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# Adoptive cell therapy using tumor-infiltrating lymphocytes for melanoma refractory to immune-checkpoint inhibitors

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## Abstract

To evaluate the feasibility of adoptive cell therapy (ACT) using ex vivo-expanded tumor-infiltrating lymphocytes (TILs) in Japanese patients with melanoma who failed immune-checkpoint inhibitor therapy, an open-label, single-arm, pilot study was conducted. We investigated the immunological and genetic factors of the pretreatment tumor and expanded TILs that may be associated with the clinical response. The treatment protocol comprised preparation of TIL culture, lympho-depleting non-myeloablative preconditioning with cyclophosphamide and fludarabine, TIL infusion, and intravenous administration of low-dose IL-2. Three patients of clinical subtypes mucosal, superficial spreading, and acral melanoma underwent TIL-ACT. Most severe adverse events, including fever and leukopenia, were manageable with the supportive regimen specified in the protocol, suggesting that the TIL-ACT regimen is suitable for Japanese patients with melanoma. One patient showed a short-term partial response, one relatively long-stable disease, and one experienced disease progression. Whole-exome and transcriptional sequencing of isolated tumor cells and immunohistochemical analyses before TIL-ACT revealed various immunostimulatory factors, including a high tumor mutation burden and immune cell-recruiting chemokines, as well as various immunosuppressive factors including TGF-β, VEGF, Wnt/β-catenin, and MAPK signaling and epithelial-to-mesenchymal transition, which might influence the efficacy of TIL-ACT. Our results imply mechanisms for the antitumor effect of and resistance to TIL-ACT. Further studies of immune-resistant mechanisms of TIL-ACT are warranted. This study is registered with the UMIN Clinical Trial Registry (UMIN 000011431).

**KEYWORDS**
adoptive cell therapy, feasibility study, immune checkpoint inhibitor, melanoma, tumor infiltrating lymphocytes

## 1 | INTRODUCTION

Melanoma is an aggressive skin cancer arising from melanocytes and its relatively immunogenic nature has led to the development of immunotherapies and reverse translational research. Immune-checkpoint inhibitors (ICIs), including anti-programmed cell death-1 (PD-1) and anti-cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) antibodies (Abs), are the standard therapies for advanced melanoma.[1] However, these have inadequate clinical efficacy. Therefore, novel immunotherapies or modulators combined with ICI are needed.

Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) has been under development since the 1980s.[2-4] In standardized TIL-ACT, TILs are ex vivo expanded from metastatic tumor in the presence of IL-2 and infused to patients along with IL-2 administration following lymphodepleting chemotherapy to support in *vivo* proliferation and persistence of the infused TILs.[4,5] TILs are a polyclonal T-cell population targeting multiple tumor antigens, including DNA mutation-derived private neoantigens and shared antigens such as cancer germ line and melanosomal antigens. Given the overall response rate of 38%-45% and durable complete response rate of 7%-20%,[6] it has been suggested that TIL-ACT for melanoma could be curative in certain patients, and clinical trials of TIL-ACT have been conducted in specialized centers worldwide.[7-10] Additionally, because of the inadequate responses to first-line ICI therapies, TIL-ACT is under evaluation for patients with melanoma who fail ICI therapy,[11] including those with acral- or mucosal-subtype melanoma, which has been reported to be resistant to ICIs.[12]

However, TIL-ACT has not been investigated in Japanese patients with melanoma. We performed a feasibility study of TIL-ACT in three Japanese patients with different clinical subtypes of melanoma who had previously received ICI therapy. Furthermore, we investigated the genomic and immunological factors of the pretreatment tumors and administered TILs that might be related to the response to TIL-ACT.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design and treatment protocol

This open-label, single-arm, feasibility study of TIL-ACT was approved by the Ethics Committee of Keio University School of Medicine and the Keio Certified Committee for Regenerative Medicine (S2015001). Patients aged 20-65 years with metastatic melanoma refractory to standard therapies, including ICIs and/or molecular target therapies, were enrolled in the study. The inclusion criteria are provided in Supporting Information File S1. Before TIL infusion ($2 \times 10^9$-$2 \times 10^{11}$ cells/200 mL, day 0), all patients received lympho-depleting non-myeloablative (NMA) conditioning treatment consisting of intravenous cyclophosphamide (60 mg/kg/d, day −7 to −6) and fludarabine (25 mg/m$^2$/d, day −7 to −3) (Figure 1). Following TIL infusion at day 0, the patients received bolus intravenous IL-2 (72,000 IU/kg) every 8 hours for 5 days or to tolerance (maximum of 15 doses). Prophylactic antiemetics (ondansetron and aprepitant) were administered. Filgrastim was administered subcutaneously beginning on day 1 and continued daily until a neutrophil count of >1000/μL for 3 consecutive days, or a count of >5000/μL. Patients were treated prophylactically with sulfamethoxazole-trimethoprim, fluconazole, and acyclovir from the beginning of treatment and during the leucopenic period. Clinical response was assessed by imaging 6-8 weeks after TIL infusion. The primary endpoint was treatment feasibility defined as completion of TIL-ACT without early cessation due to unacceptable adverse events. The secondary endpoints were safety assessed using Common Terminology Criteria for Adverse Events (CTCAE v. 4.0), clinical response; objective response rate (ORR) based on the Response Evaluation Criteria in Solid Tumors (RECIST) v. 1.1, overall survival (OS), and progression-free survival (PFS). OS was defined as the time from treatment initiation to death and PFS as the time elapsed between treatment initiation and first progression or death from any cause. Other outcome measures included immunohistochemical and gene expression analysis of tumor immune components and immunologic milieu before TIL-ACT.

### 2.2 | Preparation and evaluation of TILs

TILs were cultured in accordance with the protocol-specified guidelines and regulations (Supporting Information File S1).

### 2.3 | Flow cytometry

For surface phenotype characterization, cultured TILs were washed with Cell Staining Buffer (BioLegend) and stained with anti-human CD3 (Clone OKT-3), CD4 (Clone RPA-T4), CD8 (Clone HIT8a), and CD56 (Clone MEM-188) antibodies for 15 minutes. Next, cell-surface phenotypes were analyzed using a Beckman Coulter Gallios. Data were analyzed using FlowJo software (Becton Dickinson).

### 2.4 | T-cell receptor gene sequencing of expanded TILs

T-cell receptor (TCR) repertoire analysis (Repertoire Genesis Inc.) was performed on the cultured TILs and peripheral blood before and 4-6 weeks after TIL infusion (Supporting Information File S1).

### 2.5 | Immunohistochemistry

Formalin-fixed paraffin-embedded sections of tumor tissue sampled to generate TILs were examined for CD8, PD-L1, HLA-class I,

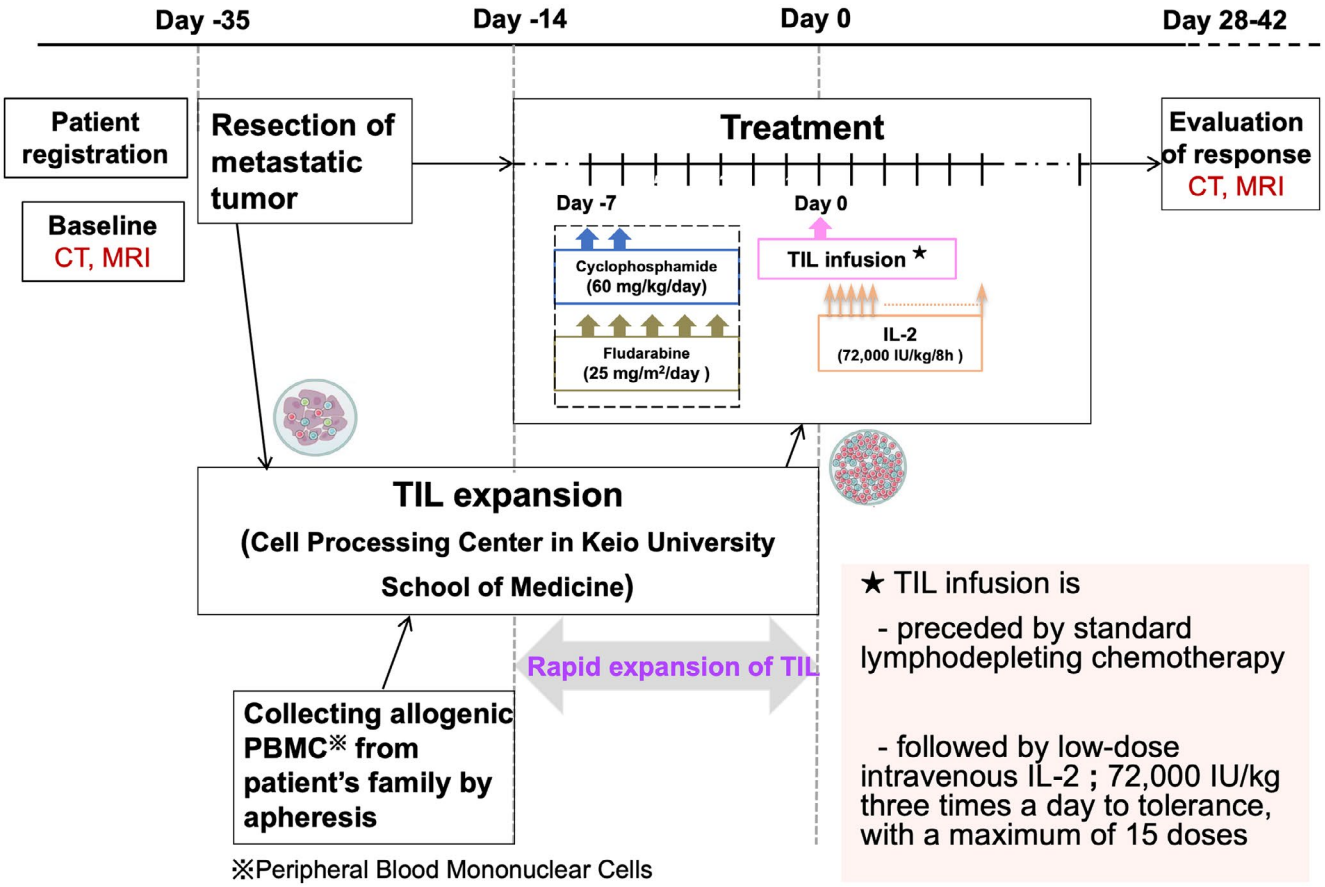



**FIGURE 1** Protocol of adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TILs). TIL infusion is preceded by standard lymphodepleting chemotherapy and followed by low-dose intravenous IL-2

and β-catenin expression. Mouse monoclonal Abs for CD8 (M7103) (clone C8/144B) (Dako), β-catenin (760-4242) (clone 14) (Roche), and HLA class I (HLA-A, B, C) (clone EMR8-5) (MBL), and a rabbit monoclonal Ab for PD-L1 (ab205921) (clone 28-8) (Abcam) were used as the primary antibodies. An alkaline phosphatase-conjugated goat monoclonal anti-rabbit Ab (Nichirei) was used as the secondary antibody.

### 2.6 | Whole-exome sequencing of isolated melanoma cells before TIL production

Genomic analysis was performed on melanoma cells from the three patients taken for TIL manufacturing and paired PBMCs.

Melanoma cells were digested using collagenase type IV and stained with an anti-CD146 Ab (clone P1H12) and anti-melanoma Ab (clone HMB45 + M2-7C10 + M2-9E3 + T311) (Abcam) to purify tumor cells. These cells were sorted using a FACS Aria II (Becton Dickinson). Genomic DNA was purified from melanoma cells and PBMCs using an AllPrep DNA/RNA Mini Kit (Qiagen) and a DNeasy Blood & Tissue Kit (Qiagen), respectively (Supporting Information File S1). The samples were subjected to whole-exome sequencing (DNA Chip Research Inc.), the data from which are available on the National Bioscience Database Center website (http://www.biosciencedbc.jp) (accession no.s GAS000249, JGAD000349, DRA010773).

### 2.7 | Microarray analysis of melanoma cells

Total RNA from isolated melanoma cells was subjected to microarray analysis using an Agilent SurePrint G3 Human GE v3 8x60K Microarray (DNA Chip Research Inc.). All of the datasets are available in the Gene Expression Omnibus repository (GSE155389).

### 2.8 | Quantitative real-time polymerase chain reaction array

Isolated RNA was subjected to cDNA synthesis using ReveTraAce qPCR Master Mix with a gDNA Remover Kit (Toyobo). qPCR was performed using a StepOne Real-Time System (Applied Biosystems/Thermo Fisher Scientific Inc.) with THUNDERBIRD Probe qPCR Master Mix (Toyobo Co., Ltd) and the indicated TaqMan probes (Applied Biosystems/Thermo Fisher Scientific, Inc.; Supporting Information File S1).

### 2.9 | Gene set enrichment analysis

Gene set activation scores for each of the subtype expression signatures in tumor samples were generated using single-sample Gene Set Enrichment Analysis (ssGSEA) (https://github.com/broadinstitute/ssGSEA2.0), which calculates a separate enrichment score for each gene set and allows assignment to the nearest or closest transcriptional subtype.

## 3 | RESULTS

### 3.1 | Patients' characteristics

Four patients with melanoma with visceral metastases who failed standard treatment (including ICIs) were enrolled. One patient was not able to continue the protocol treatment before starting NMA according to the discontinuation criteria of the protocol, due to clinical deteriorations through the appearance of new intra-atrial and intestinal metastases during TIL manufacturing. Therefore, three patients with mucosal-, superficial spreading-, and acral-subtype melanoma were treated with TIL-ACT (Table 1). These three patients had 5.2, 5.3, and 8.5 month-intervals from the last dose of ICI, respectively.

### 3.2 | Characteristics of expanded TILs

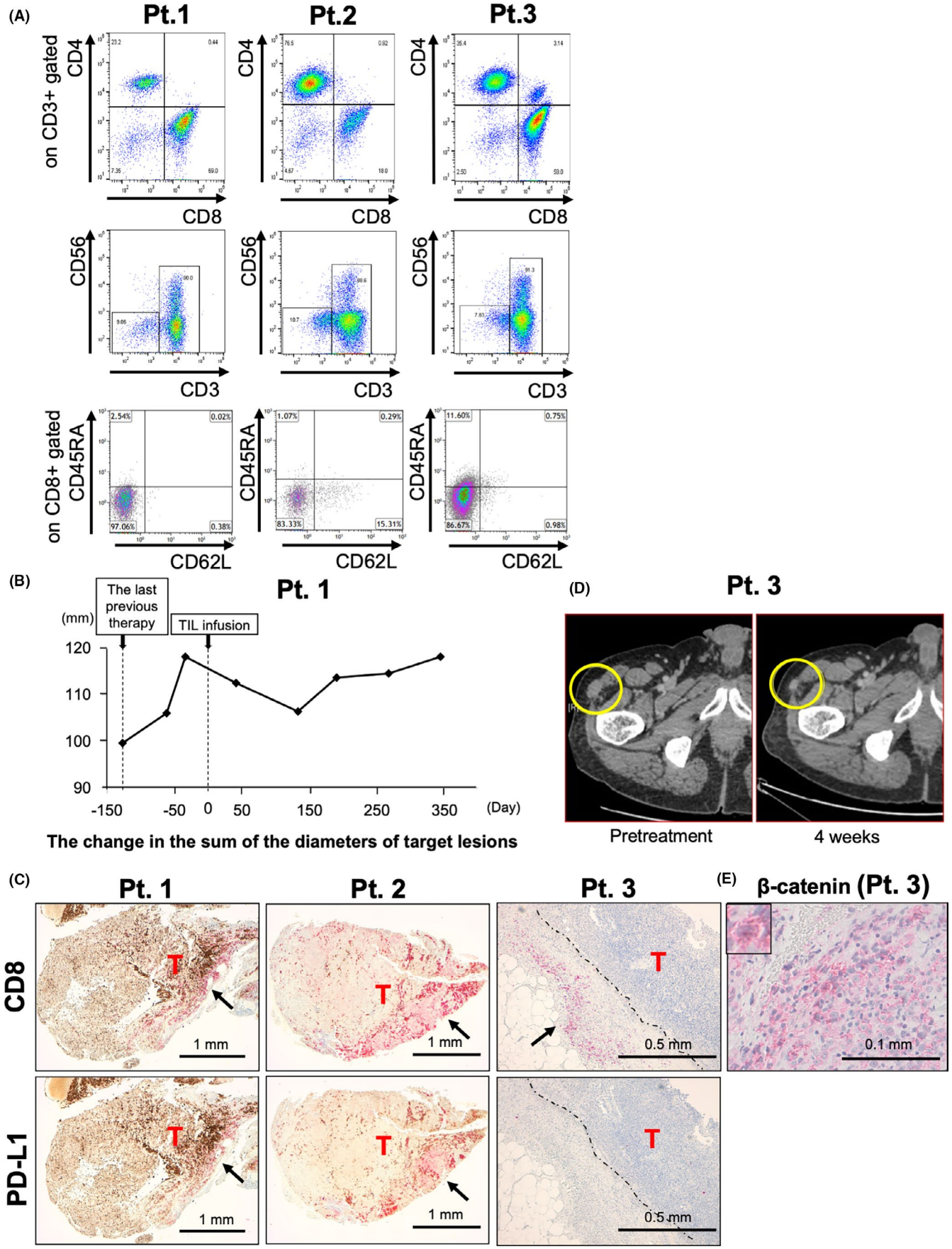
In all cases, TILs were successfully generated and passed all tests for sterility and cell viability. By flow cytometry, the expanded TILs were found to be $CD8^+$ T-cell dominant in two patients and $CD4^+$ T-cell dominant in one (Figure 2A). In all three cases, the majority of infused $CD8^+$ T cells were effector memory phenotype (CD62L-CD45RA-) (Figure 2A). All contained few $CD3^-CD56^+$ NK cells, which can suppress T-cell proliferation and effector functions.[13] The patients received an average of $6.38 \times 10^{10}$ cells ($2.7\text{-}10.2 \times 10^{10}$) (Table 1). To evaluate their viability, TCR repertoire analysis was performed on the cultured TILs (final products) and pre/post-treatment peripheral blood. In all cases, the major T-cell clones administered (which were present in small numbers in pretreatment peripheral blood) were detected at 4 or 6 weeks following TIL-ACT, suggesting that the infused TILs persisted in vivo (Figure 3).

**TABLE 1** Patient characteristics and the outcomes of TIL-ACT

| Pt. | Age | Sex | Primary site | Metastaticsite | *BRAF* status | Previous treatments | Infused cells | Dose of IL-2 | Objective response PFS/OS (month) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 59 | M | Mucosa (rectal) | LN | wt | Nivolumab | $6.23 \times 10^{10}$ | 15 | SD |
| | | | | liver | | Ipilimumab | | | PFS 9.3 |
| | | | | | | | | | OS 13.4 |
| 2 | 47 | M | Skin | LN | V600E | Nivolumab | $2.7 \times 10^{10}$ | 3 | PD |
| | | | (SSM) | Liver | | Vemurafenib | | | OS 7.0 |
| | | | | bone | | Ipilimumab | | | |
| | | | | lung | | TACE | | | |
| | | | | | | DTIC | | | |
| 3 | 44 | M | Skin | SC | wt | Nivolumab | $1.02 \times 10^{11}$ | 15 | PR |
| | | | (ALM) | IM | | Ipilimumab | | | PFS 3.0 |
| | | | | lung | | Pembrolizumab | | | OS 9.3 |

Abbreviations: ALM, acral lentiginous melanoma; DTIC, dacarbazine; IM, intramuscular; LN, lymph node; M, male; OS, overall survival; PD, progressive disease; PFS, progression free survival; PR, partial response; SC, subcutaneous; SD, stable disease; SSM, superficial spreading melanoma; TACE, trans-arterial chemoembolization; WT, wild type.

**FIGURE 2** A, Flow cytometry of infused TILs. B, D, Antitumor response induced by TIL-ACT. C, E, Immunohistochemical staining of metastatic tumor resected for TIL culturing. A, The expanded TILs were $CD8^+$ T-cell dominant in patients 1 and 3, and $CD4^+$ T cell-dominant in patient 2 (upper panel), and all contained few $CD3^-CD56^+$ NK cells, which suppress the proliferation and effector functions of T cells (middle panel). Further analysis of cryopreserved TIL products showed that the majority of infused $CD8^+$ T cells was found to have effector memory phenotype (CD62L-CD45RA-) (lower panel). B, Change in the sum of the diameters of target lesions in patient 1. The size of target lesions remained stable for 9.3 months following TIL-ACT. C, Metastatic lymph node of patient 1 (left); CD8 (red) is positive at the invasive margin (arrow in the upper left panel). Programmed death ligand 1 (PD-L1) expression (red) concentrated at the tumor margins (arrow in the lower left panel). Metastatic lymph node of patient 2 (middle); CD8 and PD-L1 were positive at the invasive margin (arrow in the upper and lower middle panels, respectively). Skin metastases in patient 3 (right); CD8 (red) was positive at the outer stroma rather than the invasive margin (arrow in the upper right panel) and there are no PD-L1-positive cells (lower right panel). T, tumor area. D, Computed tomography of patient 3 showed tumor regression of a subcutaneous metastasis on the right thigh (yellow circle) after TIL-ACT. E, β-catenin (red) was positive in tumor cells from patient 3


(A)
Pt.1
Pt.2
Pt.3
on CD3+ gated CD4
CD4
CD8
CD56
CD3
on CD8+ gated CD45RA
CD45RA
CD62L
(B)
Pt. 1
The last previous therapy
TIL infusion
(mm)
120
110
100
90
-150
-50
0
50
150
250
350
(Day)
The change in the sum of the diameters of target lesions
(D)
Pt. 3
Pretreatment
4 weeks
(C)
Pt. 1
Pt. 2
Pt. 3
CD8
PD-L1
T
1 mm
0.5 mm
(E)
β-catenin (Pt. 3)
0.1 mm

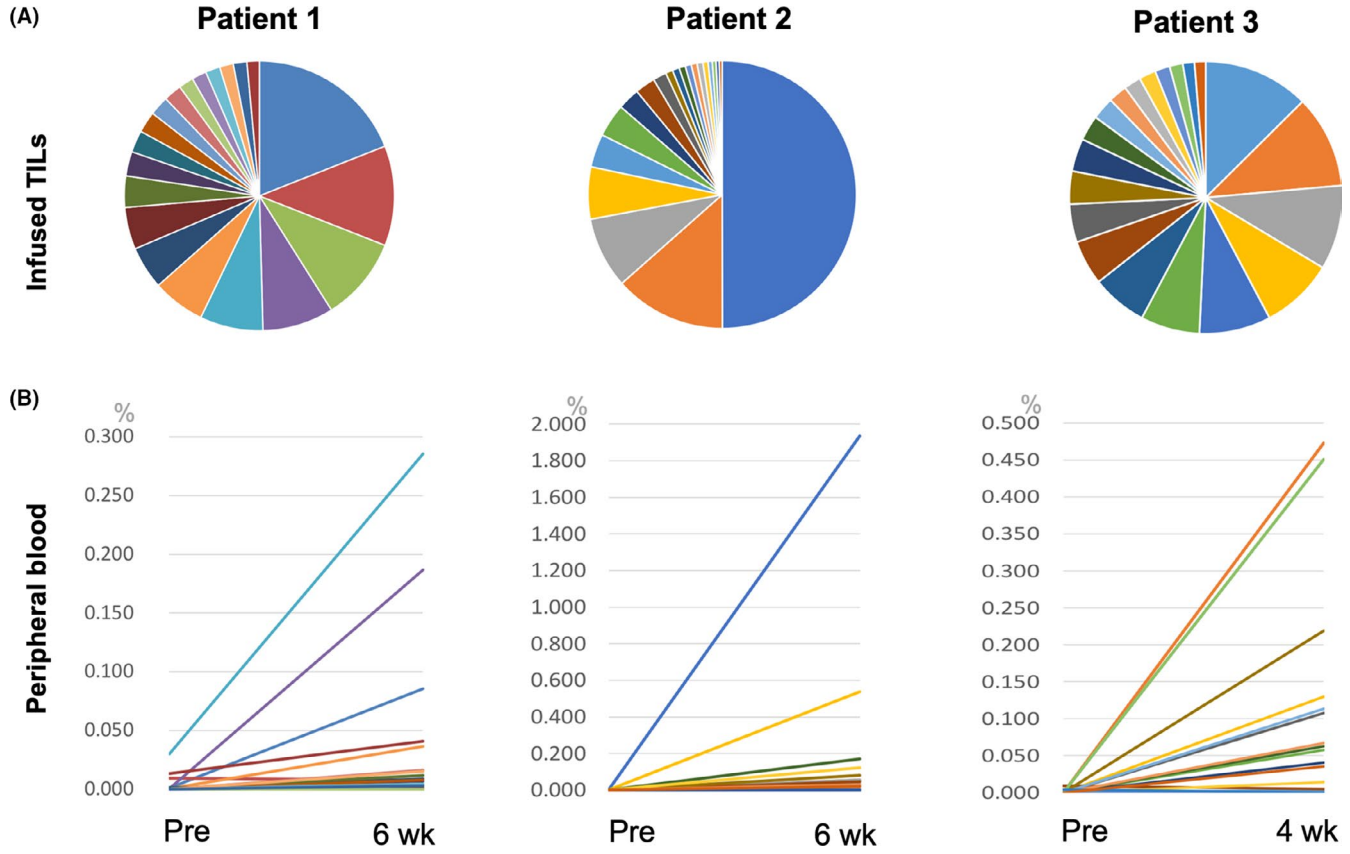



**FIGURE 3** TCR repertoire analysis of infused TILs (A) and peripheral blood before and 4-6 weeks after TIL infusion (B). A, Pie chart of the top 20 most abundant clones in infused TILs (upper panel). B, In all cases, some clones composing the majority of infused TILs that were few present in pretreatment blood were detected in peripheral blood at 4-6 weeks after TIL-ACT, eg, purple-colored line in patient 1, blue-colored line in patient 2, and orange-colored line in patient 3 (each clone is colored according to the same color as the clone in the upper pie chart)

### 3.3 | Adverse events of TIL-ACT

Grade 4 leukopenia was observed after NMA in the three patients treated with TIL-ACT, and neutropenia was managed by administration of G-CSF according to the protocol (Table 2). TIL infusion led to transient pulmonary edema in one patient, which was resolved by oxygen treatment before IL-2 administration. After initiation of IL-2 administration, grade 3 and 4 adverse events (AEs), including fever, nausea, diarrhea, hypoalbuminemia, and hypophosphatemia, were observed. The patients were treated with the supportive regimen specified in the protocol, which included acetaminophen, antibiotics, antiemetics, and electrolyte replacement. No patients needed treatment in the intensive care unit. All AEs, except for cancer-related pain such as back pain and arthralgia, were reversible. There were no treatment-related deaths.

**TABLE 2** Grade 3≥ adverse events

| | Grade 3 (N) | Grade 4 (N) |
|---|---|---|
| Hematologic toxicity | | |
| Leukopenia | | 3 |
| Non-hematologic toxicity | | |
| Febrile neutropenia | 3 | |
| Nausea | 2 | |
| Emesis | 1 | |
| Anorexia | 2 | |
| Hiccups | 1 | |
| Diarrhea | 2 | |
| Rectal stenosis | 1 | |
| Pulmonary edema | 1 | |
| Back pain | 1 | |
| Arthralgia | 1 | |
| Hypoalbuminemia | 1 | |
| Hypophosphatemia | 1 | 1 |

### 3.4 | Clinical response and correlation with immunological and genomic characteristics of pretreatment tumors and expanded TILs

In the three patients treated with TIL-ACT, one partial response (PR), one stable disease (SD), and one progression disease (PD) were observed. The clinical course and genetic and immunological characteristics of the three patients are described below.

### 3.4.1 | Patient 1: Mucosal melanoma

The patient with mucosal melanoma who failed both anti-PD-1 and anti-CTLA-4 Ab monotherapies exhibited a relatively long SD after administration of $CD8^+$ T cell dominantly expanded TILs (Figure 2A). The lesions remained stable for 9.3 months after TIL infusion, despite tumor growth after previous ICI treatment (Table 1 and Figure 2B).

Immunohistochemical analysis of pretreatment tumor tissue revealed $CD8^+$ T-cell infiltration at the invasive tumor margin, which was colocalized with PD-L1 expressed on surrounding cells (Figure 2C), and <10% of the tumor cells were positive for HLA class I (data not shown). Whole-exome sequencing (WES) of pretreatment tumor cells revealed that the total mutation burden (TMB) was extremely high (6,303 mutations), possibly as a result of mutations of DNA mismatch repair genes such as *MSH6*, *MLH1*, and *PMS1*, and proofreading genes such as *POLE* and *POLD1* (Figure 4A). Also, the tumor cells showed low expression levels of immunosuppressive factors and signaling pathways, including TGF-β, angiogenesis, VEGF signaling, Wnt/β -catenin signaling, MAPK signaling, and epithelial-to-mesenchymal transition (EMT) by ssGSEA based on microarray analysis (Figure 4A,B). Furthermore, qRT-PCR analysis of pretreatment tumor cells showed relatively high mRNA levels of $CD8^+$ T-cell-recruiting chemokines (such as CXCL9, 10) and dendritic cell (DC)-recruiting chemokines (such as CCL4) (Figure 4C). qRT-PCR analysis of TILs cultured from the tumor tissue showed relatively high expression of effector $CD8^+$ T-cell-related molecules such as IFN-γ, TNF-α, and perforin (PRF1), suggesting preexisting tumor-reactive $CD8^+$ T cells in tumors in this patient (Figure 4D).

### 3.4.2 | Patient 2: Superficial spreading melanoma

The patient with *BRAF*-mutated superficial spreading melanoma originating on the face previously failed anti-PD-1Ab and cytotoxic chemotherapies and was unable to continue BRAF inhibitor and anti-CTLA-4 Ab treatments due to immune-related adverse events (Table 1). This patient dropped out during IL-2 administration following infusion of $CD4^+$ T-cell-dominant TILs (Figure 2A) due to deterioration of bone metastases and related pain, which required radiotherapy and resulted in progressive disease (PD) (Table 1).

Immunohistochemical analysis of the pretreatment tumor showed infiltration of $CD8^+$ T cells and PD-L1 positive cells at the invasive margin (Figure 2C), but few HLA class I-positive cells. WES and gene expression analysis of pretreatment tumor cells revealed a relatively low TMB of 169 and a BRAF mutation (Figure 4A). These tumor cells showed high expression levels of immunosuppressive factors and pathways, including immunosuppressive oncogene signaling pathways such as the BRAF mutation-related MAPK signaling pathway, and immunosuppressive mesenchymal microenvironment features such as hypoxia, VEGF signaling, TGF-β signaling, and EMT (Figure 4A, B). In pretreatment tumor cells, the mRNA levels of CXCL9, 10, and CCL4 were lower than those in patient 1 (Figure 4C). The mRNA levels of Th1 cytokines such as IFN-γ, TNF-α as well as PRF1 in the $CD4^+$ T-cell-dominant cultured TILs were lower than those of patients 1 and 3 (Figure 4D), suggesting that this patient's tumor had a small number of preexisting tumor reactive CD8+ TILs.

### 3.4.3 | Patient 3: Acral melanoma

The patient with NRAS-mutated acral melanoma previously failed anti-PD-1 Ab therapy and was unable to continue anti-CTLA-4 Ab therapy because of an immune-related adverse event. Administration of $CD8^+$ T-cell-dominant expanded TILs induced a PR (Figure 2D), but tumor progression with new lesions was observed at 2 months after initial tumor regression, resulting in a short-lasting PR (Table 1).

Immunostaining revealed $CD8^+$ T-cell infiltrates at the outer stroma rather than the invasive margin, and PD-L1 staining was negative (Figure 2C). HLA class I was partially positive (20% of tumor cells, data not shown). WES and gene expression analysis of the tumor revealed a relatively low TMB (215) with NRAS mutation and highly immunosuppressive factors and pathways, including the NRAS mutation-related MAPK signaling pathway and the Wnt/β -catenin signaling pathway, which suppresses DC/T cell recruitment, as well as mesenchymal signatures such as hypoxia, VEGF, TGF-β signaling, and EMT (Figure 4A,B). Some of the tumor cells showed β-catenin nuclear positivity by immunohistochemistry (Figure 2E), and low levels of CXCL9, 10, and CCL4 by qRT-PCR (Figure 4C). The ex vivo-expanded TILs had high mRNA levels of TNF-α and PRF1, which were comparable to those of patient 1 (Figure 4D), suggesting that preexisting tumor-reactive $CD8^+$ T cells caused tumor regression on TIL administration despite the relatively low TMB and various immunosuppressive factors in the tumor.

## 4 | DISCUSSION

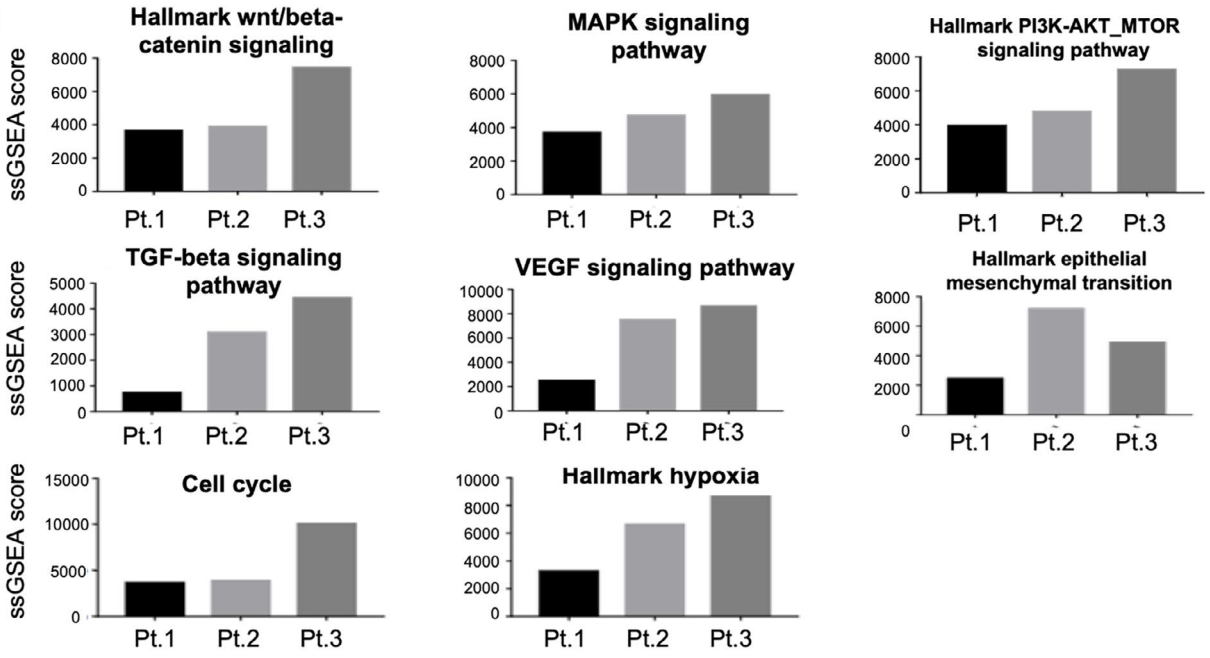
In this study of ex vivo-cultured TILs for Japanese patients with melanoma who failed previous ICI therapy, TILs were successfully expanded. The three patients who initiated NMA preconditioning completed the protocol-specified treatment course. The findings indicate a manageable safety profile. Most AEs were caused by NMA and IL-2 administration, similar to reports of TIL-ACT patients with NMA and IL-2.[4-10] Although the intravenous IL-2 dose was lower than in pivotal TIL-ACT trials, irreversible side effects such as neurotoxicity, reported with high-dose IL-2 regimens, were not observed. These results indicate that our TIL-ACT protocol is suitable for Japanese patients with melanoma.

The TIL-ACT protocol showed clinical benefit in two patients, a short PR in patient 3 and a long SD in patient 1, both of whom failed previous anti-PD-1 or CTLA-4 Ab treatment as the third-line or beyond regimen. The reasons for the resistance to ICIs and the efficacy of subsequent TIL-ACT are unclear. The remaining effects of prior PD-1 Ab treatment may be unlikely in these three patients. Free PD-1 Ab should be minimal after a 5.2-8.5 month interval following prior PD-1 Ab administration. Although infused TILs might enhance

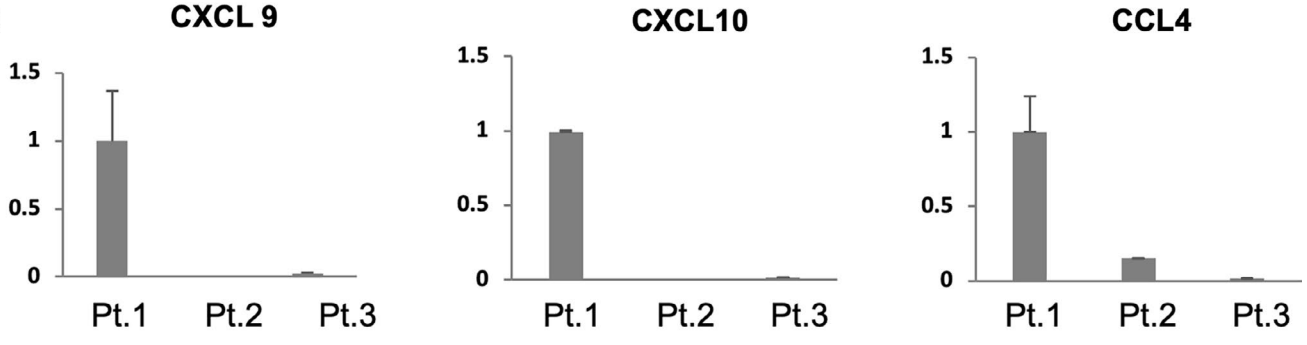
(A)

| | Pt.1 | Pt.2 | Pt.3 |
|---|---|---|---|
| Total mutation burden | 6303 | 169 | 215 |
| Mutations | MSH6, MLH1, PMS1, POLE, POLD1 | BRAF | NRAS |
| **Expression signatures** | | | |
| Wnt/β-catenin signaling pathway | low | low | high |
| MAPK signaling pathway | low | high | high |
| PI3K-AKT-MTOR signaling pathway | low | high | high |
| TGF-β signaling pathway | low | low | high |
| VEGF signaling pathway | low | high | high |
| Cell cycle | low | low | high |
| Epithelial mesenchymal transition | low | high | high |
| Hypoxia | low | high | high |

(B)




(C)




(D)

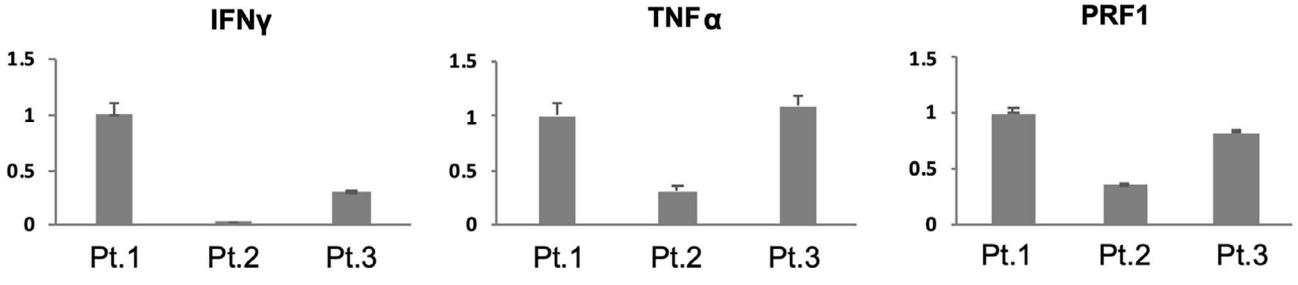


**FIGURE 4** Total mutation burden, transcriptome signature (ssGSEA score), and quantitative real-time polymerase chain reaction analysis of pretreatment melanoma cells and expanded TILs. A, To evaluate their mutation status, exome sequencing was performed on the melanoma cells used for TIL manufacturing. The numbers of mutations and the mutated genes in each tumor are shown in the upper columns. Transcriptome signatures based on single-sample gene set enrichment analysis (ssGSEA) of the tumor cells are shown in the lower columns. B, ssGSEA scores of gene sets related to tumor phenotype (based on microarray data) in the tumors of the three melanoma patients. The cut-off score was set at 4,000. C, D, Quantitative RT-PCR analysis of the expression levels of chemokines in the primary tumors (C) and of cytotoxic factors in the expanded TIL products (D). The results are fold-changes in gene expression normalized to the endogenous reference gene; error bars are standard deviations

antitumor activity of the remaining endogenous antitumor T cells which bound anti-PD-1 Ab,[14] it may also be unlikely because these patients never responded to the previous ICI treatment. One of the reasons for not durable responses in these three patients (long SD, PD, short PR) may be due to relatively low expression of MHC class-I on tumor cells. However, MHC expression needs to be evaluated after TIL treatment to confirm this possibility because MHC expression on tumor cells was known to be significantly increased following TIL-ACT.[15] In this study, our genomic and immunological analyses of the tumor microenvironment (TME) of ICI-resistant melanoma and evaluation of expanded TILs revealed that various immunostimulatory or immunosuppressive factors influence TIL-ACT therapy.

Patient 1 had mucosal melanoma, which typically has a relatively low TMB.[16] However, this patient had an extremely high TMB (6303), likely due to loss-of-function mutations of genes involved in DNA repair and proofreading, potentially generating highly immunogenic neoantigens and suggesting the presence of neo-antigen-specific T cells. Lauss et al[17] reported that a high TMB and predicted neoantigen load are significantly associated with improved PFS and OS after ACT in patients with melanoma. The reasons for the non-responsiveness of the patient to repeated treatment with anti-PD-1 and -CTLA-4 Abs are unknown, but the few $CD8^+$ TILs specific for neoantigens were not sufficiently activated in vivo by ICIs. However, ex vivo culture rendered such tumor-specific $CD8^+$ TILs active and proliferative, and infusion of expanded TILs resulted in regression of tumors that were growing despite ICI treatment. Of note, this patient's tumor cells showed relatively high levels of CXCL9 and CXCL10. In the TME, CXCL9 and 10 are produced by conventional type 1 dendritic cells (cDC1s, Batf3-related DC) to recruit $CD8^+$ effector T-cells.[18,19] CXCL9 is also produced by tumor cells and macrophages stimulated by IFN-γ, and recruits activated cytotoxic $CD8^+$ T cells into tumors.[20] Together with the data on oncogenic MAPK/β-catenin signaling and the mesenchymal environment (eg, TGF-β/VEGF, EMT, and hypoxia), our findings suggest that the TME of this patient was favorable for the recruitment and function of ex vivo-expanded effector T cells.

Patient 3, who had acral melanoma, harbored a low TMB (215), as reported previously.[16] $CD8^+$ T-cell infiltrates were observed at the outer stroma, which lacked PD-L1 expression. Furthermore, the patient's tumor cells had high scores for various immunosuppressive mechanisms, including NRAS-MAPK signaling, TGF-β signaling, and mesenchymal signatures. Activation of MAPK signaling by BRAF or NRAS mutation reportedly exerts an immunosuppressive effect in the TME by inducing production of immunosuppressive cytokines but does not prevent $CD8^+$ T-cell accumulation in tumors.[19] Melanoma with TGF-β–associated mesenchymal TME is resistant to anti-PD-1 Ab therapy.[21] TFG-β not only induces mesenchymal conditions but also inhibits immune responses by inducing immunosuppressive Tregs and MDSCs and directly inhibiting T cells.[22,23] In addition, this tumor had high Wnt/β-catenin signaling activity. Activation of β-catenin signaling is implicated in the development of non-T-cell-inflamed human melanoma via inhibiting the secretion of DC-recruiting chemokines (eg, CCL4) followed by induction of $CD8^+$ TILs, causing resistance to anti-PD-1 Ab therapy.[24] In a murine model of $BRAF^{V600E}$/$PTEN^{-/-}$/Bcat-STA melanoma, melanoma cells with activated β-catenin showed reduced production of CCL4, suppressing recruitment of Batf3-related $CD103^+$ DCs producing CXCL9/CXCL10.[18] Therefore, this patient had various immune-resistant features, including MAPK, TGF-β, and β-catenin signaling-related immunosuppression and mesenchymal TME, which might explain the non-response to ICI therapy. However, preexisting tumor-reactive TILs were sufficiently expanded under the less-immunosuppressive ex vivo culture conditions. In addition to the $CD8^+$-dominant T cells, ex vivo cultured TILs contained $CD4^+$/$CD8^+$ T-cell population in patient 3. The double positive T cells was previously reported as a tumor reactive T cells in vitro.[25] However, the roles of this population remained to be investigated, including their in vivo function, expansion, and persistence. Infusion of these TILs resulted in a PR, although tumor regression was not maintained over the long term, probably due to the immunosuppressive TME. The combination of TIL-ACT with immunomodulators may prolong the antitumor effect.

Patient 2, who failed ICIs and cytotoxic chemotherapies, did not respond to TIL-ACT. The tumor was a BRAF-mutated superficial spreading melanoma with a low TMB. The tumor cells of this patient had various immunosuppressive factors, including high BRAF-related MAPK signaling activity, TGF-β, mesenchymal TME, and very low MHC class I expression, which may explain its resistance to ICIs. Furthermore, the expanded TILs were predominantly $CD4^+$ T cells, although $CD8^+$ T cells and PD-1 positive cells were detected at the invasive margin by immunohistochemistry. In other patients who received TIL-ACT, $CD4^+$ T-cell dominant TILs resulted in an unfavorable prognosis.[26] Thus, the non-responsiveness to TIL-ACT of this patient may be explained by infusion of CD4-dominant T cells and loss of MHC class I expression, in addition to other immunosuppressive mechanisms.

We report not only the suitability of TIL-ACT for Japanese patients with melanoma of different subtypes but also the possible

mechanisms underlying the response to TIL-ACT. Our data suggest the importance of neoantigens and the balance between immunostimulatory (eg, tumor-derived immune-recruiting chemokines) and various immunosuppressive factors. Further studies of the immune-resistance mechanisms of TIL-ACT in patients with melanoma will facilitate the discovery of biomarkers and the development of effective immunotherapies comprising TIL-ACT in combination with immune modulators.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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