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Brief Communication

Tissue-resident memory T cells in human kidney transplants have alloreactive potential

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ABSTRACT

The extent to which tissue-resident memory T (T_{RM}) cells in transplanted organs possess alloreactivity is uncertain. This study investigates the alloreactive potential of T_{RM} cells in kidney explants from 4 patients who experienced severe acute rejection leading to graft loss. Alloreactive T cell receptor (TCR) clones were identified in pretransplant blood samples through mixed lymphocyte reactions, followed by single-cell RNA and TCR sequencing of the proliferated recipient T cells. Subsequently, these TCR clones were traced in the T_{RM} cells of kidney explants, which were also subjected to single-cell RNA and TCR sequencing. The proportion of recipient-derived T_{RM} cells expressing an alloreactive TCR in the 4 kidney explants varied from 0% to 9%. Notably, these alloreactive TCRs were predominantly found among CD4+ and CD8+ T_{RM} cells with an effector phenotype. Intriguingly, these clones were present not only in recipient-derived T_{RM} cells but also in donor-derived T_{RM} cells, constituting up to 4% of the donor population, suggesting the presence of self-reactive T_{RM} cells. Overall, our study demonstrates that T cells with alloreactive potential present in the peripheral blood prior to transplantation can infiltrate the kidney transplant and adopt a T_{RM} phenotype.

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; T_{RM}, tissue-resident memory T.

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1. Introduction

Tissue-resident memory T (T_{RM}) cells are long-lived immune cells that provide local immune surveillance in nonlymphoid organs.[1] Their role within solid organ transplants, where both donor- and recipient-derived T_{RM} cells may coexist, has not yet been fully elucidated.[2-8] In a previous study, we demonstrated that T_{RM} cells of both recipient and donor origin exhibit specificity against common viral pathogens.[2] Evidence from animal and human studies suggests that recipient T_{RM} cells may also play a role in allograft rejection.[5-10]

Previous studies investigating the alloreactive potential of T_{RM} cells had several methodological limitations. Murine models were investigated under sterile conditions that do not entirely mirror the complexities of the human transplant environment.[6-8] Furthermore, human T_{RM} alloreactivity was assessed by means of T cell receptor (TCR) expansion.[9,10] However, nonalloreactive T cells, such as virus-specific T cells or bystander T cells, are also abundantly present in solid organs and may also display an expanded TCR repertoire.[2,11,12] Certain investigations of alloreactive TCR repertoires within human transplanted organs employed a sophisticated TCR tracking technique that involved mixed lymphocyte reactions (MLRs) followed by TCR sequencing.[5,13-15] However, these studies predominantly relied on bulk sequencing of needle biopsy specimens, which does not provide phenotypical characteristics (ie, T_{RM} status) of the identified alloreactive clones. Consequently, the full extent of potential alloreactivity exhibited by T_{RM} cells remains unclear. Here, we used the TCR tracking method combined with a single-cell sequencing approach to examine whether T_{RM} cells can exhibit alloreactivity in kidney transplant nephrectomy specimens.

2. Materials and Methods

2.1. Study design

In this retrospective analysis, we included 4 patients whose T_{RM} cells, derived from kidney transplant nephrectomies, were previously subjected to single-cell RNA and TCR sequencing. The data for these analyses are publicly available in the National Center for Biotechnology Information's Gene Expression Omnibus database (accession number: GSE242909). The scope of the current study encompassed a reanalysis of this existing dataset, complemented by novel experimental investigations. These new experiments involved the analysis of peripheral blood mononuclear cells (PBMCs) obtained from the same 4 patients, along with splenocytes recovered from their respective donors.

2.2. Study approval

Both the previous and current study involved the use of residual and biobank materials and received approval from the Institutional Review Board of the Erasmus Medical Center, University Medical Center Rotterdam, Rotterdam, the Netherlands (MEC-2010-080, MEC-2010-022, and MEC-2020-0791). The relevant guidelines and regulations provided by the Erasmus Medical Center were followed, and all patients gave written informed consent.

2.3. MLR

MLRs were performed to identify alloreactive T cells, as described previously.[13,15] In brief, donor splenocytes and recipient PBMCs were thawed on the day of the experiment. Donor splenocytes were labeled with CellTrace Violet Cell Proliferation dye (Thermo Fisher Scientific, Waltham, Massachusetts) and subsequently exposed to gamma irradiation (40 Gy). Recipient PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific). Subsequently, 1×10^5 irradiated Violet Cell Proliferation dye-labeled donor splenocytes and 1×10^5 CFSE-labeled recipient PBMCs were plated in each well of a round-bottom 96-well plate and cultured in RPMI + 10% human serum for 6 days at 37 °C.

After 6 days of culture, cells were recovered and stained with live/dead markers 7-AAD (BD Biosciences, Franklin Lakes, New Jersey) and CD3 Brilliant Violet 510 (BioLegend, San Diego, California). Viable CD3+Violet^{low}CFSE^{low} cells were then sorted using a fluorescence-activated cell sorting Aria II cell sorter (BD Biosciences). To ensure population purity, a portion of the sorted cells was reanalyzed via flow cytometry, confirming purity levels above 97% for all samples.

2.4. Single-cell RNA and TCR sequencing

After sorting, the proliferated recipient T cells were immediately processed for single-cell RNA and TCR sequencing, following the methods previously described.[2] In brief, the Chromium Next GEM Single Cell 5' Reagent Kit V2 and the Chromium Single Cell Human TCR amplification Kit (10X Genomics, Pleasanton, California) were used for preparation of single-cell RNA and V(D)J libraries, respectively. RNA and V(D)J libraries were then sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, California). Cell Ranger Software (version 7.0.1, 10x Genomics) was used to generate binary alignement map files.[2]

2.5. Analysis of T_{RM} cells derived from kidney transplants

Isolation and single-cell analysis of T_{RM} cells in kidney transplants were performed as described previously.[2] In brief, lymphocytes were isolated from halved kidneys through mechanical and enzymatic dissociation, followed by a Ficoll procedure. T_{RM} cells were subsequently isolated by fluorescence-activated cell sorting, sorting the T cells (CD3+CD8+ or CD3+CD4+) that also expressed CD69 plus CD103 and/or CD49a. Single-cell RNA and TCR sequencing of these T_{RM} cells was executed as described above. T_{RM} cell phenotype was confirmed in the single-cell sequencing data using the automated cell annotation tool CellTypist (version 1.3.0) as well as by manual examination of the expression of T_{RM} genes.[18] The Demuxlet tool was used to identify donor and recipient origin of each individual T_{RM} cell based on human leukocyte antigen gene expression, utilizing the

available human leukocyte antigen typing of all donors and recipients.[2]

2.6. Data analysis and statistics

Analysis was performed in R (version 4.2.1; R Foundation for Statistical Computing, Vienna, Austria) with Seurat (version 4.3.0).[16] Preprocessing excluded cells with <200 transcripts or >25% mitochondrial counts. Cells with 10% to 25% of mitochondrial counts were kept for the analysis as these cells showed normal percentage of ribosomal genes and exhibited sufficient gene diversity (Supplementary Fig. S1). Genes present in <3 cells were filtered out. Data were initially normalized for sequencing depth by dividing by the total number of unique molecular identifiers in every cell and then transformed to a log scale for each cell using the NormalizeData function. Data were then integrated to remove batch effects using reciprocal principal component analysis, where anchor genes were the variable genes obtained using the variance-stabilizing transformation method. After scaling and principal component analysis, with 50 components calculated, DoubletFinder removed 1479 doublets (4.3% of cells). Cells were clustered using the shared nearest neighbor modularity optimization-based clustering algorithm with resolutions from 0.1 to 1 in steps of 0.1. Clustree guided the selection of optimal clustering resolution.[17] Lists containing differentially expressed genes within clusters were generated with Presto. Annotation of clusters was done by manual inspection of the features defining each cluster, together with the assistance of CellTypist (version 1.3.0).[18] Offset populations within the data (germinal B cells and macrophages) were removed. The remaining cells (31 311 cells) were reanalyzed following the described pipeline above. Uniform manifold approximation and projection was used for the 2-dimensional representation of the data.

Visualizations using the DotPlot function graphically represent per cluster percentage expression by dot diameter and average nonzero expression in $\log_2$ scale by dot color; data with expression <0.01 are not shown.

The immune profiling data from the TCR sequencing were analyzed and merged with the single-cell RNA data with scRepertoire (version 1.7.2).[19] Clonal diversity analysis was performed on both chains (TCR alpha and beta chain). Two metrics are reported: Shannon and inverse Pielou's. The former is an estimate of clonal diversity, and the latter is a measure of clonal evenness.[20,21]

A string-search analysis was performed for the TCR sequences shared between the alloreactive T cells and T_{RM} cells. These TCR sequences were matched with sequences published in VDJdb, PIRD, and McPAS-TCR databases.[2]

3. Results

3.1. Defining the alloreactive TCR repertoire

The aim of the present study was to assess the alloreactive potential of T_{RM} cells in human kidney transplants. The approach that was used involved generating a fingerprint of the alloreactive TCR repertoire by means of MLR, followed by single-cell RNA and TCR sequencing of proliferated recipient T cells of 4 patients, of whom a T_{RM} cell analysis had been performed previously.[2] Subsequently, the identified alloreactive clones were traced in the T_{RM} cell data (Fig. 1A, B). Baseline characteristics and description of the clinical course of these patients are provided in Supplementary Tables S1 and S2. Each of these patients experienced severe acute rejection, which ultimately resulted in graft loss and necessitated transplant nephrectomy. Their treatment for rejection included methylprednisolone in all cases. In addition, intravenous immunoglobulin was administered to patients G1 and G10, and alemtuzumab was given to patients G10 and G22 (Supplementary Tables S1 and S2).

3.2. Alloreactive TCRs were detected in donor-stimulated peripheral blood samples from kidney transplant recipients

Single-cell RNA and TCR sequencing was performed with the flow-sorted recipient T cells that proliferated during the MLR. After quality control and data processing, we analyzed over 31 000 proliferated recipient T cells, pooled from 4 patients in total. This cell population contained 12 distinct cell types, with an even distribution among these clusters of the 4 samples (Fig. 2A, B). The cell clusters included CD4+ and CD8+ cells with various phenotypes, including naïve, effector, memory, and regulatory subtypes (Fig. 2A, C and Supplementary Fig. S2). The 2 clusters that did not contain T cells, were excluded from further analysis (Fig. 2A, C). Among the T cells, TCR sequence reads were available for 78% (n = 23 130) of T cells. The TCR analysis showed variations in TCR expansion levels among the patients. Particularly, samples G1 and G9 exhibited the largest proportion of cells with hyper-expanded or largely expanded TCRs (Fig. 2D, E). In line with this, these 2 samples also had higher clonality scores and a lower number of unique alloreactive clonotypes than those in samples G10 and G22 (Fig. 2F).

3.3. Alloreactive T_{RM} cells are present in human kidney transplant nephrectomies

We proceeded to investigate whether the alloreactive TCR clones, identified by means of MLR in 4 patients, were also present in the T_{RM} cells of their respective kidney transplants. To this end, we used the single-cell RNA and TCR data of T_{RM} cells obtained from the transplant nephrectomy specimens of the respective patients, as analyzed in our prior study.[2] These 4 kidney transplant nephrectomy specimens were dominated by recipient-derived T_{RM} cells but also contained donor-derived T_{RM} cells (Supplementary Table S3). In each sample, the number of unique clonotypes was higher for recipient-derived T_{RM} cells than for donor-derived T_{RM} cells (Supplementary Table S3).

Next, we traced the alloreactive TCR clones among these T_{RM} cells. Patients G1 and G9, who also showed the most significant expansion of alloreactive TCRs after MLR (as depicted in Fig. 2D, E), exhibited the highest numbers and proportions of alloreactive clonotypes within their T_{RM} cells (Fig. 3A). Both of these patients presented clear histopathologic signs of active rejection at the

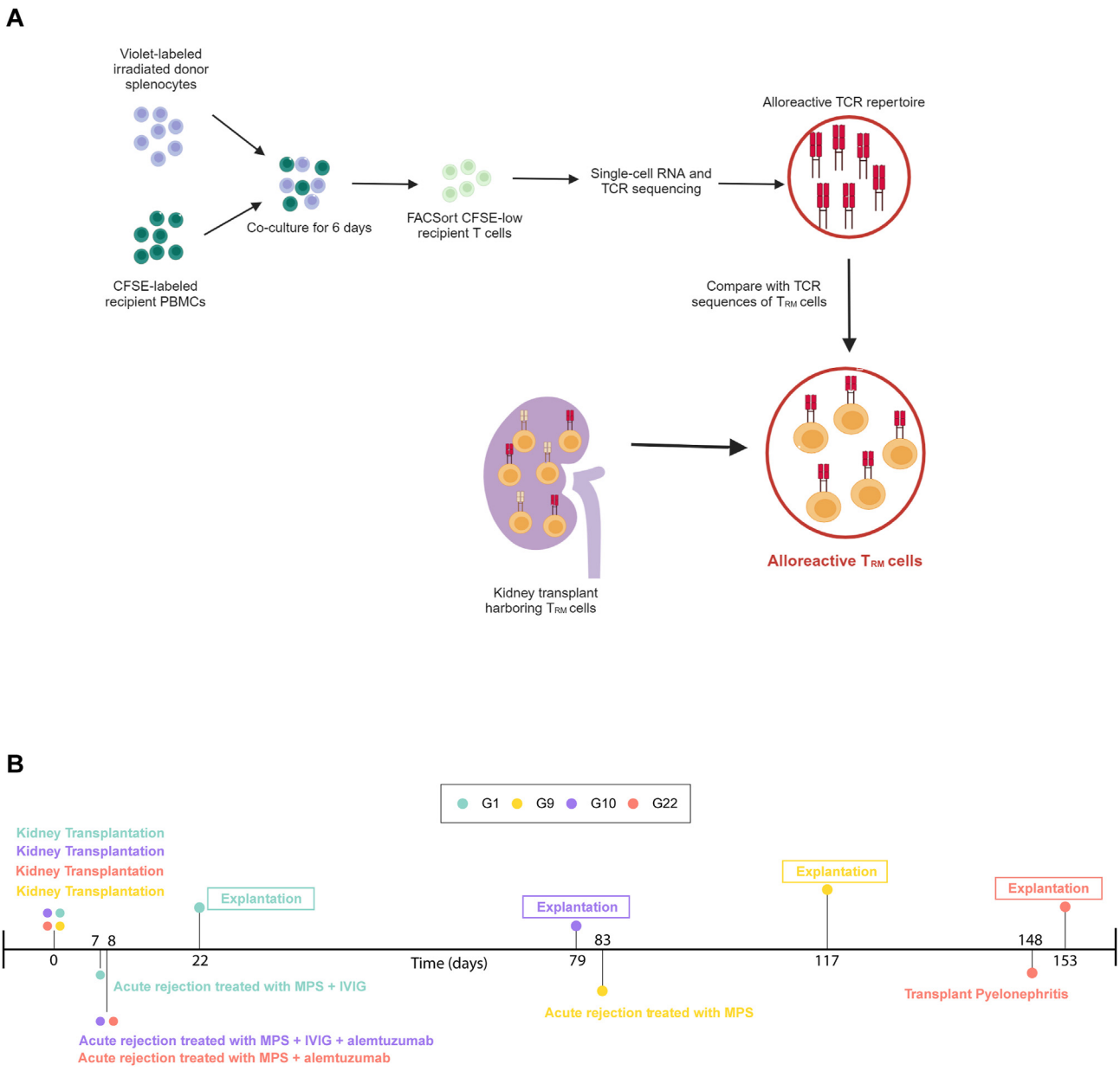

Figure 1. Study overview and relevant patient events. (A) Experimental overview of the study: Donor splenocytes and recipient peripheral blood mononuclear cell (PBMC) were cocultured for 6 days, followed by flow-sorting of proliferated recipient T cells. These sorted "alloreactive" cells were analyzed with single-cell RNA and T cell receptor (TCR) sequencing. Next, the alloreactive TCRs were compared with TCRs expressed by tissue-resident memory T (T_{RM}) cells derived from human kidney transplant nephrectomies in order to define the proportion of alloreactive T_{RM} cells. Created with BioRender.com. (B) The clinical course of the included patients (n = 4) is shown from kidney transplantation until allograft explantation. CFSE, carboxyfluorescein succinimidyl ester. FACSort, fluorescence-activated cell sorting; MPS, methylprednisolone; IVIG, intravenous immunogobulins.

time of explantation (Supplementary Table S1). In contrast, only 1 and 2 alloreactive clonotypes among T_{RM} cells were identified in samples G10 and G22 (Fig. 3A). Strikingly, the clones identified as alloreactive were not confined to recipient-derived T_{RM} cells; they were also detected within donor-derived T_{RM} cells, thus representing self-reactive clones (Fig. 3A). The absolute number of alloreactive/self-reactive clonotypes and cells was higher in recipient- versus donor-derived T_{RM} cells (Fig. 3A, B). However, the relative proportion of these alloreactive/self-reactive clonotypes was comparable between recipient and donor (Fig. 3A). The proportion of alloreactive/self-reactive cells was slightly higher in recipient-derived T_{RM} cells than in donor-derived T_{RM} cells for patient G1 (8.85% and 4.11%), G9 (3.32% and 2.14%), and G22 (0.03% and 0%), but not for patient G10 (0.07% and 0.07%; Fig. 3B; Supplementary Table S3). Notably, most T_{RM} cells expressing alloreactive/self-reactive T_{RM} clonotypes exhibited an effector phenotype (Fig. 3C).

To investigate potential cross-reactivity of T_{RM} cells against alloantigens/self-antigens and antiviral antigens, we conducted a string-search analysis across 4 public TCR databases. This analysis revealed a match for only 1 alloreactive/self-reactive clone with specificity for cytomegalovirus. This clone was

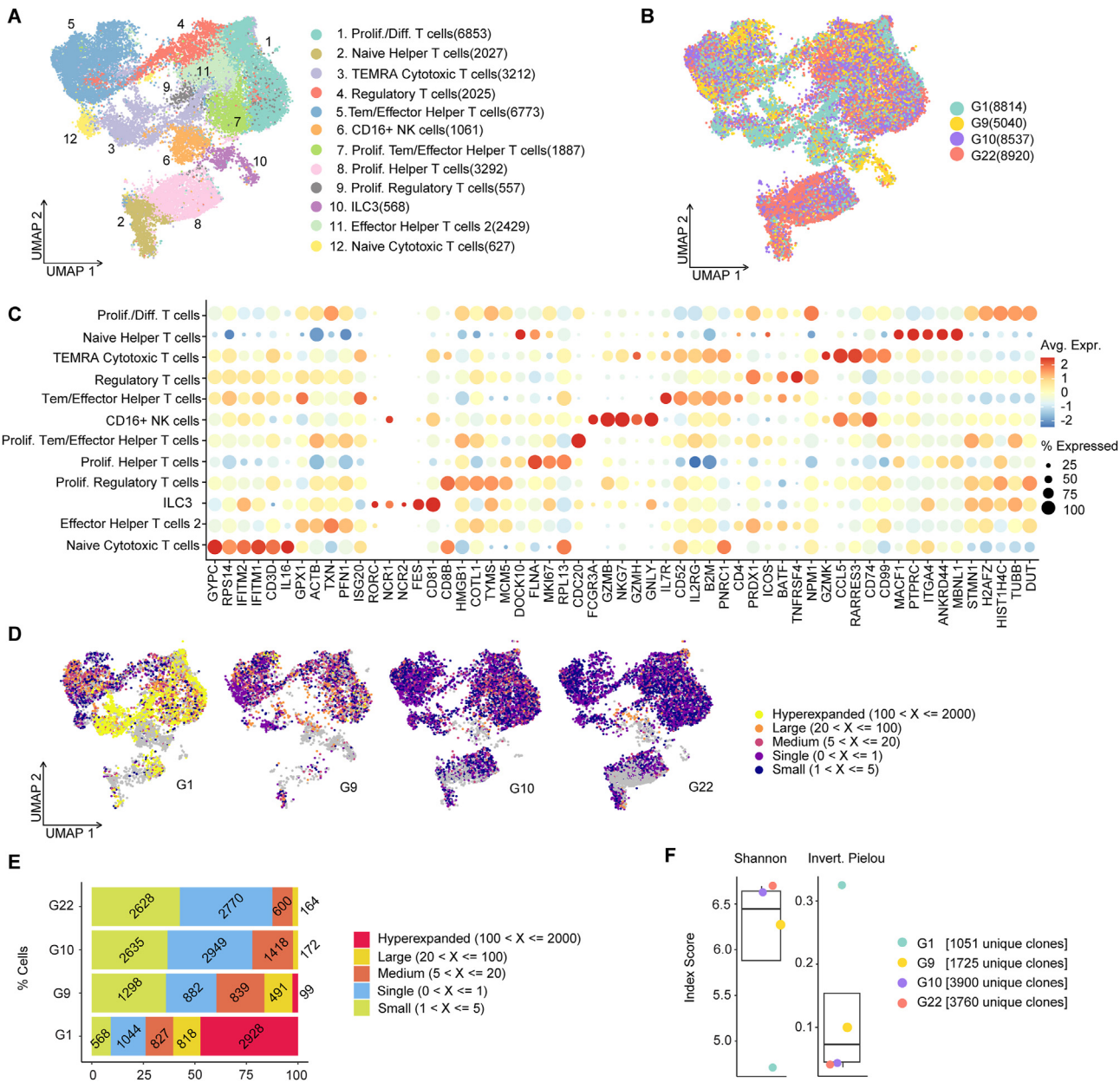

Figure 2. Exploration of cell phenotype and T cell receptor (TCR) expansion of alloreactive T cells. Paired single-cell RNA and TCR sequencing of proliferated recipient T cells (ie, alloreactive T cells) was performed. (A) Uniform manifold approximation and projection (UMAP) showing the 12 identified cell clusters. Cell numbers per cluster are shown in brackets. (B) UMAP showing the distribution of the 4 samples across cell types after batch correction. (C) Canonical and relevant genes used to identify cell populations are shown in the dot plot. (D) UMAP per sample showing the level of expansion of each individual TCR. (E) Levels of TCR expansion in relation to the cell numbers and proportions per sample. (F) Clonal diversity scores (Shannon) and clonal evenness scores (invert Pielou). The number of unique clonotypes per sample is shown in brackets. prolif., proliferating; diff.; differentiating; TEMRA, effector memory T cells re-expressing CD45RA; NK cells, natural killer cells; ILC3, type 3 innate lymphoid cells; avg., average; expr., expression.

expressed by 2 alloreactive T cells in patient G1 and in 24 T_{RM} cells (22 recipient-derived and 2 donor-derived) within the same patient. No matches were found for the remaining alloreactive clones.

4. Discussion

The present study shows that T cells with alloreactive potential detected in the peripheral blood of transplant recipients prior to transplantation can infiltrate the kidney transplant and adopt a T_{RM} phenotype. Notably, we found that the proportion of alloreactive T_{RM} cells ranged from 0% to 9%. Previously, we demonstrated the antiviral properties of T_{RM} cells, and taken together with the present findings, this underscores the multifaceted role these cells play within kidney transplants.[2]

Our methodological approach, consisting of MLR, flow-sorting, and single-cell RNA and TCR sequencing, mitigates some limitations of several prior studies with regard to the definition of T_{RM} alloreactivity. Prior methodologies relied predominantly on the level of TCR expansion and did not always confirm

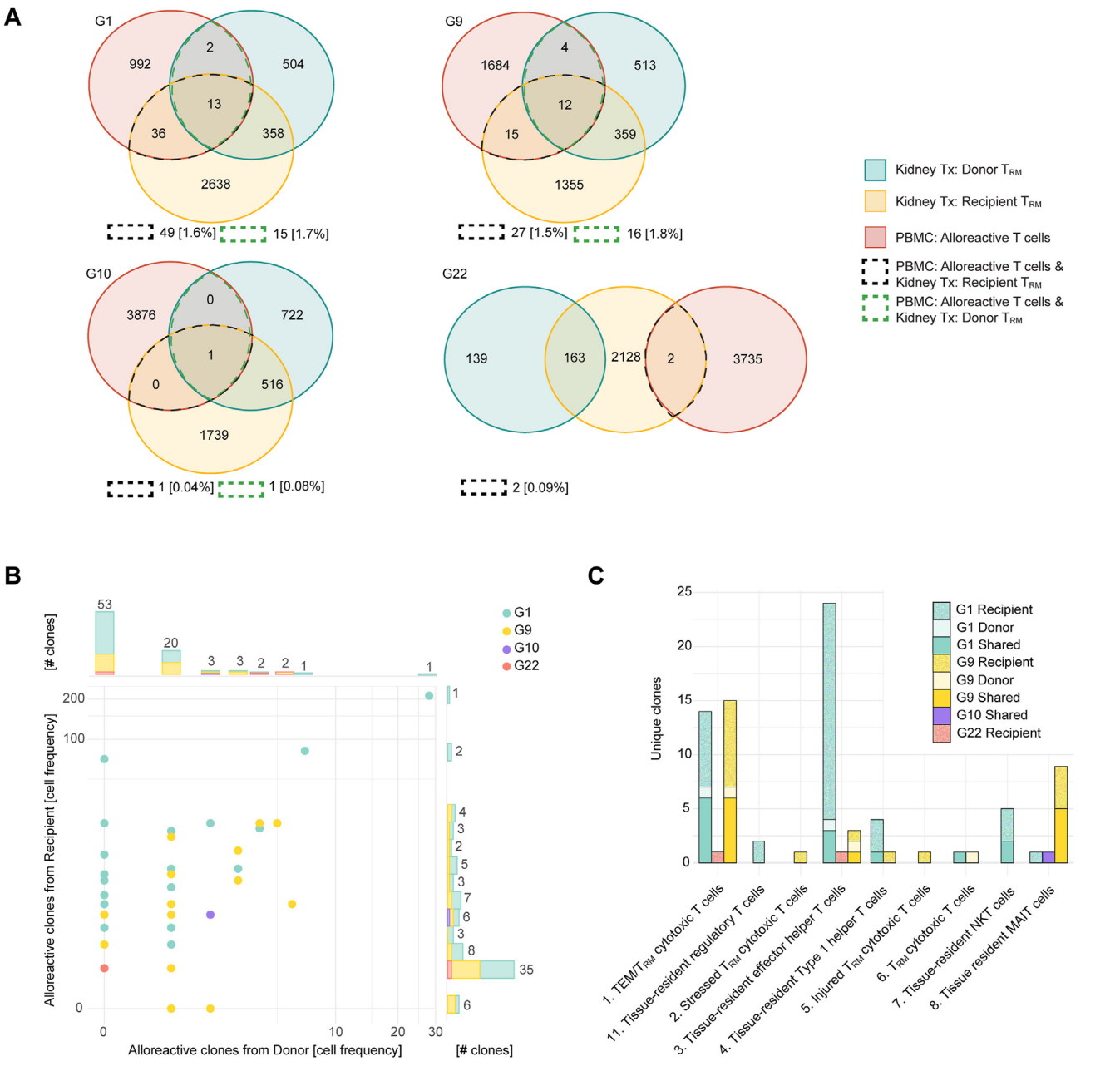

Figure 3. A proportion of tissue-resident memory T (T_{RM}) cells in explanted human kidney transplants expresses an alloreactive T cell receptor. The presence of defined alloreactive T cell receptors among T_{RM} cells in human kidney transplant nephrectomy specimens was analyzed. (A) Venn diagrams per patient show the number of clonotypes shared among the defined alloreactive T cells, recipient T_{RM} cells, and donor T_{RM} cells. The total number of alloreactive clonotypes among recipient T_{RM} cells (black) and donor T_{RM} cells (green) is annotated below the Venn diagrams, and the proportion of alloreactive clonotypes among the respective groups is shown in brackets. (B) Each dot represents an alloreactive clonotype detected among recipient and/or donor T_{RM} cells. The number of recipient cells (y-axis) and donor cells (x-axis) per alloreactive clonotype is shown. Both axes show a coordinate transformation of log (x+1). The clone distribution is shown in the histogram. (C) Distribution of donor, recipient, and shared alloreactive clones among the different T_{RM} cell clusters. PBMC, peripheral blood mononuclear cell; TEM, effector memory T cell. Tx, transplantation; NKT cells, natural killer T cells; MAIT cells, mucosal-associated invariant T cells.

the recipient origin of the cells that expressed the expanded TCRs.[9,10] Our approach combines the previously developed TCR tracking method with single-cell RNA and TCR analysis, thereby effectively overcoming these challenges.

The observed proportion of alloreactive T_{RM} cells (0%-9%) closely mirrors the prevalence of total alloreactive T cells within kidney transplants observed during acute rejection prior to treatment (2.5%-4.8% of total T cell alloreactive TCRs) using the same TCR tracking method.[13] It is important to note that all patients in the current study had received recent antirejection therapy, which might have modified the TCR repertoires. Despite this treatment, alloreactive T_{RM} cells persisted in the allograft, particularly in patients with clear histopathologic evidence of ongoing rejection at the time of explantation. This supports prior findings that alloreactive clones, while reduced after rejection treatment, are not completely eradicated and persist as T_{RM} cells.[5,9,10]

A striking observation is the presence of defined alloreactive clones among donor T_{RM} cells, suggesting the existence of self-reactive T_{RM} cells. This phenomenon may be attributed to the normal presence of self-reactive T cells in healthy individuals.[22] Thus, our findings suggest that self-reactive cells are also present among T_{RM} cells in kidney transplants. In healthy individuals, self-reactive T cells are mainly regulatory and suppress autoreactive responses.[22] However, in our study, donor-derived self--reactive T_{RM} cells predominantly exhibit an effector but not regulatory phenotype. Autoreactive immune responses have been reported to play a role in allograft rejection.[23,24] Our findings add a novel dimension to this understanding, proposing that autoreactive responses are not only limited to recipient T cells but may also be executed by donor T_{RM} cells within the transplant. While autoreactive T_{RM} cells have been implicated to play a role in the pathogenesis of various autoimmune diseases, their precise role (ie, protective or pathological) in kidney transplantation remains to be elucidated.[25,26]

The discovery of donor-derived T_{RM} cells with potential self-reactive properties in our study introduces a complex consideration for therapeutic interventions. Pretransplant elimination of these cells that could potentially harm the transplant may seem beneficial. However, in our previous study, we also identified donor-derived T_{RM} cells with antiviral specificities.[2] These cells likely play a protective role against viral infections post-transplant, indicating that their removal could increase the transplant's vulnerability to such infections. The dilemma extends to recipient-derived T_{RM} cells, as we found evidence of alloreactive as well as antiviral subsets.[2] The challenge lies in weighing the removal of cells that could be detrimental against losing those that confer protective benefits, emphasizing the urgent need for further research to guide these complex therapeutic implications.

This study is not without limitations, notably the small sample size and the absence of rejection biopsy samples in the analysis, which precludes a comprehensive assessment of alloreactivity among T_{RM} cells unaffected by immunosuppressive therapy. Our unique dataset of human transplant nephrectomy samples introduces a selection bias, as it primarily includes patients undergoing transplant nephrectomy, which is not representative of the typical course post-transplantation. Future research should also include samples from patients who experienced late rejection ($>$1 year after transplant), as T_{RM} cells might not be fully developed in the early stages. Future research should also collect kidney graft samples and conduct MLR assays across various time points within each patient to better understand T_{RM} cell dynamics over time.[27] Additionally, while effective at identifying alloreactive clones, the TCR tracking method may not fully capture the entire alloreactive T cell compartment due to multiple factors. These include limitations of single-cell sequencing, such as the limited number of cells that can be analyzed and TCR sequence reliability.[28] Integrating high-throughput sequencing and pairSEQ analysis may overcome these limitations in future studies.[29] Moreover, lineage tracing through mitochondrial mutation analysis in both epigenetic and genetic datasets could further improve our understanding of clonal relationships between cells and across donors.[30] Also, MLR predominantly activates T cells recognizing alloantigens via the direct pathway and not those activated by the indirect pathway of allorecognition. Furthermore, conducting autologous controls for the MLR experiments was not feasible due to the nature of our samples. Specifically, PBMCs could not be collected from deceased donors, and recipient splenocytes were unavailable because our study involved living patients. Our string-search analysis provides only an indication of potential cross-reactivity, necessitating future studies. For example, by cloning the identified TCR sequences into a Jurkat cell line, followed by stimulating these with self-antigens, alloantigens, and viral antigens. These experiments could also be used to investigate whether the identified specificities result in functional responses.

In conclusion, our study, together with previous findings, underscores the multifaceted roles of T_{RM} cells within kidney transplants. The current study demonstrates that some recipient T_{RM} cells in kidney transplants exhibit alloreactive potential. Moreover, our study reveals the existence of donor T_{RM} cells that may exhibit self-reactive potential within the transplant. The impact of immunosuppression on the balance between alloreactive, self-reactive, and antiviral T_{RM} cells requires future study.

Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Data availability

All single-cell sequencing data generated in this study have been deposited in the NCBI's Gene Expression Omnibus database database (GEO GSE249197).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2024.02.030.

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