Application of gamma-delta T cells obtained by ascites filtration for immunotherapy against malignant refractory ascites

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Abstract

Ovarian cancer often presents with carcinomatous ascites effusion. Cell-free and concentrated ascites reinfusion therapy (CART) provides a symptomatic treatment. The ascitic fluid contains a large number of lymphocytes including γδ T cells which are cytotoxic and used as effector cells in cancer immunotherapy. We collected ascites-infiltrating lymphocytes (AILs) from the ascitic fluid that was obtained for CART.

We examined four patients with ovarian cancer and two patients with primary peritoneal cancer. Five patients were at stage 3c, and one was at stage 4b. In patients with ascitic ovarian cancer, in which ascites is accumulated, we collected AIL from a filter and were able to culture γδ T cells. The number of cultured Vδ2⁺ T cells were 3.2 (range, 0.7–63) × 10⁵/L. We cultured AILs obtained from CART with pyrophosphomonoester or zoledronic acid (Zol) as an antigen, interleukin (IL2), and with or without IL18. In case of culture in pyrophosphomonoester, IL2, and IL18, the proportion of Vδ2⁺ T cells / CD3 positive cells was 71%, and the proliferation rate (cell number after culture/those pre-culture) of Vδ2⁺ T cells was 83. Cells cultured in Zol, IL2, and IL18 in AILs exhibited isopentenyl pyrophosphate (IPP)-dependent cytotoxicity, and the median level of it was 5.3%.

γδ T cells from AILs obtained from CART have a cytotoxic activity. However, the cytotoxic activity was low, which needs improvement. In future, we may use it as a source for adoptive immunotherapy, if we can improve the proliferation rate and cytotoxic activity.

Keywords: γδ T Cells; ovarian cancer; ascites-infiltrating lymphocyte; cell-free and concentrated ascites reinfusion therapy

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cinomatous ascites by tumor infiltrating T cells (ascites-infiltrating lymphocytes; AILs), not by the direct action for cancer cells, was anticipated. Catumaxomab was approved in Europe in 2009; however, it was withdrawn from the US market in 2013, and from the EU market in 2017. Therefore, a new treatment strategy for intractable ascites is necessary.

The existing Vγ2/Vδ2 T cells (hereafter γδ T cells) recognize nonpeptide antigens, such as pyrophosphate monoester that is produced by mycobacteria; in the peripheral blood of humans, γδ T cells exhibit considerable phylaxis for intracellular parasitism-related bacteria. In tumor cells, pyrophosphate monoester, an intermediate metabolite of the mevalonate metabolism course, accumulates in high density, is a target of γδ T cells, and is eventually damaged. Therefore, γδ T cells exhibit anti-tumor activity, and to date, various clinical trials have been conducted in this regard.

When adoptive immunotherapy is administered, peripheral blood mononuclear cells are often collected by apheresis. However, owing to the burden among patients with ovarian cancer with accumulated ascites, dehydration, and anemia to undergo apheresis, we considered collecting the samples from the lymphocytes in ascites.

The utility of immunotherapy using tumor infiltrating lymphocytes (TILs), which are tumor specific, has been reported. However, obtaining of sufficient number of TIL is technically difficult from solid tumors in patients with non-disseminated and manageable tumor. Conversely, carcinomatous ascites include a large number of lymphocytes than cancer cells and γδ T cells with anti-tumor activity have garnered attention as effector cells of cancer immunotherapy.

It was reported that the culture of γδ T cells is possible in the ascitic fluid. Initially, we collected AILs from the hollow fiber of the filtration column used at discarding ascitic filtration concentration. A sufficient number of AILs seems to be obtained easily if obtained from a filter after CART and the growth rate and the antigen-reacting fashion of cultured γδ T cells were calculated and these cells were assessed to be used as effector cells of immunotherapy. Furthermore, we inhibited the farnesyl pyrophosphate synthase in γδ T cells using bisphosphonate to multiply γδ T cells; a method enables making isopen-teryl pyrophosphate (IPP) accumulated in cells and we used zoledronic acid (Zol).

Interleukin (IL) 18 directly acts on Vδ2+ T cells through its receptors to induce the proliferation of Vδ2+ T cells and enhance the cytotoxic activity. Moreover, IL18 promotes the proliferation of Vδ2+ T cells from PBMCs in patients with ovarian cancer.

Studies have reported a method to induce 2-methyl-3-butenyl-1-pyrophosphate (2M3B1-PP), a pyrophosphonooester that acts on Vδ2+ T cells, with 100 times stronger impact than IPP, a synthetic IPP analog directly cultured using these two methods. This study reports a method to collect AILs from the ascitic fluid obtained from CART.

### Materials and Methods

#### Reagents

We prepared 2M3B1-PP at our institution, as described previously. We obtained recombinant human interleukin-2 (IL2; Proleukin, Basel, Switzerland) and Zol (Novartis, Basel, Switzerland). Human rIL18 was obtained from Medical & Biological Laboratories (Nagoya, Japan), while AILys505 NO medium (Iscove’s MEM-based serum-free medium) was obtained from the Cell Science & Technology Institute (Sendai, Miyagi, Japan). Dulbecco’s Modified Eagle’s Medium (DMEM)-high glucose was obtained from Sigma-Aldrich (St. Louis, MO). Lymphoprep™ was obtained from Fresenius Kabi Norge AS for Axis-Shield PoC AS (Oslo, Norway).

#### Antibodies

We obtained anti-Vδ2 (FITC, monoclonal mouse anti-human, Cat. No. 280798), anti-CD56 (PE, monoclonal mouse anti-human, Cat. No. A07788), and anti-CD3 (phycoerythrin-Cy5, monoclonal mouse anti-human, Cat. No. A07749 ) from Beckman Coulter (Marseille, France). Furthermore, PE mouse IgG1κ (isotype control, Cat. No. 550617) was obtained from Becton Dickinson Pharmingen (Franklin Lakes, NJ).

#### CART

CART is an instrument for collecting malignant ascites in a bag, removing the cell component in the first filter, recovering the protein component with the second concentrator, and intravenously injecting the filtered and concentrated protein component into patients (Fig. 1).

#### Culturing Caov3 cells

We obtained Caov3 (ATCC® HTB-75™), a human ovarian cancer cell line from ATCC (Manassas, VA). We cultured it according to the instructions in DMEM supplemented with 10% FBS.

#### Patients

The patients who were diagnosed as ovarian cancer (or fallopian tube cancer, peritoneal carcinoma) or treated at the Department of Obstetrics and Gynecology, Tokyo Women’s Medical University Hospital were entered in this study.

Criteria for selection was age, ≥20 years old and performance status (PS), ≥2.

Exclusion criteria were patients with HIV infection, an HTLV-1 carrier, and patients with infection, those who require continuous administration of the immunosuppressive drugs, those with pregnancy and those whom we...
are nursing.

Overall, four patients with ovarian cancer with stage 3c and two patients with primary peritoneal cancer of stage 4b were included in this study.

**Cell culture, stimulation and Vδ2⁺ T cell expansion**

In this study, patients’ ascites were obtained by filtration filter after CART. Then, the cellular component of ascites was isolated by centrifugation (2000 rpm, 10 min) and then we diluted the cellular component with PBS and layered on Lymphoprep. AILs/cancer cell mixtures were purified from ascites by centrifugation (1500 rpm, 35 min), cultured in Alys505 medium and incubated at 37°C/5% CO₂ atmosphere for 14 days.

We stimulated AILs with 2M3B1-PP (100 μM) or Zol (5 μM) to expand Vδ2⁺ T cells. 10% human-pooled type AB serum with 200 U/mL of IL2 alone or in combination with IL18 (100 ng/mL) were added after the culture for 24 hours. IL2 alone or in combination with IL18 were added every 2–3 days, and cells were cultured in Alys505 medium at 37°C/5% CO₂ atmosphere for 14 days. These cultured cells were stained with anti-Vδ2 (FITC), anti-CD56 (PE) and anti-CD3 (phycoerythrin-Cy5) for 20 min on ice[13]. Finally, these cells were subjected to flow cytometry (FACSCalibur, Becton Dickinson Biosciences) and we used mouse IgGs as isotype controls.

**Cytotoxicity assay**

We performed the cytotoxicity assay using the N-SPC Non-Radioactive Cellular Cytotoxicity Assay Kit (Technosuzuta, Nagasaki, Japan)[14] according to protocol. Caov3 cells were used as a target. In addition, we used tetrakis-pivaloyloxymethyl 2-(thiazole-2-ynamino) ethylidene-1,1-bisphosphonate (PTA; active bisphosphonate prodrug, Technosuzuta) at a concentration of 500 nM[15, 16] to detect internal IPP-dependent cytotoxicity. Vδ2⁺ T cells (14-day culture with Zol, IL2 and IL18, as described above) were used as effector cells. We performed the cytotoxicity assay at E:T ratios 40:1–0.625:1 in triplicate culture. Time-resolved fluorescence was measured using an ARVO multiplate reader (2030 ARVO™ X; PerkinElmer, Waltham, MA). All procedures were performed along with the manufacturer’s instructions.

**Statistical analysis**

In this study, data analysis was performed using the Wilcoxon signed-rank test. We considered $P < 0.05$ as statistically significant. We used JMP version 13.0 software for statistical analyses. The low number of subjects ($N = 6$) may be attributable to a lack of power in the study.

**Ethics**

We obtained informed written consent from all donors as per the Declaration of Helsinki guidelines regarding the use of their ascites for research purposes. This study protocol was approved by the Review Board of the Tokyo Women’s Medical University (approval number: 3126, 3784).

**Study Design**

This trial was a prospective study. Primary outcomes were proportion and number of γδ T cells in ascites under various culture conditions: changing the culture conditions and increasing the proportion of γδ T cells in ascites before and after culture. The samples were collected from October, 2015 to October, 2016.


Table 1. Number of collected AILs

| Case | Ascites (L) | Count of AILs (×10^7 cells) | Ratio Vδ2+ T cells/CD3+ cells (%) | Count of Vδ2+ T cells (×10^6 cells) | Count of AILs (×10^7 cells/L) |
|------|-------------|-----------------------------|----------------------------------|----------------------------------|-----------------------------|
| 1    | 1.28        | 38                          | 2.1                              | 81                               | 30                          |
| 2    | 3.97        | 4.0                         | 1.2                              | 4.8                              | 1.0                         |
| 3    | 3.83        | 8.8                         | 0.3                              | 2.5                              | 2.3                         |
| 4    | 2.31        | 15                          | 1.6                              | 24                               | 6.4                         |
| 5    | 1.37        | 6.7                         | 0.9                              | 6.0                              | 4.9                         |
| 6    | 4.32        | 4.0                         | 2.3                              | 9.0                              | 0.9                         |

Results

Obtained from a filter after CART

We examined four patients with ovarian cancer and two patients with primary peritoneal cancer in this study. The median patient age was 69 (range: 57–77) years, with stage 3c cancer in five patients and stage 4b in one patient. After performing paracentesis when carcinomatous ascites accumulated in six patients, we obtained a median 3.1 (range: 1.3–4.3) L of the ascitic fluid and performed concentrated ascitic fluid filtration. The ascitic barrier filter was washed with saline after the treatment and AILs were collected (Fig. 1). The number of AILs were 7.7 (4–38) × 10^7 cells and 3.6 (0.9–30) × 10^7 cells/L (ascites) were collected. The number of Vδ2+ T cell was 7.5 (2.5–81) × 10^5 cells, and 3.2 (0.7–63) × 10^5/L (ascites) (Table 1).

The growth rate and antigen-reactive comparison of Vδ2+ T cells

The proportion of Vδ2+ T cell/CD3+ cells was 1.2% median before culture (Fig. 2A). We cultured AILs obtained from CART filter with 2M3B1-PP or Zol because immunotherapy was provided using these cells. The proportions of Vδ2+ T cells in AILs were tended to be higher with 2M3B1-PP and IL2 with or without IL18, although this difference was not statistically significant (Wilcoxon signed-rank test, N = 5, df = 4, two-sided test, p = 0.067; Fig. 2A).

The proportions of Vδ2+ T cells in AILs with 2M3B1-PP, IL2 and IL2 and IL18 were higher than those of pre-culture cells (Wilcoxon signed-rank test, P < 0.05; Fig. 2A). The significant differences between 2M3B1-PP and Zol were not seen in these experiments.

In the case of culture in 2M3B1-PP or Zol and IL2, the median proliferation rates of Vδ2+ T cells were 1.3 and 6.6, respectively, and those with IL18, the rates increased by 83 and 23, respectively. There was no significant difference in proliferation rates between 2M3B1-PP and Zol. Despite a limited number of cases, there was no significant difference (Fig. 2B and C).

Cytotoxocities of ovarian cancer cell line

We examined the cytotoxocities of Vδ2+ T cells that we obtained from a CART filter and cultured using N-SPC. In a prior study, we cultured AILs with Zol and found that cultured AILs had few residual carcinoma cells, then we assumed that Zol was more desirable antigen than 2M3B1-PP. Cytotoxocities of cultured AILs obtained from a CART filter in Zol, IL2 and IL18 were examined with E:T ratio of 40:1 (effector cells, 2 × 10^6; target cells, 5 × 10^5). Cells cultured in Zol, IL2 and IL18 in AILs exhibited IPP-dependent cytotoxicity, and the median of the four cases was 5.3% (Fig. 3A). The cytotoxicity was E:T ratio-dependent (Fig. 3B).

Discussion

This study described a method to expand AILs easily from the ascites filtration filter after CART. We originally collected AILs from the hollow fiber of the filtration column easily and these cells were available for the culture of γδ T cells.

We obtained PBMCs with a median of 8.50 (range: 3.6–15.3) × 10^6 cells when we applied 1.2 ± 0.1 L apheresis to six patients with ovarian cancer and one patient with peritoneal carcinoma (data not shown). Because patients with ascitic ovarian cancer often have anemia and dehydration, apheresis is sometimes harmful to these patients.

The number of mononuclear cells obtained from 4L of ascitic fluid was equivalent to the number obtained from 200 mL of peripheral blood, and these cells were obtained without any suffering of the patients. Of note, γδ T cells, which are AILs obtained from CART, are ideal as a cellular source when used as immunotherapy.

It is reported that the ratio of Vδ2+ T cells in CD3+ cells of PBMC in 24 patients with ovarian cancer, fallopian tube cancer and peritoneal carcinoma were 1.42%8). In this study, the ratio Vδ2+ T cells in CD3+ cells in the ascitic fluid obtained from a CART filter was 1.2%, which was similar to the ratio of cells obtained from the peripheral blood. Reportedly, the growth rate of γδ T cells in the ascitic fluid of patients with ovarian cancer who underwent direct paracentesis with 2M3B1-PP and IL2 was 1.9 times and 3.8 times with Zol and IL28).
γδ T cells by ascites filtration for immunotherapy

Fig. 2  Ratio V\(\delta^2\) T cells / CD3\(^+\) cells

The V\(\delta^2\) T cells/CD3\(^+\) cell ratio and the proliferation ratio of V\(\delta^2\) T cells in ascites collected from filtration filter after cell-free and concentrated ascites reinfusion therapy (CART). The proportion of V\(\delta^2\) T cells in CD3\(^+\) cells in ascites from three patients with ovarian cancer and two patients with primary peritoneal cancer (A). The proportion of V\(\delta^2\) T cells relative to CD3\(^+\) cells after culture with each antigen (A). In case of culture in 2M3B1-PP and IL2, the proportion of V\(\delta^2\) T cells/CD3\(^+\) cells (27%) tended to be higher than that of pre-culture cells (1.2%; Wilcoxon signed-rank test, N = 5, df = 4, two-sided test, p = 0.067). In case of culture in 2M3B1-PP, IL2, and IL18, the proportion of V\(\delta^2\) T cells/CD3\(^+\) cells (71%) was higher than that of pre-culture cells (1.2%; Wilcoxon signed-rank test, N = 5, df = 4, two-sided test, p = 0.029). In case of culture in Zol and IL2, the proportion of V\(\delta^2\) T cells/CD3\(^+\) cells (49%) was higher than that of pre-culture cells (1.2%; Wilcoxon signed-rank test, N = 5, df = 4, two-sided test, p = 0.029). The proportions of V\(\delta^2\) T cells in AILs tended to be higher with 2M3B1-PP and IL2 and IL18, although this difference was not statistically significant (Wilcoxon signed-rank test, N = 5, df = 4, two-sided test, p = 0.067; Fig. 2A). The proportions of V\(\delta^2\) T cells in AILs with 2M3B1-PP and IL2 and IL18 tended to be higher and with Zol significant (Wilcoxon signed-rank test, P < 0.05; Fig. 2A). The proliferation of V\(\delta^2\) T cells from ascites after 14 days in culture with 2M3B1-PP or Zol (B) and (C). Wilcoxon signed-rank test, significant * P > 0.05.

Fig. 3  Cytotoxicity of V\(\delta^2\) T cells

Cytotoxicity of V\(\delta^2\) T cells in ascites collected from a filtration filter after cell-free and concentrated ascites reinfusion therapy (CART). V\(\delta^2\) T cells from three patients with ovarian cancer and one patient with primary peritoneal cancer (A). We performed cytotoxicity assays at E:T ratios ranging from 40:1-0.625:1 in triplicate culture. Representative data from one patient with stage 3c ovarian cancer (B).
which is similar to the growth rate we obtained from a CART filter. Compared with peripheral blood γδ T cells, the proliferation potency of γδ T cells was remarkably impaired in the ascitic fluid\(^6\).

The growth rates of cells cultured from ovarian cancer patients’ PBMCs with 2M3B1-PP, IL2 and IL18 were reported to significantly increase than those with IL2 alone\(^7\). It is suggested that IL18 increased growth rates of mononuclear cells that we collected from CART filter in this study. Vδ2+ T cells that we cultured with Zol, IL2 and IL18 from AILs obtained from a CART filter exhibited endogenous pyrophosphate-dependent cytotoxic activity. It is challenging to obtain cells for TILs therapy using tumor antigen-specific lymphocytes but is effective; γδ T cells are not tumor antigen-specific. However, we applied the characteristics of tumors, which was aggravated by the mevalonic acid pathway with tumor cells and can target γδ T cells by inhibiting Farnesyl pyrophosphate (FPP). Thus, it is advantageous because we can culture all γδ T cells and obtain tumor-specific T cells.

Besides ascitic lymphocytes, cancer cells are also included. Despite performing centrifugation using a lymphocyte separation solution, both AILs and cancer cells are contained in the AILs layer, and separation is difficult unless sorting is performed. Although mixed cells of AILs and cancer cells were cultured in this study, some cancer cells disappeared during culture. The lymphocytes were cultured and expanded, and these cells might increase cytotoxicity and possibly killed cancer cells.

Regarding γδ T cells, culture efficiency are lower in the ascitic fluid (approximately 1/50) than γδ T cells\(^7\) from PBMC of patients with ovarian cancer. The number of these cells is not sufficient for clinical use, especially for clinical trials at this moment even if we culture all these cells is not sufficient for clinical use, especially from PBMC of patients with ovarian cancer. The number of these cells is not sufficient for clinical use, especially during adoptive immunotherapy using γδ T cells, γδ T cells are not tumor antigen-specific. However, we applied the characteristics of tumors, which was aggravated by the mevalonic acid pathway with tumor cells and can target γδ T cells by inhibiting Farnesyl pyrophosphate (FPP). Thus, it is advantageous because we can culture all γδ T cells and obtain tumor-specific T cells.

During adoptive immunotherapy using γδ T cells, γδ T cells in ascites can also may be sufficiently expanded. Perhaps, γδ T cells in ascites can be cultured and used as a source of cells.

In patients with ascitic ovarian cancer, which accumulates in CART, we collected AIL from a filter and were able to culture γδ T cells. Additionally, we found that proliferation was efficient when we used IL18. γδ T cells from AILs obtained from CART have a cytotoxic activity after the culture for 14 days. Furthermore, we may use these cells as a source for adoptive immunotherapy in future if the proliferation rate and cytotoxicity can be increased.

However, only six cases were examined in this study and more cases must be accumulated to further clarify this point. The proportion, proliferation, and cytotoxicity of γδ T cells collected from CART filter were not satisfactory for the immunotherapy at present and it is necessary to study how to efficiently expand these cells using new antigens or by removing immunosuppressive cells.

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### Conflicts of Interest:
The Authors have no financial conflicts of interest regarding this study.

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