Characterization of the cryoablation-induced immune response in kidney cancer patients

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ABSTRACT
Cryoablation is one of treatment modalities for kidney cancer and is expected to induce strong local immune responses as well as systemic T-cell-mediated immune reactions that may lead to the regression of distant metastatic lesions. Thus, the characterization of T cell repertoire and immune environment in tumors before and after treatment should contribute to the better understanding of the cryoablation-induced anticancer immune responses. In this study, we collected tumor tissues from 22 kidney cancer patients, before cryoablation and at 3 mo after cryoablation. In addition, blood samples were collected from 14 patients at the same time points. We applied a next generation sequencing approach to characterize T cell receptor β (TCRB) repertoires using RNAs isolated from tumor tissues and peripheral blood mononuclear cells. TCRB repertoire analysis revealed the expansion of certain T cell clones in tumor tissues by cryoablation. We also found that proportions of abundant TCRB clonotypes (defined as clonotypes with ≥ 1% frequency among total TCRB reads) were significantly increased in the post-cryoablation tissue samples than those of pre-cryoablation tumor samples. Some of these TCRB clonotypes were found to be increased in peripheral blood. Expression analysis of immune-related genes in the tissues of pre- and post-cryoablation showed significantly elevated transcriptional levels of CD8+, CD4+, Granzyme A (GZMA), and CD11c along with a high CD8/FOXP3 ratio in the post-cryoablation tissue samples. Our findings revealed that cryoablation could induce strong immune reactions in tumors with oligoclonal expansion of antitumor T cells, which circulate systemically.

Abbreviations: CDR3, complementarity determining region 3; HLA, human leucocyte antigen; PBMC, peripheral blood mononuclear cells; TCR, T cell receptor; TCRB, T cell receptor β.

Introduction
Cryoablation is one of promising cancer treatments and has some advantages over other modalities such as minimum invasiveness and less damage to surrounding structures. As this technology has been improved over the decades, cryoablation is now widely used in the treatment of various types of cancer including kidney cancer. Interestingly, the treatment causes not only local elimination of tumors, but also sometimes causes regression of distant and untreated tumor legions after certain time lag. These findings evoke a hypothesis that the cryoablation may induce strong and long-lasting anticancer immune responses in the body. So far, this unique feature of cryoablation has been reproduced in animal models in which the prevention effects for re-challenge of tumor cells or regression of untreated tumors were mainly examined. In human, only a few studies have examined the cryoablation-induced immune response showing an increase of interferon-γ-producing T cells against human cancer cell lines. However, the molecular mechanisms of the immune response, particularly for T cells that play fundamental roles in attacking cancer cells, still remain unclear.

T cell receptors (TCR), most of which are a heterodimer of TCR α (TCRA) and β (TCRB) chains, are present on the surface of T cells. To recognize a huge number of antigens, TCR genes undergo somatic V-(D)-J recombination that generates a huge diversity of T cell repertoire. To obtain comprehensive T cell repertoire information in various disease conditions including cancer, we recently established a TCR analysis method with next-generation sequencers using RNAs extracted from blood, ascites, and tumor tissues.

In the present study, we demonstrated that cryoablation could induce strong immune reactions characterized by the expansion of oligoclonal T cells in tumor tissues. Interestingly, an increase of some T cell clonotypes, which were expanded in tumor tissues, was also detected in peripheral blood, indicating a possibility of systemic antitumor response that might attack...
cancer cells in distant tumor lesions (and possibly circulating tumor cells). Furthermore, we found the infiltration of a large number of CD11c-positive cells, probably representing macrophages and dendritic cells into the post-cryoablation tissues. Collectively, TCR repertoire analysis and immune profiling of cryoablation-treated cancer patients may lead to a better understanding of the cryoablation-induced anticancer immune response.

Results

Clonal T-cell expansion in tumors after cryoablation

To examine the effect of cryoablation on T cell repertoire in tumor tissues, we performed TCRB sequencing on pre- and post-cryoablation tissue samples obtained from 22 kidney cancer patients. The clinical characteristics of all patients are summarized in Table 1. Through cDNA sequencing of TCRB, we obtained total sequence reads of 1,132,906 ± 815,953 (average ± one standard deviation) mapped to V, D, J, and C segments for TCRB (Table S1). From these TCRB reads, we identified unique TCRB complementarity determining region 3 (CDR3) clonotypes of 34,487 ± 41,724 in individual tissues. After sorting out CDR3 clonotypes according to their frequencies in tumor tissue samples, we counted the proportions of abundant CDR3 clonotypes, which were defined as the clonotypes with the frequency of 1% or higher, in each tumor tissue, and found that the sum of the abundant CDR3 clonotypes was increased by cryoablation in 15 of the 22 cases (Fig 1A); in the remaining seven tumor tissues, four patients (CK1, CK7, CK30 and CK31) revealed the very strong clonal expansion of T cells in pre-cryoablation tumor tissues. Overall, on average, the sum of abundant TCRB CDR3 clonotypes (defined by frequency of ≥ 1%) was significantly increased from 27.3% ± 27.4% in pre-cryoablation tumor tissues to 46.4% ± 31.0% in post-cryoablation tissues (p = 0.024, Fig 1B). We also measured the TCR diversity by calculating the inverse Simpson’s diversity index (1/Ds) that becomes high when TCR clones are evenly distributed whereas becomes low with the enrichment of certain T cell clones. As expected, the diversity indexes for TCRB were significantly lower in tissues after cryoablation in comparison with those of pre-treatment tumor samples (p = 0.021, Fig 1C). We further investigated whether TCRB CDR3 clonotypes enriched in post-cryoablation tissues were present in pre-cryoablation tumors in 15 cases that had significant increase in certain CDR3 clonotypes by cryoablation. As shown in Fig 2A and Table S2, most of the enriched CDR3 clonotypes in post-cryoablation tissues were absent (bars in red color) or rarely present (bars in green color) in pre-treatment tumors, suggesting that the death of cancer cells induced by the cryoablation activated T lymphocytes that probably recognize cancer-specific antigens.

We subsequently examined the presence of common CDR3 clonotypes among patients by comparison of 20 most abundant CDR3 clonotypes of each tissue sample. We found that one TCRB CDR3 clonotype was shared in post-cryoablation tissues of patients who have the same HLA class I subtype, HLA-A24:02 (Fig 2B).

Unique TILs are also detected in systemic circulation

Accumulating evidences have suggested that cryoablation can stimulate adaptive and systemic antitumor immune responses.11,13-15 We further examined the frequencies of TCRB CDR3 clonotypes, which were enriched in post-cryoablation tissues, in peripheral blood samples. Among 14 patients from which we obtained PBMC samples, we examined 10

Table 1. Clinical information of 22 kidney cancer patients performed cryoablation

| Case | Age at cryoablation (yr) | Gender | Affected side | Tumor size (mm) | Stage | Histology | pre-eGFR (mL/min/1.73m²) | post-eGFR (mL/min/1.73m²) | Recurrence | Status | Follow-up (mo) |
|------|-------------------------|--------|--------------|-----------------|------|-----------|--------------------------|---------------------------|------------|--------|----------------|
| CK1  | 80                      | M      | Lt           | 17              | pT1a | Clear Cell RCC | 71.4                      | 66.5                      | --         | Alive  | 26.7           |
| CK3  | 69                      | M      | Lt           | 22              | pT1a | Clear Cell RCC | 38.3                      | 35.4                      | --         | Alive  | 24             |
| CK4  | 80                      | M      | Lt           | 20              | pT1a | Clear Cell RCC | 98.2                      | 86.6                      | --         | Alive  | 24             |
| CK5  | 75                      | F      | Lt           | 17              | pT1a | Clear Cell RCC | 67.7                      | 63.1                      | --         | Alive  | 21.5           |
| CK6  | 81                      | M      | Rt           | 28              | pT1a | Clear Cell RCC | 63.2                      | 45                        | --         | Alive  | 19.6           |
| CK7  | 76                      | M      | Lt           | 25              | pT1a | Clear Cell RCC | 44.2                      | 30.7                      | --         | Alive  | 15.7           |
| CK8  | 78                      | M      | Lt           | 21              | pT1a | Clear Cell RCC | 30.7                      | 20.8                      | --         | Alive  | 18.2           |
| CK9  | 74                      | M      | Rt           | 22              | pT1a | Clear Cell RCC | 46.2                      | 40.6                      | --         | Alive  | 13.8           |
| CK11 | 60                      | M      | Autologous   | 22              | pT1a | Clear Cell RCC | 50.9                      | 39.6                      | --         | Alive  | 18.4           |
| CK12 | 67                      | M      | Lt           | 30              | pT1a | Clear Cell RCC | 34.9                      | 27.6                      | --         | Alive  | 15.3           |
| CK13 | 77                      | M      | Lt           | 38              | pT1a | Clear Cell RCC | 49.8                      | 44.9                      | --         | Alive  | 12.5           |
| CK14 | 71                      | M      | Rt           | 29              | pT1a | Clear Cell RCC | 61.8                      | 64.1                      | --         | Alive  | 6.2            |
| CK15 | 77                      | M      | Lt           | 32              | pT1a | Clear Cell RCC | 73.2                      | 63.9                      | --         | Alive  | 15.5           |
| CK17 | 73                      | F      | Lt           | 14              | pT1a | Chromophobe RCC | 83.8                      | 79.9                      | --         | Alive  | 3.8            |
| CK19 | 87                      | M      | Rt           | 32              | pT1a | Clear Cell RCC | 65.2                      | 44.8                      | --         | Alive  | 12.4           |
| CK22 | 82                      | F      | Rt           | 35              | pT1a | Clear Cell RCC | 68.0                      | 66.9                      | --         | Alive  | 12.6           |
| CK23 | 68                      | M      | Lt           | 22              | pT1a | Clear Cell RCC | 46.1                      | 48.7                      | --         | Alive  | 9.4            |
| CK28 | 66                      | M      | Lt           | 20              | pT1a | Clear Cell RCC | 52.8                      | 53.4                      | --         | Alive  | 3.8            |
| CK29 | 88                      | M      | Rt           | 26              | pT1a | Clear Cell RCC | 28.7                      | 28.5                      | --         | Alive  | 3.7            |
| CK30 | 62                      | F      | Rt           | 23              | pT1a | Clear Cell RCC | 51.7                      | 50.2                      | --         | Alive  | 3.9            |
| CK31 | 80                      | M      | Lt           | 38              | pT1a | Clear Cell RCC | 31.5                      | 24.5                      | --         | Alive  | 8.6            |
| CK32 | 52                      | M      | Lt           | 20              | pT1a | Clear Cell RCC | 65.7                      | 62.8                      | --         | Alive  | 4.0            |

M: male, F: female, Lt: left kidney, Rt: right kidney, Autologous: A kidney after autologous transplantation, pT: pathologic primary tumor stage, RCC: renal cell carcinoma, eGFR: estimated glomerular filtration rate.
patients (CK3, CK5, CK8, CK9, CK14, CK15, CK17, CK22, CK28, and CK32) who showed the expansion of oligoclonal TCRB CDR3 clonotypes (frequency of ≥1%) in post-cryoablation tissues. As a result, certain TCRB CDR3 clonotypes in each sample were significantly increased at 3 mo after the cryoablation compared with pre-cryoablation samples of PBMC (p values less than $3.6 \times 10^{-4}$ were considered to be significant as described in Materials and methods, Fig 3 and Fig. S1). These findings may indicate the presence of the cryoablation-induced T cell-mediated systemic anticancer immune responses.

**Antitumor immune-related genes are upregulated after cryoablation**

To characterize changes of T cell subtypes in the tissues, we examined transcriptional levels of CD3, CD4, CD8, FOXP3, GZMA, PRF1, IDO1, IL10, TIM3, CD11C, and HMGB1 in the tumors with the real-time RT-PCR method (Fig 4A). Higher expression levels of CD8, GZMA, and PRF1 are considered as higher cytolytic activity in tumor tissues, while those of FOXP3, IDO1, IL10, and TIM3 represent higher immunosuppressive

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**Figure 1. Clonal T cell expansion in post-cryoablation tissues.** (A) The distribution of the abundant TCRB CDR3 clonotypes with the read frequency of 1% or higher is presented in pie charts (green color: pre-cryoablation; blue color: post-cryoablation). Gray color indicates portion of CDR3 clonotypes below the read frequency of 1%. (B) The total proportion of the abundant TCRB CDR3 clonotypes with the frequency of 1% or higher was significantly increased in post-cryoablation tissues ($p = 0.024$). (C) After cryoablation, tissue samples revealed significantly lower TCRB diversity index compared with that in pre-cryoablation ($p = 0.021$). The Mann—Whitney U test was performed for the comparison of total proportion of the most abundant CDR3 clonotypes (frequency of ≥1%) and the diversity index of TCRB between pre- and post-cryoablation.
activity. For each of these molecules, we calculated the ratio of post/pre-cryoablation expression levels. In the tissues after cryoablation, we found significant increase of CD4, CD8, and GZMA expression levels, implying that CD4+ or CD8+ T cells were strongly infiltrated and/or expanded by cryoablation. The ratio of CD8/FOXP3 expression was significantly elevated, while that of FOXP3/CD4 became significantly decreased, in post-cryoablation tissues compared with the pre-treatment tumors. With respect to the immunosuppressive markers, IDO1 expression was significantly decreased after cryoablation, whereas TIM3 was increased. We also evaluated correlation between CD8 and HLA-A expression levels as a representative of HLA class I molecule as HLA class I molecules are known to play essential roles in cancer cells for the presentation of tumor-associated antigens. As shown in Fig 4B, there was a significant correlation (p = 0.0085) between CD8 and HLA-A expression levels in pre-cryoablation tumor tissues, although the correlation between CD4 and HLA-DRB1 (one of the three major HLA class II molecules) was not observed (p = 0.47, Fig 4C). We also found that the post/pre-cryoablation ratio of the sum of abundant TCRB CDR3 clonotypes (defined by frequency of ≥ 1%) in tissues was significantly correlated with HLA-A expression in pre-cryoablation tumor tissues (p = 0.046, Fig 4D). In addition, these HLA-A expression levels in pre-cryoablation tumor tissues were higher in tissues sample where the expansion of certain TCRB CDR3 clonotypes was observed.

Figure 2. Cryoablation stimulated expansion of unique T cell repertoires in tumor tissues. (A) The 20 most abundant TCRB CDR3 clonotypes newly appeared or drastically increased in the tissues after cryoablation (green bars: clonotypes that were rarely present in pre-cryoablation tissues; red bars: clonotypes that were completely absent in pre-cryoablation tissues; blue bars: 20 most abundant clonotypes observed in post-cryoablation tissues). Figures are from 15 representative cases that revealed a drastic increase of oligoclonal CDR3 sequences in post-cryoablation tissues in Fig 1A. (B) One TCRB CDR3 clonotype was shared in two patients with HLA-A24:02 genotype. Yellow-color bars indicate the identical CDR3 clonotype.
observed ($p = 0.032$, Fig. 4E). These results indicate that the basal expression level of HLA class I molecules is an important factor to effectively induce clonal expansion of T cells by cryoablation.

**Drastic increase of CD11c$^+$ cells (macrophages and dendritic cells) in post-cryoablation tissues**

To further investigate the changes of antigen-presenting cells in the tissues, we evaluated CD11c$^+$ cells that represent macrophages and dendritic cells by transcriptional levels and immunohistochemical analysis. As shown in Fig 4A, we found that CD11C transcriptional levels were drastically increased in the post-cryoablation tissues, indicating that a large number of macrophages and dendritic cells infiltrated into tumor tissues by the stimulation of cryoablation. We also found the increase of HMGB1 expression level probably through the induction of damage-associated molecular patterns (DAMPs) that were released from the dead/damaged cancer cells. Through the immunohistochemistry (IHC) analysis of CD8 and CD11c on a subset of samples (Fig 5, Fig S2), we confirmed the significant increase of infiltrated CD8$^+$ T and CD11c$^+$ cells in post-cryoablation tissues.

**Discussion**

Significant improvement in cryoablation devices has provided some advantages in cryoablation treatment compared with the standard surgical treatment; cryoablation reveals minimum invasiveness with the preservation of organ function. In addition to these benefits, cryoablation sometimes induces the shrinkage of untreated tumor lesions. However, the molecular mechanism of anticancer immune reaction generated by cryoablation is still largely unknown. For the better understanding of the cryoablation-induced anticancer immune reactions, many animal models have been tested; for example, Gazzaniga et al. reported the infiltration of macrophages and dendritic cells into the peritumoral areas at an early time point after cryoablation. In addition, cryoablation was indicated to activate tumor-specific T cells in tumor draining lymph nodes. In this manuscript, we performed TCRB repertoire profiling combined with the expression analysis of immunorelated genes in tumor tissues treated with cryoablation to investigate the local and systemic T cell responses after cryoablation in human patients.

Through TCRB repertoire analysis in this study, we have demonstrated several novel findings that may explain cryoablation-induced anticancer immune responses. First, we found
that a small subset of T cells was significantly expanded in tumor tissues after cryoablation. Most of these T cell clones were not detected in pre-cryoablation tissues, suggesting that cryoablation strongly stimulated local immune responses, possibly against cancer cells, in tumor tissues. Interestingly, some of these T cell clones were also detected in peripheral blood, indicating that clonally expanded T cells can circulate systemically in the body. These findings may partly explain the mechanism why cryoablation can sometimes induce the regression of untreated tumor lesions, and propose future application of cryoablation in the cancer patients having metastatic lesions, preferably in the combination of cancer vaccine treatment and/or immune checkpoint blockade. Second, we detected drastic increase of CD11c+ cells (macrophages and dendritic cells) in post-cryoablation tumor tissues. By freezing and thawing cycles of cancer tissues, extracellular and intracellular ice crystals mechanically cause the collapse of cancer cells. However, unlike the radiofrequency ablation therapy, cancer cell death by freezing may preserve the antigenicity of cancer-cell-specific molecules, possibly due to the minimum effect on the denaturation of proteins in cancer cells.27-30 In addition, tissue damages may have produced DAMPs, as evidenced by the increased level of HMGB1, which can activate immune-response characterized by the infiltration of macrophages and dendritic cells.31,32

![Graph showing expression of immune-related genes in tumor tissues.](image)

**Figure 4.** Expression of immune-related genes in tumor tissues. (A) Expression level of each gene was calculated relative to that of GAPDH. The x-axis indicates the ratio of post-cryoablation/pre-cryoablation in the log2 transformed values. *p < 0.01, **p < 0.05 indicates the significant difference by the Mann—Whitney U test. (B) and (C) Correlation analysis between HLA-A and CD8 expression or HLA-DRB1 and CD4 expression in pre-cryoablation tumor tissues. HLA-A and HLA-DRB1 represent one of the major HLA class I and class II molecule, respectively. (D) Correlation analysis between HLA-A expression in pre-cryoablation tumor tissues and the post/pre-cryoablation ratio of the sum of the abundant TCRB CDR3 clonotypes (frequency of ≥ 1%). (E) Comparison of basal HLA-A expression levels in the pre-cryoablation tumor tissues where total proportion of the abundant TCRB CDR3 clonotypes (frequency of ≥ 1%) were decreased (left) or increased (right) by cryoablation.
post-cryoablation tissues in our study. Third, we found that HLA-A expression levels in the cancer tissues were significantly correlated with CD8⁺ expression levels (= the extent of CD8 T cell infiltration) in cryoablation tumor tissues. It is well known that CD8⁺ T cells play a central role in cancer immunity surveillance and the loss or downregulation of HLA class I molecule expression is one of the major mechanisms that cancer cells escape from the host immune system. We previously reported that intratumoral high expression of HLA-A might be one of the predictive markers for clinical responses to anti-PD-1 therapy in metastatic melanoma. In the present study, the high expression level of HLA-A in tumor tissues also revealed correlation with the cryoablation-induced oligoclonal expansion of T cells. These results further support the hypothesis that HLA class I expression levels might be one of important determinants for strong T cell-mediated anticancer immune response by cryoablation.

Finally, we found that the expression of TIM3, which is an immune suppressive molecule against T cell responses, was significantly increased in post-cryoablation tissues. Several studies involving mice or human have shown that chronic infections induced high expression levels of TIM3 as the negative feedback system after the activation of CD8⁺ and CD4⁺ T cells. Because these tissue samples in our study were collected 3 mo after cryoablation, the increased expression of TIM3 might reflect the activation of the negative feedback mechanism in tumor tissues at the time of tissue collection.

In summary, our results imply that cryoablation could induce both local and systemic immune responses associated with the infiltration of CD11c-positive cells and oligoclonal expansion of (possible) antitumor T cells. For the translation into clinics, unique TCR sequences identified in post-cryoablation tissues in individual patients can be applied to personalized TCR-engineered T cell therapy.

Materials and methods

Study design

Between June 2014 and August 2016, a total of 22 kidney cancer patients were enrolled in this study. All patients were confirmed to have kidney cancer by needle biopsy before cryoablation. Computed tomography (CT)-guided percutaneous cryoablation was performed using CRYO-HIT System (Galil Medical Ltd, Yokneam, Israel) under local anesthesia. Multiple (typically, three) 17 gauge probes were percutaneously inserted under CT fluoroscopy guidance, and ice ball formation was monitored by CT imaging to ensure a > 5 mm safety margin around the renal tumor. Two freezing and thawing cycles of cryoablation were routinely repeated to increase the tumor cell injury. At 3 mo after cryoablation, all patients underwent follow-up dynamic CT and needle biopsy to confirm no residual tumors.

Tumor tissues were collected at the time points of pre-cryoablation and 3 mo after cryoablation. In addition, peripheral blood samples were obtained from 14 patients (CK1, CK3, CK5, CK8, CK9, CK14, CK15, CK17, CK19, CK22, CK28, CK30, CK31, and CK32) at the time points of pre- cryoablation and 3 mo after cryoablation. The study protocol was approved by the Institutional Review Board of University of Chicago (approval number 13—0797) and Osaka University (approval number 14265). All patients provided written informed consent.

Patient characteristics

The clinical characteristics of all patients are summarized in Table 1. Twenty-one tumors were histologically diagnosed as clear cell renal cell carcinoma and one was as a chromophobe renal cell carcinoma. Patients’ ages were 52—88. The median size of tumor was 23 mm with a range of 14—38 mm at clinical stage I. No recurrence was detected with the median follow-up period of 13.2 mo (range: 3.7—26.7 mo). To evaluate kidney function, we calculated the estimated glomerular filtration rate (eGFR) before and 3 mo after cryoablation. The mean rate of kidney functional deterioration was 6.2%. Perirenal hematoma developed in two patients that spontaneously regressed without any interventions.

TCR sequencing

Total RNAs from tumor tissues and PBMC were isolated using AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA).
Sequencing libraries of TCRB were prepared as described previously, and subjected to sequencing on the Illumina MiSeq platform, using 600 cycles MiSeq Reagent Kit V3 (Illumina, San Diego, CA).

**Sequence analysis**

To identify V, D, J, and C segments in individual TCRB sequencing reads, each of the sequence reads in FASTQ files was mapped to the reference sequences provided by IMGT/GENE-DB using Bowtie2 aligner (Version 2.1.0). Raw FASTQ files were analyzed using Tcrip software.

**Human leukocyte antigen (HLA) typing**

Genomic DNA was isolated from PBMC using AllPrep DNA/RNA Mini kit (Qiagen). PCR amplicon-based high-resolution typing on MiSeq (Illumina) was performed for HLA-A, HLA-B, and HLA-C, in Scisco Genetics Inc. (Seattle, WA). HLA genotypes of all patients were summarized in Table S3.

**Gene expression analysis**

cDNA was synthesized from RNA using Superscript III first-strand synthesis kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using Taqman gene expression assay (Life Technologies, Grand Island, NY) in the ABI ViiA 7 system (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. The TaqMan probes are listed in Table S4. Since we used total amount of mRNA for TCR sequencing in two cases (CK1 and CK4), these were excluded from the analysis.

**Immunohistochemical studies**

Paraffin-embedded tissues were cut into 5-μm-thick sections and tissue slides were stained on Leica Bond RX automatic stainer (Leica Biosystems, Wetzlar, Germany). Epitope retrieval solution 1 (Leica Biosystems, Wetzlar, Germany) was used for 20 min treatment. Anti-human CD8 antibody (clone C8/144B, Dako, Carpinteria, CA) or anti-human CD11c antibody (clone 5D11, Leica Biosystems) were applied on tissue sections for 25 min incubation and the antigen-antibody binding was visualized by Bond polymer refine detection solution (Leica Biosystems) according to manufacturer’s protocols.

**Statistical analysis**

The diversity index (inverse Simpson’s index) in CDR3 sequences was calculated as follows:

\[
1 / D_3 = \left[ \sum_{i=1}^{K} n_i (n_i - 1) \right]^{-1} / N(N - 1)
\]

where \( K \) is the total number of CDR3 clonotypes, \( n_i \) is the number of sequences belonging to the \( i \)th clonotype, and \( N \) is the total number of identified CDR3 sequences.

The Mann—Whitney U test (two-tailed) was performed for the comparison of total proportion of the most abundant CDR3 clonotypes (≥1% frequency among total TCRB reads), the diversity index of TCRB, gene expression levels between pre- and post-cryoablation, correlations between HLA-A and CD8 or HLA-DRB1 and CD4 expression in pre-cryoablation tissues and the correlation between HLA-A expression and post/pre-cryoablation ratio of total proportion of the most abundant CDR3 clonotypes (frequency of ≥1%) using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA). To analyze the changes of CDR3 clonotypes in PBMC, we focused on a total of 139 abundant CDR3 clonotypes (≥1% frequency among total TCRB reads) that were expanded in post-cryoablation tissues in 10 cases (CK3, CK5, CK8, CK9, CK14, CK15, CK17, CK22, CK28, and CK32). Then, we applied Fisher’s exact test to examine the significance in the increase of each of these CDR3 clonotype in PBMC samples. In this analysis, we set a significant level of \( p \) value < 3.6 × 10^{-4} (0.05 of 139) according to the Bonferroni correction. In other analyses, differences were considered significant at \( p \) value < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author Contributions**

Y.N. planned and supervised the entire project; J.P. provided the study design and the working hypothesis and completed the article; T.K. designed the study, conducted experiments, performed data analysis, and drafted the article; N. and U.M. provided the study design and completed the article; T.I. conducted experiments and performed data analysis; Y.I. and K.K. conducted data analysis and provided scientific advice; A.N. and H.H. performed data analysis and provided scientific advice; H.H. and K.O. performed cryoablation and completed the article.

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