

# Complete Remission of Lung Metastasis Following Adoptive Immunotherapy Using Activated Autologous $\gamma\delta$ T-cells in a Patient with Renal Cell Carcinoma

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**Abstract.** *Background:*  $\gamma\delta$  T-cells have recently attracted considerable attention in the development of novel cancer immunotherapy, and several different approaches have been designed and employed in clinical trials. *Case Report:* A patient with lung metastasis after radical nephrectomy for renal cell carcinoma had six cycles of adoptive immunotherapy using autologous *in vitro*-activated  $\gamma\delta$  T-cells followed by low-dose interleukin-2 and zoledronic acid intravenous infusion. Complete remission was achieved which has been maintained for 2 years without any additional treatment. Immunological analysis demonstrated a high level of interferon-gamma four hours through one day following the transfer and peripheral blood  $\gamma\delta$  T-cells increased 10-fold from the baseline value, 7 days after the transfer. No serious adverse events were observed. *Conclusion:* Adoptive immunotherapy using  $\gamma\delta$  T-cells was shown here to be clinically beneficial and safe, and may become a therapeutic option for patients with advanced RCC.

It remains a challenge to achieve a complete response (CR) in metastatic renal cell carcinoma (RCC) even since the development of new anti-angiogenic drugs. Human gamma-delta T-cells comprise less than 10% of peripheral blood T-cells. Seventy percent of peripheral blood  $\gamma\delta$  T-cells express V  $\gamma$ 2 and V  $\delta$ 2 from among the variable elements of T-cell receptors (V $\gamma$ 2V $\delta$ 2 T-cells) (1).  $\gamma\delta$  T-cells have recently

attracted considerable attention in the development of novel cancer immunotherapy, and several different approaches using them have been designed and employed in clinical trials (2-4). We showed that 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP) and interleukin-2 (IL-2) developed within bulk-culture promote V $\gamma$ 2V $\delta$ 2 T-cells (5). We reported a pilot study of adoptive immunotherapy using V $\gamma$ 2V $\delta$ 2 T-cells and demonstrated possible antitumor effects against RCC(2). We also reported that activated V $\gamma$ 2V $\delta$ 2 T-cells show strong potent cytotoxic activity against tumor cell lines sensitized with aminobisphosphonate (6).

In the present case, multiple lung metastases of RCC disappeared after six courses of adoptive immunotherapy using autologous *in vitro*-activated V $\gamma$ 2V $\delta$ 2 T-cells followed by low-dose IL-2 and zoledronic acid administered as aminobisphosphonate intravenous infusion.

## Case Report

A 61-year-old apparently healthy man was diagnosed with right-sided RCC with lung metastases and underwent a radical nephrectomy at Tokiwakai Iwaki Hinyoukika hospital. The pathological examination showed clear cell type, grade 3, pT1b. He was treated with 600 $\times$ 10<sup>6</sup> units of interferon alpha (IFN- $\alpha$ ) twice a week for three months. However, Computed tomography (CT) imaging demonstrated no change in the tumor burden. The patient applied to enter our clinical trial (<http://www.cancer.gov/clinicaltrials/TRIC-CTR-GU-05-01>). Written informed consent that fulfilled the committee's guidelines was obtained from the patient.

One month after the washout period for IFN- $\alpha$ , the patient underwent leukopheresis for the harvest of peripheral blood mononuclear cells (PBMCs) which were then stimulated with 100  $\mu$ M of 2M3B1PP and 100 units/ml of IL-2 for 11 days. All procedures were performed in our cell processing room at Tokyo Women's Medical University Hospital.

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**Key Words:** Renal cell carcinoma, gamma-delta T-cells, adoptive immunotherapy.

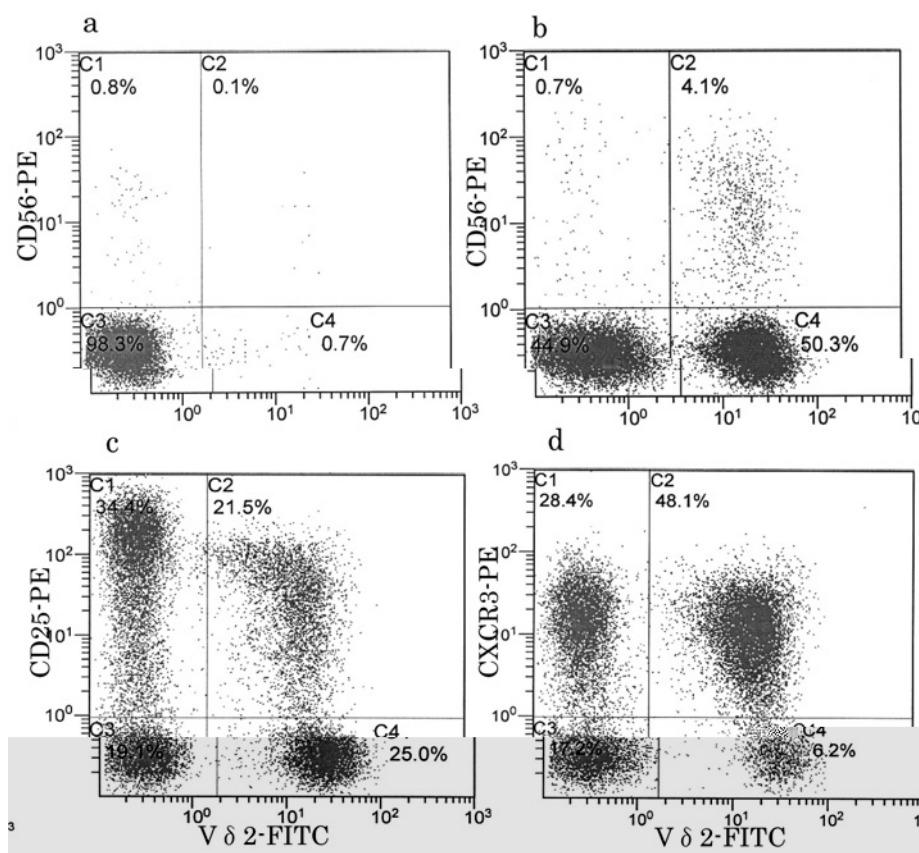


Figure 1. Flow cytometric analysis of cell surface markers of fresh isolated PBMCs(a) and in vitro-cultured Vγ2Vδ2 T-cells(b,c,d). PBMCs and cultured Vγ2Vδ2 T-cells were stained with PC5-conjugated CD-3 mAb and FITC-conjugated-Vδ2 chain mAb, and then with PE-conjugated-CD56 mAb(a,b), PE-conjugated-CD25 mAb(c), and PE-conjugated-CD25-CXCR3 mAb(d). The stained cells were first gated by CD3 and then analyzed by box plots. The percentage of CD56<sup>+</sup>, CD25<sup>+</sup>, and CXCR3<sup>+</sup> Vγ2Vδ2 T-cells was calculated by dividing the numbers of CD56<sup>+</sup>, CD25<sup>+</sup> and CXCR3<sup>+</sup> cells by Vδ2-chain<sup>+</sup> cells.

The patient received 1.4 million units of IL-2 for 2 hours and 4 mg of zoledronic acid for 30 minutes, intravenously, at the same time. Activated Vγ2Vδ2 T-cells were then injected intravenously. IL-2 at  $1.4 \times 10^6$  units was administered for four consecutive days. The patient received these treatments once a month for a period of six months.

Immunological monitoring was carried out by flow cytometric analysis and examination of cytotoxic activity and serum cytokine levels. The following monoclonal antibodies were used for flow cytometric analysis: anti-Vδ2 chain, anti-CD3, anti-CD25, anti-CXCR3, anti-IFN-γ and anti-IL-4 (BD Biosciences, California, USA).

Intracellular staining of IFN-γ and IL-4 of cultured Vγ2Vδ2 T-cells was performed using IntraPrep™ permeabilization reagent purchased from Beckman Coulter(CA, USA).

A standard <sup>51</sup>Cr release assay was performed to determine the cytotoxic activity of activated Vγ2Vδ2 T-cells. Two RCC cell lines (VMRC-RCW and Caki-1) were used as target cells and Daudi cells for the positive control. The effector to target (E:T) ratio was 40:1.

The patient's blood was collected at seven different time points after the transfer of Vγ2Vδ2 T-cells. Serum cytokine levels were measured using the BD™ Cytometric Beads Assay (BD Biosciences, USA) according to the manufacturer's instructions.

Antitumor efficacy was evaluated by CT imaging. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria, Version 3.0 (CTCAE ver.3.0).

The level of Vγ2Vδ2 T-cells in peripheral blood at the time of harvest was only 0.8%, but climbed to 54.4% after culture (Figure 1). The absolute number of Vγ2Vδ2 T-cells was  $7.0 \times 10^6$  before and  $1636.9 \times 10^6$  after the culture at the first therapy; 7.5% of Vγ2Vδ2 T-cells expressed CD56, over 46% expressed CD25 and 88.6% expressed CXCR3 (Figure 1). We stained the intracellular cytokines of activated γδ T-cells to assess the potency of cytokine production. Over 95% of the γδ T-cells showed a potent production of IFN-γ (Figure 2). Almost none of the Vγ2Vδ2 T-cells produced high levels of IL-4 (data not shown).

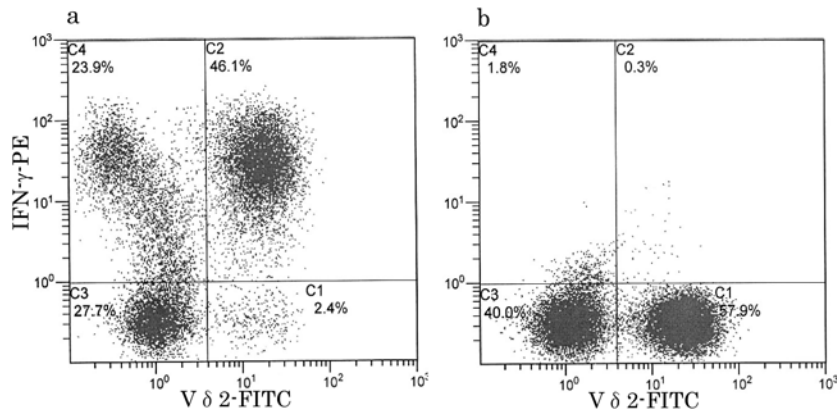


Figure 2. Flow cytometric analysis of IFN- $\gamma$  intracellular staining of *in vitro*-cultured V $\gamma$ 2V $\delta$ 2 T-cells. V $\gamma$ 2V $\delta$ 2 T-cells were stimulated by calcium ionomycin and PMA for 4 hours. After the culture, V $\gamma$ 2V $\delta$ 2 T-cells were stained with V $\delta$ 2-FITC mAb, and with IFN- $\gamma$ -PE(a) or without IFN- $\gamma$ -PE as a control(b) by IntraPrep™ permeabilization reagent purchased from Beckman Coulter.

The patient received 6 cycles of V $\gamma$ 2V $\delta$ 2 T-cells by intravenous injection and the average number of V $\gamma$ 2V $\delta$ 2 T-cells was  $1.7 \times 10^9$ . Less than 10/ $\mu$ l of V $\gamma$ 2V $\delta$ 2 T-cells were seen until 4 days after the transfer, but increased up to 50/ $\mu$ l at 7 days after the transfer (Figure 3).

Cytotoxicity assays demonstrated that activated V $\gamma$ 2V $\delta$ 2 T-cells exhibited strong lytic activity against Daudi (60%) and VMRC-RCW (60%) cells, and intermediate cytotoxicity against Caki-1 (26%) cells (data not shown).

Levels of cytokines in the patients serum showed a transient rise in IFN- $\gamma$  and IL-6. Peak levels of IFN- $\gamma$  were 175.8 pg/ml at day 1 and returned to baseline 3 days after the transfer. In the case of IL-6, peak levels were 130.6 pg/ml at 4 hours after the transfer and returned promptly to baseline. Levels of IL-5 rose more slowly from 4 hours to 4 days, but were undetectable 7 days after the transfer. IL-12p70, IL-10 and IL-5 remained at low levels during the whole course of therapy (Figure 4).

CT imaging demonstrated multiple lung metastases that responded following 3 cycles of therapy and disappeared completely following 6 cycles of therapy. Complete Response (CR) has been maintained for 2 years following the last cycle of therapy, without any additional therapy for RCC (Figure 5).

No severe adverse events (AEs) were observed during the therapy and all AEs resolved. Grade 2 AEs included transient depletion of lymphocytes and hypocalcemia. Grade 1 AEs included fever, hypoalbuminemia, elevated serum creatinine and eosinophilia.

## Discussion

Anti-angiogenic drugs have shown much higher response rates and clinical benefits compared to immunotherapies, such as IFN- $\alpha$  and IL-2, but have rarely induced CR, and AEs were often more severe than those produced by

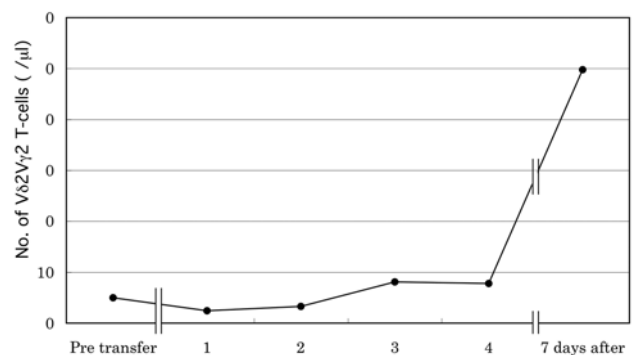


Figure 3. Peripheral blood samples were obtained just before the transfer, at 1 day through 4 days and at 7 days after the transfer. PBMCs were purified and stained with CD3-PC5 and V $\delta$ 2-FITC, and were subjected to flow cytometric analysis. Absolute numbers of V $\gamma$ 2V $\delta$ 2 T-cells were calculated using the following formula: Number of white blood cells (cell number/ $\mu$ l)  $\times$  % fraction of mononuclear cells  $\times$  % of CD3<sup>+</sup> cells  $\times$  % of V $\delta$ 2<sup>+</sup> cells.

immunotherapies (7, 8). However, low-dose IL-2 therapy induces durable CR in about 2% of cases and less severe AEs.

We previously reported that activated V $\gamma$ 2V $\delta$ 2 T-cells show strong potent cytotoxic activity against tumor cell lines sensitized with aminobisphosphonate (6). In the setting of a clinical trial, we administered activated V $\gamma$ 2V $\delta$ 2 T-cells followed by zoledronic acid and low-dose IL-2. Eleven advanced RCC patients were enrolled in the clinical trial and we obtained the results of 1 CR, 5 stable disease and 5 progressive disease. We report the immunological monitoring data of one patient who showed CR of lung metastases.

The percentage of V $\gamma$ 2V $\delta$ 2 T-cells before culture was only 0.8% but 10 days after the culture, the percentage rose to 54.4%. V $\gamma$ 2V $\delta$ 2 T-cells expanded more than 200 times *in*

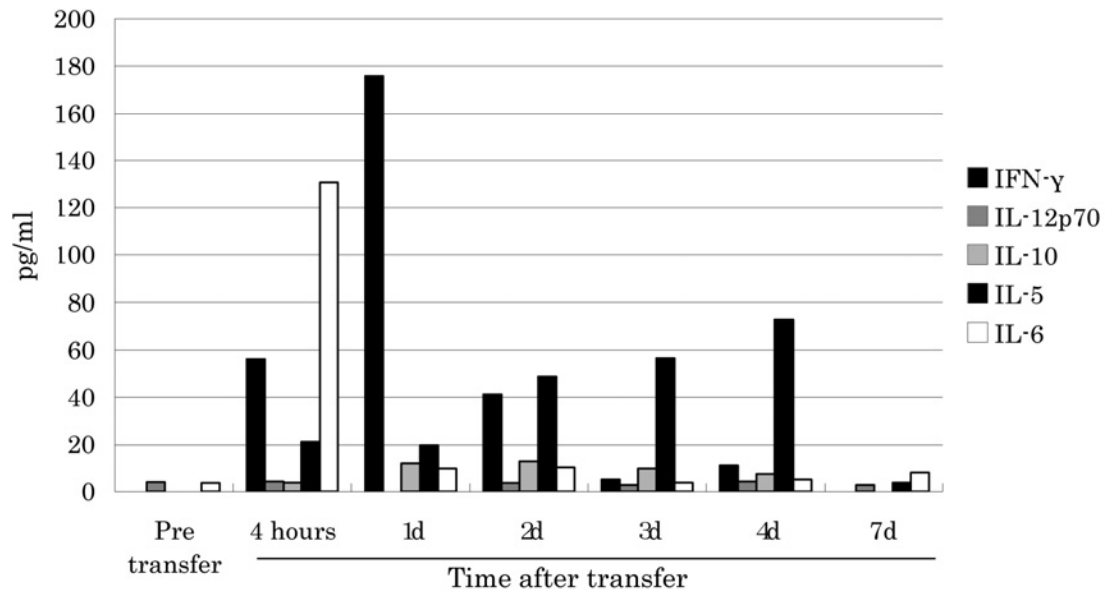


Figure 4. Serum from the patient was obtained at 7 time points during the first course of the therapy, namely just before the transfer, and at 4 hours, 1 day, 2 days, 3 days, 4 days and 1 week after the transfer. The patients serum was stored at  $-70^{\circ}\text{C}$  before analysis. Serum cytokine levels were measured by BD™ Cytometric Beads Assay (BD Biosciences, CA, USA) according to the manufacturer's instructions.

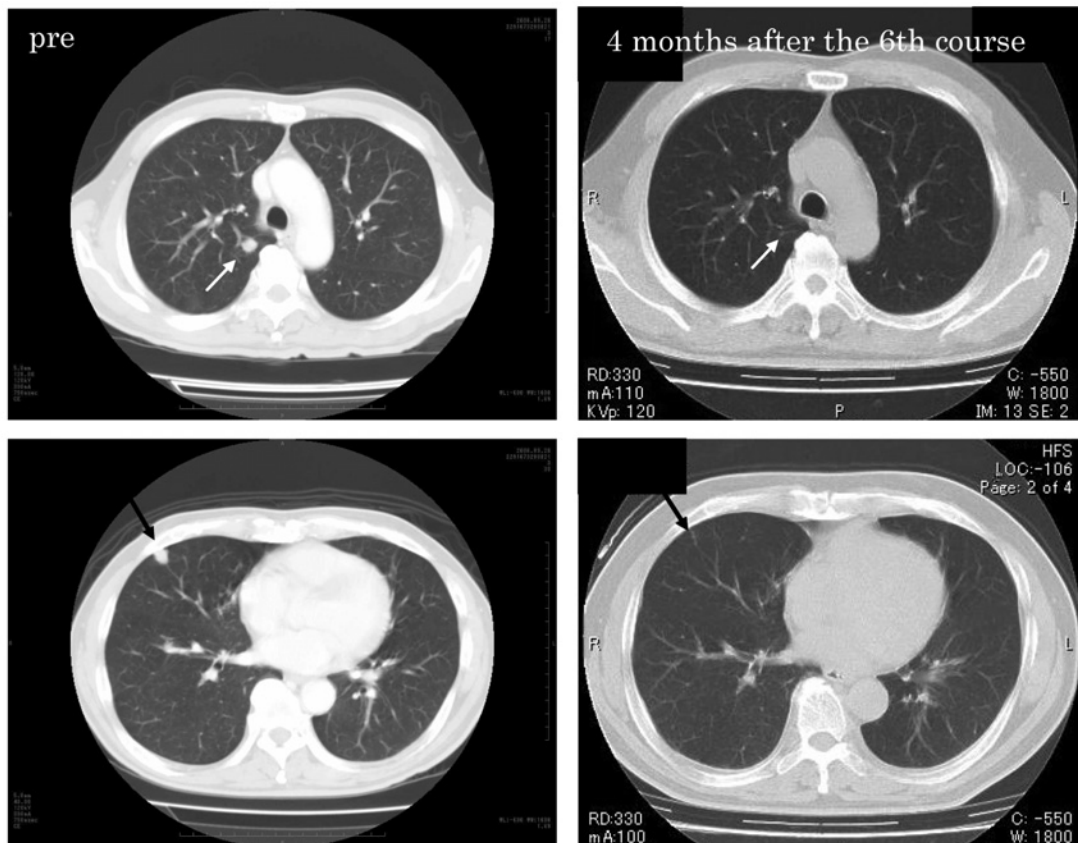


Figure 5. Chest CT examinations of the patient were taken at 4 time points, namely, before entry into the clinical trial, after 3 courses of the therapy, and at 1 and 4 months after the 6th course of the therapy. In this figure we show representative CT images obtained before entry into the clinical trial and 4 months after the 6th course of the therapy.



*vitro* and the numbers of peripheral blood V $\gamma$ 2V $\delta$ 2 T-cells also increased 10-fold before the transfer. We believe that CD25-expressing V $\gamma$ 2V $\delta$ 2 T-cells responded to the systemic administration of IL-2 by increasing *in vivo*.

Almost all the V $\gamma$ 2V $\delta$ 2 T-cells showed potent production of IFN- $\gamma$  *in vitro*; moreover, high levels of IFN- $\gamma$  were measured in the patient's serum after the therapy. We consider that transferred V $\gamma$ 2V $\delta$ 2 T-cells might release IFN- $\gamma$  after recognition of RCC cells exposed by zoledronic acid. High levels of IL-6 were also measured but the mechanism of IL-6 production was not clear. It is well known that many macrophages infiltrate into tumor sites(9) and macrophages produce IL-6 by stimulation of IFN- $\gamma$  (10). High levels of IFN- $\gamma$  and IL-6 were seen even 4 hours after the transfer. IFN- $\gamma$  released by transferred V $\gamma$ 2V $\delta$ 2 T-cells might stimulate macrophages to produce IL-6. The level of IL-5 rose up to 4 days after the transfer. High levels of IL-5 were seen during IL-2 therapy but returned to baseline after IL-2 administration ceased.

The number of peripheral blood V $\gamma$ 2V $\delta$ 2 T-cells increased 4 days after the transfer and the levels of IFN- $\gamma$  and IL-6 returned to baseline before this time. High levels of IFN- $\gamma$  induce chemokines, such as monokine induced by IFN- $\gamma$  (Mig) and interferon-inducible protein-10 (IP-10). These chemokines induce CXCR3 expression on V $\gamma$ 2V $\delta$ 2 T-cells that mediate trafficking to the tumor site. Kondo *et al.* reported that high levels of Mig and IP-10 in tumor sites are related to a good prognosis (11). Activated V $\gamma$ 2V $\delta$ 2 T-cells show potent cytotoxic effects against RCC cell lines *in vitro* (12). These results may support our data showing that transferred V $\gamma$ 2V $\delta$ 2 T-cells remained and infiltrated into the tumor sites, showing cytotoxic activity, and about 4 days later, they left the tumor sites and moved into the peripheral circulation. No severe AEs were seen during the therapy, we expected that almost all AEs were brought about by IL-2 and/or zoledronic acid.

We conclude that immunotherapy using V $\gamma$ 2V $\delta$ 2 T-cells may have clinical utility, appears safe, and may become a therapeutic option for advanced RCC.

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