the number and type of TILs in ovarian cancer (10); however, the proportion of V$\delta_2$$^+$ T cells in TILs has not been studied. Here it is shown that the proportion of V$\delta_2$$^+$ T cells in AILs is 1.35%. This ratio increased significantly in peripheral blood after culture in Zol compared with culture in 2M3BI1-PP, and the proliferation rate also increased. The same tendency was observed in ascites. The mechanism of activation of V$\delta_2$$^+$ T cells by Zol was slightly more complex; Zol inhibits farnesyl pyrophopate synthase in mevalonic acid metabolism in normal monocyte lineage cells and tumor cells, which results in intracellular accumulation of IPP. IPP binds to the B30.2 intracellular domains of BTN3A and activates V$\delta_2$$^+$ T cells.

Our findings and previous studies suggest that Zol acts on co-cultured tumour cells and antigen presenting cells V$\delta_2$$^+$ T cells in ascites, and it is thought to further increase γδ T cell proliferative capacity (11, 12). In turn, activated V$\delta_2$$^+$ T cells showed potent cytotoxic activity to tumour cells. In ascites, no significant increase in the median proportion or proliferation rate was observed when Zol was used compared with 2M3B1-PP. It may depend on the patients; a review of individual cases demonstrated that 43% (six of 14 cases) of patients showed increased number of V$\delta_2$$^+$ T cells, which was greater than 50 times the number when Zol was used. Recent studies report that Zol and IL-18 promote vigorous proliferation of V$\delta_2$$^+$ T cells (13, 14), and IL-18 will be useful for the expansion of V$\delta_2$$^+$ T cells in AILs. However, it is unclear which patients showed vigorous increase in V$\delta_2$$^+$ T cells in AILs and, therefore, further examination is needed.

Regarding clinical application, peripheral blood V$\delta_2$$^+$ T cells have a higher median proliferation, but the amount of peripheral blood V$\delta_2$$^+$ T cells that can be taken from one apheresis is less and invasive; however, there is a greater amount of V$\delta_2$$^+$ T cells in ascites that can be recovered by a single ascitic puncture. There is a possibility that more V$\delta_2$$^+$ T cells may be obtained by preselecting and culturing cases that grow well in V$\delta_2$$^+$ T cells in ascites than in peripheral blood.

In addition, when using AILs for immunotherapy, it is difficult to remove all CCs from ascites before the culture. In the present study, even if the CCs were mixed before the culture, during the culture of V$\delta_2$$^+$ T cells stimulated by Zol, the CCs acted as antigen presenting cells to V$\delta_2$$^+$ T cells and were in turn injured as target cells by V$\delta_2$$^+$ T cells, and

finally, they disappeared from the culture. In bacterial peritonitis, there is a possibility of bacterial contamination of ascites. When using AIL for the treatment of cancer, the prevention of infection of recipients can be achieved by sterility and endotoxin testing of the material at the time of shipment. Such treatments hold promises for treating other cancer types such as ovarian cancer, gastric cancer, and colon cancer, that are likely to accumulate malignant ascites (2, 15).

In conclusion, $V\delta_2^+$ T cells in ascites can be sufficiently expanded, depending on the patient, opening up the possibility of culturing and usage as a source of cells for adoptive immunotherapy.

Conflicts of Interest

The Authors have no financial conflicts of interest regarding this study.

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