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Dual Receptor T Cells Mediate Pathologic Alloreactivity in Patients with Acute Graft-Versus-Host Disease

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Abstract

Acute graft-versus-host disease (aGVHD) results from a robust response of donor T cells transferred during hematopoietic stem cell transplantation (HSCT) to allogeneic peptide-major histocompatibility complex antigens. Previous investigations have not identified T cell subsets that selectively mediate either protective immunity or pathogenic alloreactivity. We demonstrate that the small subset of peripheral T cells that naturally express two T cell receptors (TCRs) on the cell surface contributes disproportionately to aGVHD in patients after allogeneic HSCT. Dual TCR T cells from patients with aGVHD demonstrate an activated phenotype and produce pathogenic cytokines *ex vivo*. Dual receptor clones from a patient with symptomatic aGVHD responded specifically to mismatched recipient human leukocyte antigens (HLAs), demonstrating pathologic alloreactivity. Human dual TCR T cells are strongly activated and expanded by allogeneic stimulation *in vitro*, and disproportionately contribute to the repertoire of T cells recognizing both major (HLA) and minor histocompatibility antigens, providing a mechanism for their observed activity *in vivo* in patients with aGVHD. These results identify dual TCR T cells as a target for focused analysis of a T cell subset mediating GVHD and as a potential prognostic indicator.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) is caused by alloreactive donor T cells, which are transferred into the recipient during hematopoietic stem cell transplantation (HSCT) (1–3). However, the presence of T cells in the hematopoietic graft is a dichotomous proposition: Although T cell alloreactivity drives aGVHD, pan-T cell depletion results in decreased protective immunity, delayed engraftment, and increased rates of malignant disease relapse (4, 5). Therefore, there is much interest in identifying T cell subsets that predominantly mediate either protective immunity or pathologic GVHD after transplantation, with the goal

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SUPPLEMENTARY MATERIALS

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Fig. S1. Generation of mAbs recognizing human TCRV α 4 and TCRV α 9.

Fig. S2. Robust measurement of dual TCR T cells in PBLs.

Table S1. Patient characteristics.

Table S2. HLA typing of T cell clones and allogeneic PBL panel.

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of either selective T cell enrichment or depletion, respectively, in HSC grafts. These subsets could also be used for developing biomarkers for immune competence and GVHD risk. However, to date, no specific determinants of T cell predisposition toward GVHD have been identified (6, 7).

T cell function is primarily determined through sensitive and specific recognition of peptide-MHC (major histocompatibility complex) through the T cell receptor (TCR) (8). A small population of T cells in mice and humans expresses two TCRs because of incomplete allelic exclusion of TCR α loci during thymocyte development (9, 10). This produces two TCR α chains capable of pairing with a single TCR β to form functional TCRs. Both TCRs are capable of participating in immune responses, and it could be expected that expression of a second TCR would double the antigenic reactivity of a T cell. However, we hypothesize that there may be qualitative differences in secondary TCRs because only one TCR is required to mediate positive selection (11–14) and expression of dual TCRs can mask a potentially autoreactive TCR from deletion during thymic development (13, 15–16). This process would result in a T cell subset having TCRs less stringently shaped by thymic selection to ensure recognition of self-MHC and avoid cross-reactivity or strong reactivity to self.

Our previous investigations in mice demonstrated that dual TCR T cells have an atypically high frequency of response to alloantigens (14). Murine dual TCR T cells are preferentially activated and expanded by allogeneic stimulation either in vitro or in vivo in an MHC-mismatched model of aGVHD. Strikingly, genetic elimination of secondary TCRs, eliminating less than 10% of the peripheral TCR repertoire, resulted in a nearly 50% reduction in the frequency of T cells responding to allogeneic stimulation. This demonstrated a significantly disproportionate contribution of secondary TCRs to the alloreactive T cell repertoire in mice and indicated that dual TCR T cells are critical contributors to the alloreactive T cell repertoire. We hypothesized that human dual TCR T cells may have similar responses to allogeneic stimulation and may be important in driving pathologic alloreactivity producing aGVHD.

RESULTS

Generation of monoclonal antibodies recognizing human TCRV α 4 and TCRV α 9

The existence of T cells simultaneously expressing two different receptors was proven by pairwise labeling of human peripheral blood leukocytes (PBLs) with TCRV α monoclonal antibodies (mAbs) (9). However, subsequent functional investigations of human dual TCR T cell biology have been limited by difficulty in detecting sufficient numbers of rare dual TCR T cells by flow cytometry. Currently, mAbs are available for 3 of the 48 functional V gene segments in the TCR α locus: TCRV α 2 (*TRAV12*), TCRV α 12 (*TRAV19*), and TCRV α 24 (*TRAV10*). This limits analysis to only three pairwise combinations of TCRV α . To address this limitation, we generated two mAbs specifically recognizing TCRV α 4 (*TRAV26*) and TCRV α 9 (*TRAV16*). B cell hybrids generated by fusion of splenocytes from mice immunized with soluble TCRs cloned from healthy donors with a mouse B cell lymphoma cell line and cloned via limiting dilution were screened for production of mAbs specifically recognizing TCRV α by enzyme-linked immunosorbent assay (ELISA) (fig. S1A). Two clones, 2B2 recognizing TCRV α 9 and 5B2 recognizing TCRV α 4, were selected, and specificity was confirmed by flow cytometry of T cell clones with known TCR α (fig. S1B). The mAbs labeled PBLs from healthy donors, at frequencies consistent with other TCRV α mAbs (fig. S1, C and D).

Assessment of dual TCR T cells in peripheral blood by multiparameter flow cytometry

Development of mAbs recognizing human TCRV α increases the ability to examine T cell TCRV α repertoire (Fig. 1A) and expands the number of pairwise TCRV α combinations from 3 to 10 (Fig. 1B). This improves the ability to identify rare dual TCR T cells from limiting patient samples. With this approach, examination of healthy donor PBLs ($n = 12$) demonstrated low but consistent frequencies of dual TCR T cells among TCRV α mAb⁺ T cells (4.3 ± 0.8 per 10^3 T cells, mean $\pm$ SEM, Fig. 1C). Other studies have attempted to extrapolate the total frequency of dual TCR T cells by evaluating the numbers of dual TCR T cells identified as a percentage of all possible dual receptor T cells that could be identified with the pairwise labeling approach. Evaluation of dual TCR T cells by this calculation (focusing on TCRV α 12⁺ dual TCR T cells because V α 12 was consistently the most frequently expressed V α ; fig. S1D) demonstrated that our approach estimated total dual TCR T cell frequencies comparable with previous estimates ($25.7 \pm 4.8\%$, mean $\pm$ SEM, Fig. 1D).

Multiparameter flow cytometry analysis of TCRV α expression to identify dual TCR T cells was robust, demonstrating consistency over time and analysis parameters. Analysis of PBLs from healthy donors at different time points demonstrated consistent frequencies of dual TCR T cells (fig. S2A). Addition of the two TCRV α mAbs increased the total number of dual TCR T cells identified but did not bias measurement of dual TCR T cell frequencies (fig. S2B, $r = 0.666$, $P < 0.001$). Furthermore, changes in interpretive criteria (such as using a more conservative gating strategy limiting TCRV α mAb labeling compared to a more liberal gating strategy) similarly changed the absolute number of dual TCR T cells identified but did not significantly alter measured dual TCR frequencies (fig. S2, C and D; $r = 0.815$, $P < 0.001$). Together, these data demonstrate that our multiparameter flow cytometry approach provides a robust assay to examine dual TCR T cells.

Increased numbers and activity of dual TCR T cells in allogeneic HSCT patients with aGVHD

Our previous investigation of dual TCR T cells in mice demonstrated that dual TCR T cells are important contributors to the alloreactive T cell repertoire (14). We hypothesized that human dual TCR T cells are similarly important contributors to alloreactive T cell responses, particularly in aGVHD. To investigate this, we examined the frequency of dual TCR T cells in patients after allogeneic HSCT. Peripheral blood samples were collected from healthy volunteer donors and patients after allogeneic HSCT, either at follow-up after HSCT or at presentation with symptomatic aGVHD (table S1). Dual TCR T cells were identified by flow cytometry as described in Fig. 1. Analysis of PBLs from patients with symptomatic aGVHD ($n = 20$) revealed significant increases in dual TCR T cells ($23.0 \pm 7.9/10^3$, mean $\pm$ SEM) compared to healthy controls ($P = 0.001$) and allogeneic HSCT patients who did not develop aGVHD ($n = 6$, $4.9 \pm 1.5/10^3$, $P = 0.012$) (Fig. 2A). The increase in dual TCR T cells was related to the degree of mismatch between the donor and recipient. Patients developing aGVHD after human leukocyte antigen (HLA)–mismatched HSCT had significantly more dual TCR T cells ($n = 8$, $40.5 \pm 13.6/10^3$, $P = 0.012$) than those developing aGVHD after HLA-matched HSCT ($n = 12$, $13.2 \pm 2.6/10^3$). The increased numbers of dual TCR T cells in patients with aGVHD were dependent on transferred donor T cells; patients with aGVHD that had received antithymocyte globulin (ATG) at the time of transplantation ($n = 5$) had fewer dual TCR T cells (5.60 ± 1.89 per 10^3 T cells) compared to patients with aGVHD who did not receive ATG (22.95 ± 7.91 per 10^3 T cells, $P = 0.040$). Estimation of the total dual TCR T cell frequency in patients with aGVHD indicated that dual TCR T cells were substantially expanded in patients developing aGVHD after allogeneic HSCT ($58.6 \pm 6.4\%$, Fig. 2B).

The *in vivo* activity of dual TCR T cells in patients with aGVHD was evaluated by analyzing expression of CD69 and CD45RO. Dual TCR T cells from patients with symptomatic aGVHD demonstrated significantly increased frequencies of cells expressing CD69 compared to HSCT patients without aGVHD (Fig. 3A, $P = 0.041$) or healthy controls ($P = 0.004$). In individual samples from patients with aGVHD, dual TCR T cells were significantly more likely to express CD69 than cells labeled with a single TCRV α mAb (Fig. 3B, $P = 0.005$). This preferential activation of dual TCR T cells was not observed in healthy controls or in allogeneic HSCT patients without aGVHD. Conversely, no significant differences in CD45RO expression were observed between dual TCR T cells from patients with aGVHD and those from allogeneic HSCT recipients without GVHD ($P = 0.620$) or healthy controls ($P = 0.222$), with no significant differences between single and dual TCR T cells (Fig. 3, C and D). This indicates that the preferential activation of dual TCR T cells is not a general predisposition to activation, but rather specifically temporally related to alloreactivity during aGVHD.

The pathologic potential of dual TCR T cells in aGVHD was examined by analyzing *ex vivo* cytokine production. Intracellular cytokine labeling of PBLs demonstrated significantly increased production of interferon- γ (IFN- γ) (Fig. 3E, $P = 0.016$) and interleukin-17a (IL-17a) (Fig. 3G, $P = 0.020$) by dual TCR T cells from patients with aGVHD compared to those from healthy controls. Dual TCR T cells from allogeneic HSCT patients without GVHD did not demonstrate similarly increased production of IFN- γ ($P = 0.143$) or IL-17a ($P = 0.230$). Significant differences in cytokine production between single and dual TCR T cells in aGVHD patients were not observed (Fig. 3, F and H). We propose that the *ex vivo* stimulation required for cytokine analysis obfuscated differences between single and dual TCR T cells actively producing cytokines *in vivo*. Nonetheless, these results demonstrate production of known mediators of aGVHD by dual TCR T cells, indicating their pathogenic potential.

In vitro alloreactive response of dual TCR T cells from aGVHD patients

To examine the antigenic specificity of dual TCR T cells mediating GVHD, we generated dual TCR T cell clones from patients after allogeneic HSCT and tested their alloreactivity *in vitro* (Fig. 4). T cell clones were generated by single-cell fluorescence-activated cell sorting (FACS) dual TCR T cells from PBLs with dual receptor expression confirmed by FACS and PCR (polymerase chain reaction) (Fig. 4, B and C). Clones were tested against a panel of allogeneic PBLs (table S2), with reactivity measured by IFN- γ production after 24 hours of culture (Fig. 4, A and D). Dual TCR T cell clones from a GVHD-free patient were responsive to stimulation but demonstrated minimal reactivity against the allogeneic cell panel. Conversely, dual TCR T cell clones from a patient with aGVHD demonstrated significant IFN- γ production in response to multiple allogeneic stimulator cells (Fig. 4A). The alloreactivity of dual TCR T cell clones from the aGVHD patient was specific for alloantigens mismatched during HSCT, reacting specifically to cells expressing HLA-A*02:01 (Fig. 4D). These results directly demonstrate alloreactivity of dual TCR T cells to alloantigens mismatched in HSCT.

Expansion of dual TCR T cells resulting from allogeneic stimulation

The increased frequencies of dual TCR T cells in patients with aGVHD suggest that donor dual TCR T cells from HSC grafts preferentially expanded in response to allogeneic stimulation after transplantation. We tested directly whether dual TCR T cells are expanded by measuring dual TCR T cell frequencies from healthy donor PBLs cultured for 5 days with irradiated autologous or allogeneic PBLs (Fig. 5). Allogeneic stimulation resulted in expansion of dual TCR T cells (Fig. 5A, $3.5 \pm 0.4\%$ TCRV α -labeled T cells, mean $\pm$ SEM, $P = 0.029$), resulting in a 1.75-fold increase in dual TCR T cell frequency compared to

autologous cell culture ($2.0 \pm 0.2\%$). This demonstrates a preferential expansion of dual TCR T cells in response to allogeneic stimulation. Within individual alloreactive cultures, a significantly larger proportion of dual TCR T cells produced IFN- γ compared to all T cells (Fig. 5B, $P = 0.001$). A similar difference was not observed for T cells cultured with autologous PBLs. These results directly demonstrate the predisposition of dual TCR T cells to respond to allogeneic stimulation.

Recognition of both major and minor histocompatibility antigens by dual TCR T cells

The observation that dual TCR T cells are similarly increased in patients with aGVHD after either HLA-matched or HLA-mismatched HSCT (Fig. 2) suggests that dual TCR T cells are likely involved in responses to both minor and major histocompatibility antigens. To examine whether dual receptor T cells have increased recognition of minor as well as major histocompatibility antigens, we examined the frequency of recognition of the HLA-A*02:01-restricted alloantigen HA-1 (VLHDDLLEA) (17). Using HLA-A*02:01-HA-1 tetramers, we examined the frequency of recognition of a minor histocompatibility antigen (by HLA-A*02:01⁺ HA-1⁻ cells, which recognize the HA-1 minor alloantigen) and a major histocompatibility antigen (by HLA-A*02:01⁻ cells, which recognize the HLA-A*02:01-HA-1 complex as a major histocompatibility antigen) (table S2) with a single system. Dual TCR T cells more frequently recognized the allogeneic tetramer ligand in the setting of both a minor (Fig. 6, A and B; $P = 0.008$) and a major histocompatibility antigen (Fig. 6, D and E; $P = 0.008$), compared to T cells labeled with a single TCRV α mAb. Analysis of all tetramer-labeled T cells demonstrated that $25.8 \pm 3.6\%$ of T cells recognizing a minor histocompatibility antigen and $14.1 \pm 3.2\%$ recognizing a mismatched HLA antigen expressed dual TCR T cells, compared to $3.3 \pm 1.3\%$ of total T cells in the assay (Fig. 6, C and F). These results demonstrate that dual receptor T cells are principal contributors to the alloreactive T cell repertoire responding to both minor and major histocompatibility antigens.

DISCUSSION

Our investigations demonstrate that the small subset of T cells that naturally express two TCRs is involved in pathologic alloreactivity in aGVHD. Although aGVHD is known to result from a robust oligoclonal response of donor T cells transferred in the HSC graft to recipient alloantigens (18–21), specific T cell subsets that selectively mediate protective immunity or pathologic alloreactivity after HSCT have not been identified. The increases in dual TCR T cell frequencies observed specifically in patients with aGVHD, combined with ex vivo and in vitro evidence of preferential responses to alloantigens, suggest that dual TCR T cells may be considered a T cell subset specifically associated with pathologic alloreactivity.

Our investigations of human dual TCR T cells use multiparameter flow cytometry to identify dual TCR T cells by pairwise labeling with TCRV α mAbs, combined with analysis of markers of T cell function. However, even with the addition of two mAbs, we were still only able to interrogate a fraction of the TCR repertoire. It is likely that this results in underestimation of the size of the dual TCR T cell populations. Although markedly increased frequencies of dual TCR T cells were detected in most (14 of 20 with more than twofold increase) of the patients with symptomatic aGVHD, we observed only modest (2 of 20 with less than twofold increase) or no increase (4 of 20) in some patients. We propose that the limited number of TCRV α mAbs missed some oligoclonal expansions of dual TCR T cells in these patients. Additionally, dual TCR T cell function was evaluated by comparison to T cells that were labeled with a single TCRV α mAb, an approach that enabled direct comparison of dual TCR T cells to conventional T cell populations and

provided internal controls for all experiments. However, there are certainly dual TCR T cells within subsets labeled with only a single TCRV α mAb. This likely underestimates differences between single and dual TCR T cells. Nevertheless, our data demonstrate the benefit of examination of dual TCR T cells both as targets for functional examination of T cells mediating aGVHD and as a potential indicator for aGVHD development.

The observation that dual TCR T cells recognize allogeneic peptide-HLA tetramers, as either major or minor histocompatibility antigens, as much as eight times more frequently than conventional T cell populations demonstrates the disproportionate contribution of dual TCR T cells to the alloreactive T cell repertoire. This is in agreement with our previous mouse model investigations indicating that dual TCR T cells may contribute as much as 50% of the alloreactive T cell repertoire, despite compromising only a small portion of the peripheral TCR repertoire (14). The functional relevance of this predisposition toward recognizing allogeneic ligands is reflected by in vivo and in vitro evidence of preferential activation and expansion of dual TCR T cells in response to allogeneic stimulation.

We hypothesize that the increased alloreactivity of dual TCR T cells may be a result of a lack of stringent imposition of the requirements of thymic selection on secondary TCRs. Thymic selection normally ensures that T cells that successfully develop have a TCR capable of interacting with self-MHC and not strongly reactive to self-peptide-MHC ligands (22). However, secondary TCRs have less stringent requirements for selection during development, with the primary TCR capable of solely mediating positive selection (11–14) and potentially masking autoreactive TCRs from deletion during negative selection (13, 15, 16). The relationship between thymic selection and alloreactivity is not entirely defined. There is increasing evidence that the ability to respond to MHC is not imparted by positive selection, but rather an inherent property of TCR (23–26), whereas negative selection eliminates TCRs that are highly cross-reactive or respond too strongly to shared self-peptide antigens (27).

We have recently defined polyclonal alloreactive T cell responses as limited by a requirement for specific recognition of endogenous peptides presented by allogeneic MHC (28). This observation reconciles the notion of germ line-encoded affinity of TCRs for MHC with the observed frequencies of alloreactive T cells and the experimental evidence for TCR contact with presented peptide antigens. Our findings regarding the disproportionate contribution of dual TCR T cells to the alloreactive T cell repertoire fit well with a peptide-dependent model of alloreactivity (29). Secondary TCR that escapes negative selection may be responsive to shared endogenous antigen epitopes or highly cross-reactive, enabling responses to a larger number of endogenous antigens. The potential alloreactivity of nonthymically selected TCR is particularly relevant in light of recent engineered antigen receptor systems designed for clinical antitumor therapy (30–32). These systems have demonstrated unpredictable alloreactivity due to pairing between transgenic and endogenous TCR chains (33, 34). Dissecting the interactions between secondary TCR and allogeneic ligands and their relationship with thymic selection may have important implications in understanding the interactions that promote pathologic alloreactivity.

Because our hypothesis regarding the mechanism of dual TCR T cell alloreactivity is related to their exemption from strict requirements of thymic selection, it is intriguing to speculate regarding dual TCR T cells that develop de novo after HSCT and their possible contribution to chronic GVHD. We hypothesize that dual TCR T cells that develop after HSCT would exhibit alloreactivity and autoreactivity similar to those in an unperturbed system. However, the thymus is subject to inflammatory damage from pre-HSCT conditioning regimens and thymic GVHD, and thus presents a markedly altered environment for thymocyte development, likely with substantial consequences to the repertoire of T cells generated (35,

36). It would be reasonable to consider that this could result in large changes to the dual TCR T cell repertoire, with relevant functional consequences. Of particular interest, it has been suggested that a large portion of regulatory T cells in humans express dual TCRs (37). Because regulatory T cells are thought to develop in the thymus within a very narrow window of TCR affinity for self-ligands (38), it is possible that this relationship is markedly altered in the disrupted thymic environment that exists after HSCT. It will be of great interest to carefully examine dual TCR T cell development and function after HSCT to better examine the relationship between thymic selection, TCR biology, and T cell function.

MATERIALS AND METHODS

Patient samples

Patients receiving allogeneic or autologous HSCT at the Siteman Cancer Center at Washington University School of Medicine and healthy volunteer donors were recruited for participation. HSCT patients received donor products collected by HSC mobilization with granulocyte colony-stimulating factor and apheresis. Pretransplant conditioning regimens are indicated in table S1. All patients and healthy donors donated 10 to 30 ml of peripheral blood collected by peripheral venipuncture. HSCT patient samples were collected at routine follow-up after transplantation or at admission for symptomatic aGVHD. All patients with aGVHD received standard of care therapy. GVHD-free patients were defined as patients who received allogeneic HSCT and did not develop symptomatic GVHD requiring adjustment of immunosuppressive regimen within 100 days after HSCT. All samples were collected and deidentified before analysis. Healthy donors were HLA-typed for HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB by DNA sequence analysis with uTYPE software (Life Technologies). Healthy donors were typed for HA-1 minor histocompatibility antigen by primer-specific PCR (17). All collection, storage, and analysis of samples and patient data were performed under the approval of the Washington University Human Studies Institutional Review Board.

Generation of TCRV α mAbs

mAbs specific for human TCRV α were generated by cloning TCR α transcripts from healthy donor complementary DNA into pET30 expression constructs containing the human TCR α region (39). Soluble cloned TCR α and LC13 (TCRV β 7) TCR β were expressed in BL21 DE3 pLysS *Escherichia coli* (Life Technologies) and refolded in 8 M urea, and TCR $\alpha\beta$ heterodimers were isolated by fast protein liquid chromatography. B6 and CBA mice were immunized with soluble TCR by standard protocols, and splenocytes were fused with P3Ag8.6.5.3 B cell fusion partner (40). B cell hybrids were screened for production of TCRV α mAb by ELISA against soluble TCR bound to Immulon 2 plates. mAbs were purified with protein A or protein G (Thermo Scientific) and labeled with Pacific Blue and Pacific Orange fluorescent dyes (Molecular Probes).

Flow cytometric analysis of PBLs

PBLs were labeled for cell viability with LIVE/DEAD fixable ultraviolet blue fluorescent reactive dye (Molecular Probes). PBLs were labeled for multiparameter flow cytometry with anti-CD4-allophycocyanin (APC)/Cy7 or anti-phycoerythrin (PE)/Alexa Fluor 610 (OKT4), anti-CD8-peridinin chlorophyll protein (PerCP)/Cy5.5, anti-CD45RO-Alexa Fluor 700 (UCHL1), CD69-PE/Cy5 (FN50) (BioLegend), TCRV α 12-FITC (fluorescein isothiocyanate) (6D6), TCRV α 2 (F1) (Thermo Scientific)-Alexa Fluor 647 or Alexa Fluor 594 (Molecular Probes), TCRV α 24-PE (C15, Beckman Coulter), and anti-TCRV α 4-Pacific Orange (5B2) and TCRV α 9-Pacific Blue (2B2). For intracellular cytokine labeling, 10^7 PBLs were stimulated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) and 1 μ M ionomycin for 4 hours at 37°C in the presence of brefeldin A (10 μ g/ml). Cells were washed

and labeled with surface mAbs for 30 min at 4°C, fixed with 50 μ l of BD Cytotfix (BD Bioscience), permeabilized with permeabilization buffer (BioLegend), and labeled with anti-IFN- γ -PE/Cy7 (4S.B3) and anti-IL-17a-APC/Cy7 (BL168) (BioLegend). Isotype controls MPC-11 [mouse immunoglobulin G2b (IgG2b)] and MOPC-21 (mouse IgG1 κ) (BioLegend) were used for analyzing TCRV α and intracellular cytokines. Tetramer analysis was performed on CD3⁺-enriched PBLs [10 ml of PBLs enriched with Human T Cell Enrichment Cocktail (Stemcell Technologies) and Ficoll] by labeling as described, followed by incubation with HLA-A*02:01–HA-1 (VLHDDLLEA) Alexa Fluor 680 tetramer [National Institutes of Health (NIH) Tetramer Facility] for 30 min at 4°C. Non-tetramer-labeled T cells were used as a control. Labeled cells were analyzed on an LSRII (BD Biosciences) with FACSDiva software. Data were analyzed with FlowJo 7.6.4 (Tree Star).

Mixed lymphocyte reactions

MLR was performed by culture of 10⁶ PBLs with 10⁶ irradiated autologous or allogeneic PBLs in 2 ml of RPMI 1640 supplemented with 10% fetal calf serum (HyClone), 1% Hepes, 2 mM GlutaMAX, gentamycin (50 μ g/ml) (Life Technologies), and 2% human IL-2 (gift of M. Cella, Washington University) (R10 + 2% IL-2) at 37°C. Stimulation with PMA (20 ng/ml) and 1 μ M ionomycin was used as positive control. Cultures were analyzed at 24 hours by measurement of IFN- γ production by ELISA and at 4 days by FACS analysis.

IFN- γ ELISA

IFN- γ production in triplicate 24-hour culture supernatants was measured by ELISA with MD-1 capture, 4S.B3-biotin (BioLegend), and streptavidin–horseradish peroxidase (Southern Biotech) label, developed with 1-Step Ultra tetramethylbenzidine substrate. Absorbance was measured with a Victor3 plate reader (PerkinElmer), and IFN- γ concentrations were calculated by linear regression from concurrent standard curves.

Generation and culture of T cell clones

Dual TCR T cell clones were generated from two patients after allogeneic HSCT by single-cell FACS sorting of pairwise TCRV α mAb-labeled PBLs with a FACSaria II (BD Bioscience). Cells were sorted into 96-well round-bottomed plates containing R10 (100 μ l) + 2% IL-2, irradiated allogeneic PBLs (2×10^6 /ml), irradiated Epstein-Barr virus-transformed allogeneic B cell line E02 (2×10^5 /ml) [derived from donor C02 PBLs cultured with B95-8 cell line supernatant in the presence of cyclosporine A (1 μ g/ml)], and phytohemagglutinin (1 ng/ml) (Remel) and cultured at 37°C. Clones were monitored visually for growth, passaged every 15 days, and expanded to culture of 10⁵ to 10⁶ cells/ml. Dual TCR expression was confirmed by FACS and by PCR with forward primers specific for TCRV α 2 and TCRV α 12, with TCRV α 1- and TCRV α 24-specific primers as negative controls, in combination with a TCRC α reverse primer. T cell clones were examined for alloreactivity by triplicate culture of 5×10^4 T cells with 10⁵ irradiated allogeneic PBLs in 200 μ l in 96-well round-bottomed plates for 24 hours at 37°C. Stimulation with PMA (100 ng/ml) and 1 μ M ionomycin was used as positive control. Responses were assessed by measurement of IFN- γ production by ELISA.

Statistical analyses

Data were analyzed with Prism 5 software (GraphPad). Nonparametric analyses between groups were performed with Mann-Whitney test. Intrasample comparisons between single and dual TCR T cells were performed with ratio paired *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Measurement of dual TCR T cells in peripheral blood

(A) Peripheral blood from healthy donors was analyzed for TCRVα expression by flow cytometry. PBLs were gated on live singlet CD4⁺ or CD8⁺ cells, and Vα expression was examined using simultaneous labeling with Vα2, Vα4, Vα9, Vα12, and Vα24 mAbs. Isotype controls were used to set Vα gates. Representative FACS plots from healthy donor shown, with percentages and total numbers of Vα⁺ T cells identified. (B) Dual TCR T cells were identified among live CD4⁺ and CD8⁺ T cells in PBLs by pairwise labeling with Vα mAbs. FACS plot of representative healthy donor PBLs is shown, with numbers of dual TCR T cells identified. (C) Dual TCR T cell numbers evaluated as a frequency of measured T cells. Total numbers of dual TCR T cells were divided by the total number of T cells labeled with Vα mAbs. Measured dual TCR T cell frequencies from healthy donors ($n = 12$), mean $\pm$ SEM. (D) Dual TCR T cells evaluated as a calculated percentage of the possible observable dual TCR T cells using pairwise Vα mAb analysis. Frequencies were calculated by dividing the observed number of Vα12⁺ dual TCR T cells by the possible number of dual TCR T cells identifiable among Vα12⁺ T cells (number of Vα12⁺ T cells multiplied by the frequency of labeling with Vα2, Vα4, Vα9, and Vα24 mAbs). Calculated dual TCR T cell frequencies from healthy donors ($n = 12$), mean $\pm$ SEM.

Fig. 2. Dual TCR T cells are increased in patients with aGVHD

Dual TCR T cells were measured in patients after allogeneic HSCT by flow cytometry of peripheral blood as described in Fig. 1. **(A)** Measured frequencies of dual TCR T cells from healthy donors ($n = 12$, shown in Fig. 1C), allogeneic HSCT patients who did not develop aGVHD ($n = 6$), patients developing aGVHD after transplant with an HLA-matched graft ($n = 12$), and patients developing aGVHD after transplant with an HLA-mismatched graft ($n = 8$). Data are expressed as the number of dual TCR T cells observed per 10^3 T cells labeled with TCRV α mAbs. Each point represents a single patient. Bars are means $\pm$ SEM. Data were compared nonparametrically by Mann-Whitney test. **(B)** Dual TCR T cell frequency was calculated as described in Fig. 1 for patients with aGVHD ($n = 20$). Bars are means $\pm$ SEM.

Fig. 3. Dual TCR T cells from patients with aGVHD are activated and produce pathogenic cytokines

(A to D) Dual TCR T cells from healthy controls ($n = 9$) and allogeneic HSCT patients without ($n = 5$) or with aGVHD ($n = 11$) were analyzed by flow cytometry for (A) CD69 and (C) CD45RO expression. Data are means $\pm$ SEM; groups compared nonparametrically by Mann-Whitney test. Expression of (B) CD69 and (D) CD45RO was compared on single and dual TCR T cells within samples from patients with aGVHD. Paired sample measurements indicated by connecting lines (ratio paired t test). (E to H) Production of (E) IFN- γ and (G) IL-17a by PBLs from healthy controls ($n = 5$) and allogeneic HSCT patients without ($n = 3$) or with aGVHD ($n = 4$) was measured by intracellular flow cytometry after 4 hours of ex vivo stimulation with PMA (0.1 μ g/ml) and ionomycin (1 μ M). Data are means $\pm$ SEM; Mann-Whitney test. Production of (F) IFN- γ and (H) IL-17a was compared between

single and dual TCR T cells within samples. Paired sample measurements indicated by connecting lines (ratio paired t test).

Fig. 4. Dual TCR T cells respond to mismatched HLA antigens in aGVHD

Dual TCR T cell clones were generated by single-cell FACS sorting of PBLs from patients after allogeneic HSCT. (A) T cell clones were tested for alloreactivity by measuring IFN- γ production by ELISA after 24 hours of culture of 5×10^4 clone T cells with 1×10^5 irradiated allogeneic PBLs in triplicate. Stimulation with PMA (100 ng/ml) and ionomycin (1 μ M) was used as a positive control. Reactivity was assessed compared to positive controls and represented as a percentage of the maximal response. Data are averages of at least three independent experiments for each T cell clone for each condition. (B to D) Example of representative dual TCR T cell clone from patient with aGVHD. Dual TCR expression of clones was confirmed (B) by FACS, with gates determined by labeling with isotype controls, and (C) by PCR with primers specific for TCRV α gene segments. (D) Alloreactivity was assessed by ELISA measurement of culture supernatants after 24 hours of mixed lymphocyte reaction (MLR). Data are means $\pm$ SEM of representative experiment.

Fig. 5. Dual TCR T cells preferentially respond to allogeneic stimulation

(A) Dual TCR T cells expand in response to allogeneic stimulation in vitro. Healthy donor PBLs were cultured for 5 days in MLR with autologous or two different allogeneic donors. Dual TCR T cell frequency was assessed by FACS analysis. Data are means $\pm$ SEM from four independent experiments ($n = 4$); Mann-Whitney test. (B) Comparison of IFN- γ production by single TCR or dual TCR T cells in individual cultures. IFN- γ production measured by intracellular cytokine staining after 5 days of autologous (gray symbols) or allogeneic (black symbols) MLR. Each point represents a single culture from independent experiments ($n = 4$), with paired sample measurements indicated by connecting lines. Data analyzed by ratio paired t test.

Fig. 6. Dual TCR T cells disproportionately recognize major and minor histocompatibility antigens

T cell recognition of HLA-A*02:01–HA-1 tetramer measured by flow cytometry. (**A** to **C**) HLA-A*02:01⁺ HA-1⁻ cells recognize HA-1 as a minor histocompatibility antigen. (**A**) Representative example of recognition of HLA-A*02:01–HA-1 tetramer by single and dual TCR T cells in a single sample. FACS plot of live singlet T cells gated on cells labeled with TCRV α mAbs. (**B**) Recognition of minor histocompatibility antigen tetramer by single and dual TCR T cells for individual donors ($n = 4$). Paired measurements indicated by connecting lines; Wilcoxon matched pairs test. (**C**) Comparison of average frequency of dual TCR T cells among all T cells and minor histocompatibility antigen tetramer-binding T cells. Data are means $\pm$ SEM of all donors ($n = 4$); Mann-Whitney test. (**D** to **F**) HLA-A*02:01⁻ HA-1⁻ cells recognize HLA-A*02:01 as a major histocompatibility antigen. (**D**) Representative example of recognition of HLA-A*02:01–HA-1 tetramer by single and dual TCR T cells in a single sample. (**E**) Recognition of major histocompatibility antigen tetramer by single and dual TCR T cells for individual donors ($n = 4$). Paired measurements indicated by connecting lines; Wilcoxon matched pairs test. (**F**) Comparison of average frequency of dual TCR T cells among all T cells and major histocompatibility antigen tetramer-binding T cells. Data are means $\pm$ SEM of all donors ($n = 4$); Mann-Whitney test.