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Proinflammatory Dual Receptor T Cells in Chronic Graft-versus-Host Disease

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ABSTRACT
Defective post-transplantation thymopoiesis is associated with chronic graft-versus-host disease (GVHD), a multiorgan pathology affecting up to 80% of patients after allogeneic hematopoietic stem cell transplantation (HSCT). Previous work demonstrated that the subset of T cells expressing 2 T cell receptors (TCRs) is predisposed to alloreactivity, driving selective and disproportionate activity in acute GVHD in both mouse models and HSCT patients. Here we investigate a potential role for this pathogenic T cell subset in chronic GVHD (cGVHD). HSCT patients with cGVHD demonstrated increased numbers of dual TCR cells in circulation. These dual receptor cells had an activated phenotype, indicating an active role in cGVHD. Notably, single-cell RNA sequencing identified the increased dual TCR cells in cGVHD as predominantly expressing Tbet, indicative of a proinflammatory phenotype. These results identify dual TCR cells as specific mediators of pathogenic inflammation underlying cGVHD and highlight Tbet-driven T cell function as a potential pathway for potential therapeutic targeting.

INTRODUCTION
Allogeneic hematopoietic stem cell transplantation (HSCT) presents a medically relevant example of the double-edged nature of T cell function. Post-allogeneic HSCT mortality is driven by 3 main causes: malignancy relapse, graft-versus-host disease (GVHD), and opportunistic infection [1,2]. Opportunistic infection and malignant disease relapse represent, in part, a failure of T cells to mediate protective immunity [3,4]. Conversely, GVHD is unwanted or misdirected T cell function that results in multiorgan immune-mediated damage [5,6]. Acute GVHD (aGVHD) is caused by a robust response of donor T cells in the hematopoietic stem cell graft against recipient alloantigens [6], while increasing evidence indicates that chronic GVHD (cGVHD) represents a failure of effective self-tolerance by the newly developed immune system [5-12]. It is a goal in HSCT to differentiate T cells capable of mediating effective post-transplantation protective immunity from those promoting GVHD, with the aim of identifying specific mechanisms driving pathology.

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in normal physiology from simultaneous rearrangement of both TCRα loci in CD4+CD8+ double positive thymocytes as a mechanism to maximize positive selection efficiency [23]. However, promotion of positive selection comes with the cost of reduced stringency of thymic selection requirements for dual receptor thymocytes; only 1 of the TCRs on a dual receptor cell is required to mediate positive selection [24,25] and expression of a second receptor can mask TCRs that would otherwise be eliminated by negative selection [26-28]. This results in emergence of populations of dual TCR cells containing unique TCRα clonotypes that would not be present under more stringent selection [23]. The presence of these unique TCRs does not appear to affect responses to foreign antigens but is associated with increased frequency of cells reactive against alloantigens and autoantigens [23,29]. These reactivities underlie the potential for dual TCR T cells to initiate pathologic alloreactive and autoimmune responses observed in patients and animal models [25,28,30,31].

We hypothesized that dual TCR cells could be a source of potentially pathogenic T cells in cGVHD. To test this, we examined dual TCR cells in peripheral blood samples from allogeneic HSCT patients with or without symptomatic cGVHD. Using a previously utilized multiparameter flow cytometry approach, we identified that dual TCR cells were increased in patients with cGVHD compared with those in healthy controls or allogeneic HSCT patients without cGVHD. Dual TCR cells in cGVHD patients were disproportionately activated, indicating participation in disease pathology. Utilizing a cutting-edge single-cell sequencing approach, we confirmed the increased frequencies of dual TCR cells in patients with cGVHD and identified expression of Tbet, a transcription factor associated with proinflammatory Th1 and cytotoxic T cell function, as a predominant population associated with cGVHD. These findings support the idea of dual TCR cells as a selectively pathogenic subset in transplantation and highlight Tbet-mediated proinflammatory pathways as a potential therapeutic target in cGVHD.

MATERIALS AND METHODS
Human Subjects
Peripheral blood leukocytes were collected from consenting healthy adult volunteers during apheresis platelet donation at the San Diego Blood Bank. WBCs were recovered from apheresis filters. Adult patients receiving allogeneic HSCT at the University of California San Diego Moores Cancer Center were recruited for participation and provided informed consent. Patients received standard of care therapy, including pretransplantation conditioning and granulocyte colony-stimulating factor–mobilized HSCs or bone marrow as indicated (Table 1). Peripheral blood samples from HSCT patients (10 mL to 30 mL) were collected by peripheral venipuncture at routine follow-up visit or at admission for treatment of symptomatic cGVHD. cGVHD was assessed using National Institutes of Health (NIH) diagnosing and staging criteria [32]. All samples and data from healthy donors and HSCT patients were deidentified and assigned a study-unique identifier, linking data and samples.

Leukocytes were collected by density-gradient centrifugation (lymphoprep, Stem Cell Technologies, Vancouver, BC) and stored at −80°C until analysis. All collection, storage, and analysis of samples and patient data were performed under the approval of the University of California San Diego human institutional review board.

Flow Cytometry
Human peripheral blood T cells were labeled with Live/Dead Yellow viability dye (Thermo-Fisher Scientific, Waltham, MA) before labeling with TruStain Fc block, anti-CD3 (HIT3a)-PE, anti-C57 (Oxford), anti-CD8 (HT1a)-PerCP-Cy5.5, anti-CD45RA (H100)-BV421, anti-CD45R0 (UCHL1)-AF700, anti-TCRαv7.2 (C15)-PE (Beckman Coulter, Brea, CA), anti-TCRVα2 (F1) (Thermo-Fisher Scientific) labeled with AF647 (Molecular Probes, Eugene, OR), and anti-TCRVα4 (5B2) and anti-TCRVα9 (2B2) [31] labeled with Pacific Orange and AF594, respectively (Molecular Probes). Samples were analyzed on a FACSCanto or LSR II instruments (BD Biosciences, San Jose, CA), with FACSDiva software. Samples were run in batches containing both control and experimental samples. Cutoffs for defining positive labeling were determined using fluorescence minus-1 controls for surface labeling and isotype controls for intracellular labeling. Data were analyzed using Flowjo v10 (Tree Star, Ashland, OR).

Single-Cell TCR Clonotype and Transcription Factor Analysis
Human peripheral blood T cells were isolated using a FACS ARIA II cell sorter into a 96 well PCR plate (Axygen, Corning, NY), directly into 11 μL of 1x One-Step RT-PCR buffer (Qiagen, Hilden, Germany). Barcoded single-cell libraries of TCRα, TCRβ, and effector genes were generated by independent nested PCR amplification of cDNA using HotStar Taq (Qiagen) [33]. The nested amplification protocol was modified from the original version, reducing the reaction volumes by 50% and separating TCRα and TCRβ amplification to improve efficiency. The final products were pooled, gel purified, and sequenced using 500 cycle v2 MiSeq reagents (Illumina, San Diego, CA). Demultiplexing data for individual wells and counting effector gene transcripts was performed using previously described algorithms [33]. TCR sequence data were analyzed using MiTR software [34].

Statistical Analysis
Data were analyzed using Prism 6 software (GraphPad). Nonparametric analyses were performed using Mann-Whitney test. Intrasample comparisons of phenotype for single and dual TCR cells were performed using paired t-test. Frequencies of categorical data were compared using Fisher’s exact test.

RESULTS
Flow Cytometry Identified Increased Frequencies of Dual TCR Cells in Patients with cGVHD
Given the ability of dual TCR T cells to selectively mediate pathologic alloreactive and autoimmune responses in patients and animal models [25,28,30,31], we examined T cells in peripheral blood samples from patients developing cGVHD 19 to 48 months after allogeneic HSCT (n = 9), allogeneic HSCT patients not developing cGVHD within a comparable post-transplantation time period (n = 4), and age-matched healthy controls (n = 5) (Table 1). We utilized our previously described approach of flow cytometry analysis for pair-wise TCRα expression [31]. Using antibodies recognizing TCRVα2, TCRVα4, TCRVα7, TCRVα9, TCRVα12, and TCRVα24 enables examination of approximately 15% of the TCRα repertoire (Figure 1A).

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HSC indicates hematopoietic stem cell; RIC, reduced-intensity conditioning; PBSC, mobilized peripheral blood stem cells; MAC, myeloablative conditioning; BM, bone marrow; n/a, not available. HLA matching refers to allele-level match for HLA-A, -B, -C, -DRB1, and -DQB1. cGVHD severity was assessed using NIH criteria.
and identification of 15 pair-wise combinations of TCRVα expression (Figure 1B). Using this flow cytometry approach, dual TCR cell frequency was evaluated both as absolute number of dual TCR cells among all cells labeled with anti-TCRVα mAbs and as a percentage of dual TCR cells potentially identifiable (using the most consistently abundant TCRVα examined, TCRVα12, to normalize between samples) (Figure 1C). These analyses enabled normalization for differential use of TCRVα gene segments between individuals. Although increased frequencies of T cells expressing the 6 TCRVα segments that we are capable of examining by flow cytometry increased the absolute number of dual TCR cells identifiable, there was no bias in the frequency of identifiable dual TCR cells caused by use of these differential TCRVα (Figure 1D). Blinded independent analysis of dual TCR T cell frequencies by 2 different investigators demonstrated excellent correlation (Figure 1E), indicating the robustness of this approach. Although the frequency of dual TCR cells identifiable using the method is low,
Dual TCR Cells Are Selectively Activated in cGVHD

The use of multiparameter (13-color) flow cytometry enables phenotypic examination of dual TCR cells and comparison of their phenotype with that of other cells in the same sample. Dual TCR cells did not evidence any difference in total TCR expression as measured by CD3 expression (Figure 3A,B). Dual TCR cells were also equally likely to have a naïve (CD45RA⁻) phenotype as the T cell population in general (41.1 ± 7.1% and 38.8 ± 5.9% respectively, \(P = .311\)) (Figure 3C,D). However, dual receptor cells were much more likely to be activated than all TCRα mAb⁻ cells in patients with cGVHD, as evidenced by expression of CD69 (24.3 ± 10.0% of dual TCR cells compared with 8.1 ± 4.6% all TCRα⁺ cells, \(P = .004\)) (Figure 3E,F).

**Figure 3.** Phenotypic examination of dual TCR cells in patients with cGVHD. Phenotype of dual TCR T cells identified by pair-wise TCRα mAb labeling was compared with all TCRα⁺ cells in samples. (A) Representative example of CD3 expression by all TCRα⁺ cells and dual TCR cells. (B) Comparison of CD3 expression by all TCRα⁺ cells and dual TCR cells in samples from healthy donors, allogeneic HSCT patients without cGVHD, and patients with cGVHD. (C) Representative example of CD45RA expression by all TCRα⁺ cells and dual TCR cells. (D) Comparison of CD45RA expression by all TCRα⁺ cells and dual TCR cells in samples from patients with symptomatic cGVHD. Data shown are individual samples with groups linked by line. Data compared using ratio paired t-test. (E) Representative example of CD69 expression by all TCRα⁺ cells and dual TCR cells identified by pair-wise labeling. (F) Comparison of CD69 expression by all TCRα⁺ cells and dual TCR cells in patients with symptomatic cGVHD. Data shown are individual samples with groups linked by line. Data compared using ratio paired t-test.
**Single-Cell RNA Sequencing Confirms Increased Dual TCR Cells in cGVHD**

Identification of dual TCR T cells by flow cytometry is critically restricted by a paucity of available reagents. This significantly limits our ability to examine the entire potential dual TCR repertoire. Furthermore, the flow cytometry approach does not unambiguously identify T cells expressing a single TCR, as it would be expected that a number of cells express secondary TCRs that cannot be measured by available reagents. To overcome this, we adopted a single-cell barcoded PCR strategy that enables identification of TCRαβ clonotypes combined with selected transcription factor/effector gene expression by multiplexed next-generation DNA sequencing [33] and MiTCR gene rearrangement analysis software [34]. Individual T cells (176/sample) from healthy donors (n = 4) and HSCT patients with symptomatic severe cGVHD (n = 4, Patients 6, 14, 15, and 18) were isolated by flow cytometry into 96-well plates for single-cell analysis. A stringent cutoff of 10^5 TCR sequence reads was applied to minimize the possibility for cross-contamination resulting in false-positive TCR sequences for a given well. Using this cutoff, we identified in-frame TCRβ rearrangements in 75.0% and TCRα in 70.0% of sorted wells (Figure 4A). Further refinement of the data was performed by eliminating cells with 2 in-frame TCRβ transcripts to avoid the possibility for having sorted 2 cells into a single well as well as eliminating TCRα transcripts paired with more than 1 TCRβ from a given patient to minimize the possibility for cross-contamination resulting in false-positive identification of dual TCR cells. Wells with 2 TCRα transcripts demonstrated a range of ratios between the transcripts (Figure 4B). Together, these analyses confidently identified TCRαβ clonotypes in 49.6% of wells tested.

Using the single-cell sequencing strategy, we identified TCRαβ clonotypes from 429 T cells from healthy donors and 422 T cells from patients with cGVHD (Figure 5A). Two in-frame TCRα transcripts were identified in 68 (18.8%) of cells from healthy donors, consistent with other reports as well as the estimate from our flow cytometry data (Figure 2C). Single-cell TCR sequencing of peripheral blood T cells from 4 patients with severe cGVHD (NIH grading criteria) demonstrated a significantly higher percentage of cells with 2 in-frame TCRα transcripts (23.4%, P = .019) compared with single TCR T cells, though this is possibly attributable to the relatively limited number of dual TCR clones identified. TCRs from dual receptor cells did not demonstrate structural abnormalities in the CDR3α or CDR3β, with CDR3 lengths comparable to single TCR cells from cGVHD patients as well as single or dual TCR cells from healthy donors (Figure 5C). Similarly, TCRs from dual receptor cells did not demonstrate preferential use of basic (R, H, K), acidic (D, E), small (G, A), nucleophilic (S, T, C), hydrophobic (V, L, I, M, P), aromatic (F, Y, W), or amide (N, Q) amino acids in the CDR3α or CDR3β regions (Figure 5D). The single-cell sequence data provide additional confidence in our observation of increased dual TCR T cell frequencies in HSCT patients with cGVHD by flow cytometry and unambiguously identify TCRαβ clonotypes from dual receptor cells for examination of their activity in cGVHD.

**Dual TCR Cells in cGVHD Are Proinflammatory**

A key benefit of the multiplexed single-cell sequencing approach is the ability to link TCR clonotypes with phenotype. Using the described multiplex phenotype PCR primers [33] we examined expression of 14 key transcription factors and effector molecules. However, T cells were not restimulated ex vivo, which limited information from cytokine and effector molecule analysis. Therefore, we focused phenotypic analysis on transcription factors capable of differentiating T cell functional subsets, including RUNX1, RUNX3, TBX21, RORC, GATA3, BCL6, and FOXP3 [35]. The nested PCR approach used for single-cell library construction provides qualitative but not quantitative analysis of gene expression. Read count cutoff values for positive gene expression were determined by analysis of read count distribution. CD4+ (RUNX1+) and CD8+ (RUNX1+) dual TCR T cells from patients with cGVHD (Figure 6A) demonstrated frequent expression of TBX21 (Tbet), a promoting factor for proinflammatory Th1 CD4+ and cytotoxic CD8+ T cell function [36]. TBX21+ expressing cells were significantly more common in both single TCR (59.8% CD4+ cells, 17.6% CD8+ cells) and dual TCR cells (70.0% CD4+ cells, 18.8% CD8+ cells) from patients with cGVHD compared with healthy donors (50.4% CD4+ cells, P = .009, 8.5% CD8+ cells, P = .054), indicating involvement of these cells in cGVHD (Figure 6B). GATA3 and BCL6 were also commonly expressed by dual TCR cells. No difference in expression of these factors was observed between T cells from patients with cGVHD and healthy controls. Few FOXP3+ CD4+ regulatory T cells (Tregs) were observed in patients with cGVHD (1.4%), though, the number of cells identified was not statistically different from healthy donors (3.8%, P = .261).
Comparison of transcription factor expression by single TCR and dual TCR cells did not identify specific variances, suggesting differences in function associated with cGVHD. However, dual TCR T cells in patients with active cGVHD were significantly more likely to express at least 1 of the effector function–driving \( \text{TBX21} \), \( \text{GATA3} \), or \( \text{BCL6} \) compared with single TCR cells (92.5% of dual TCR CD4\(^+\) cells compared with 76.6% of single TCR CD4\(^+\) cells, \( P = .034 \); 81.2% of dual TCR CD8\(^+\) cells versus 67.6% of single TCR CD8\(^+\) cells, \( P = .371 \)). A similar difference was not observed when comparing dual TCR and single TCR cells from healthy donors and cGVHD patients. Data are shown as relative frequency for CDR3 amino acid length by TCR clonotypes within each group. (D) CDR3\(^\beta\) and CDR3\(^\alpha\) amino acid use by single and dual TCR cells from healthy donors and cGVHD patients. Data are shown as relative frequency for CDR3 amino acid length by TCR clonotypes within each group.

These results are in line with our observation of selective activation of dual TCR T cells as evidenced by CD69 expression (Figure 3E) and suggest that dual TCR T cells are selective mediators of disease.

**DISCUSSION**

The pathogenic link between altered post-HSCT thymopoiesis and development of systemic inflammatory disease characterizing cGVHD led us to investigate the potential involvement of the subset of T cells naturally coexpressing 2 TCRs. Dual TCR cells have been shown to selectively mediate alloreactive responses and initiate the earliest phases of aGVHD [23,25,31]. Using both a previously described multiparameter...
flow cytometry assay as well as a single-cell sequencing approach, we provided proof of concept that dual TCR T cells are selectively increased and active in patients with cGVHD. Although the number of subjects in this study is small, limiting the ability to draw larger conclusions regarding the magnitude of the role of dual TCR cells in pathogenesis and the potential utility for measurement of these cells as a prognostic biomarker, the single-cell DNA sequencing approach provided significant novel information. The single-cell analysis performed in this study has identified over 400 αβ TCR (and ααβ TCR) clonotypes associated with cGVHD for further functional investigation. Importantly, the single-cell sequencing approach provided a method to not only unambiguously identify single TCR and dual TCR cells and provide additional confidence in the results from the flow cytometry-based studies, but also to link TCR clonotypes to effector function. This is an important area for investigation, as the mechanisms driving cGVHD pathology remain enigmatic.

Single-cell sequencing enabled focused examination of suspected pathogenic cells, permitting separation of population-specific signal from background bulk population noise. Our analysis of transcription factors associated with driving T cell effector phenotypes demonstrated a predominance of CD4+Th1 and CD8+Tbet cells. This is consistent with other observations of cGVHD driven by proinflammatory T cells [37]. We also observed frequent expression of GATA3 and BCL6, though the relationship to function is not directly evident, as they have multiple effects on T cell function. GATA3 is associated with driving CD4+Th2 responses, but is also involved in T cell homeostasis and Th9 differentiation [38]. BCL6 is associated with differentiation of follicular helper T cells, which have recently been identified as having a role in antibody-mediated cGVHD [39,40], and is also associated with multiple other effects, including CD8+ T cell proliferative burst and promoting memory T cell development [41-43]. The pleiotropic functions of BCL6 likely obfuscate any differences in follicular helper T cells between single and dual TCR cells in our patients. Further study with additional phenotypic or gene expression markers would possibly identify these differences. Regardless, it is likely that multiple T cell effector subsets contribute both independently and synergistically to multimodal cGVHD pathology. An overarching feature may be defective post-transplantation Treg production and function, which enable immune dysregulation and subsequent cGVHD pathogenesis [44,45]. To this end, we observed decreased frequencies of CD4+FOXP3+ cells in patients with cGVHD compared with that observed in healthy controls, though the low numbers of cells precluded statistical confidence in the data. Future

Figure 6. Dual TCR cells in cGVHD are preferentially activated with a proinflammatory phenotype. Expression of lineage-directing transcription factors and effector molecules by peripheral blood T cells from allogeneic HSCT patients with symptomatic cGVHD and age-matched healthy controls were linked to TCRαβ clonotypes, including dual TCR cells, by barcoded sequence analysis. (A) Expression of lineage-directing transcription factors by single and dual TCR cells identified from single-cell sequencing. Red denotes detected expression above threshold. (B) Comparison of gene expression by single TCR and dual TCR cells from healthy donors and cGVHD patients. Data indicate percentage of RUNX1+ (CD4+) and RUNX1− (CD8+) cells expressing indicated gene. Data between groups compared by Fisher’s exact test.
focused examinations of post-transplantation Treg TCR repertoires using the single-cell approach could be insightful in evaluating changes in the Treg TCR repertoire associated with cGVHD.

Increases in dual TCR cell frequency associated with symptomatic cGVHD observed in allogeneic HSCT patients must result from either expansion of donor cells transferred as part of the hematopoietic stem cell allograft or from T cells derived from post-transplantation thymopoiesis. Our previous examinations indicated that allogeneic HSCT patients did not evidence expansion of donor dual TCR cells peritransplantation (90 days) in the absence of GVHD [31]. Here, patients developing cGVHD were 9 to 67 months after transplantation, a period where thymic production of new T cells would be expected [20]. These data suggest that the dual TCR cells associated with cGVHD may have arose from post-transplantation thymopoiesis rather than expansion of donor cells, though it is impossible to definitively determine the origin of these cells. It has become increasingly evident that cGVHD is a consequence of defects in thymic regeneration of the T cell repertoire after transplantation. We propose that dual TCR T cell production could be a mechanism linking qualitatively defective thymopoiesis and cGVHD. Dual TCR thymocytes have an advantage during positive selection in the thymus [23], which could combine with defective post-transplantation negative selection to generate a specific subset of cells with high risk for the types of immune responses driving cGVHD [7-12]. A direct link between thymic dysfunction and cGVHD pathogenesis in human subjects has proven elusive because of the substantial heterogeneity of the T cell compartment. Longitudinal analyses of post-HSCT T cell repertoire formation will be essential to truly understand the origin and natural progression of pathogenic cells in cGVHD. Our data presented here indicate dual TCR T cells may be a specific and useful marker to examine effects of HSCT on the generation of T cells with increased pathogenic potential.

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Authorship statement: A.B. and G.P.M. designed and performed experiments. N.G., E.D.B., and G.P.M. managed patient recruitment, sample collection and processing, clinical record collection, and analyzed clinical data. R.S. performed computational analysis of single-cell sequencing data. A.B. and G.P.M. analyzed data and wrote the manuscript.

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