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*J Immunol* 2009; 182:6639-6643; doi: 10.4049/jimmunol.0900638

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Cutting Edge: Highly Alloreactive Dual TCR T Cells Play a Dominant Role in Graft-versus-Host Disease

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Alloreactivity is the response of T cells to MHC molecules not encountered during thymic development. A small population (1–8%) of peripheral T cells in mice and humans express two TCRs due to incomplete allelic exclusion of TCRα, and we hypothesized they are highly alloreactive. FACS analysis of mouse T cell MLR revealed increased dual TCR T cells among alloreactive cells. Quantitative assessment of the alloreactive repertoire demonstrated a nearly 50% reduction in alloreactive T cell frequency among T cells incapable of expressing a secondary TCR. We directly demonstrated expansion of the alloreactive T cell repertoire at the single cell level by identifying a dual TCR T cell with distinct alloactivities for each TCR. The importance of dual TCR T cells is clearly demonstrated in a parent-into-F1 model of graft-vs-host disease, where dual TCR T cells comprised up to 60% of peripheral activated T cells, demonstrating a disproportionate contribution to disease. The Journal of Immunology, 2009, 182: 6639–6643.

A n unresolved question in alloreactivity involves how the naive T cell repertoire, shaped during thymic development to interact with self-MHC, reacts 100- to 1000-fold more frequently with allogeneic MHC. The strong reactivity toward allogeneic MHC has led to many models proposed to explain the nature of this interaction, ranging from degenerate recognition of MHC by germline TCR elements (1, 2) to a focus on presented peptides (3). Several studies have provided evidence for TCR germline affinity for MHC (1, 2, 4) although we have shown that alloreactive TCRs are capable of polyspecificity, recognizing multiple distinct peptides presented by allogeneic MHC (5). The few available TCR-peptide-allogeneic MHC cocystal structures demonstrate that alloreactivity is generally similar to conventional recognition of a foreign peptide presented by self-MHC (6, 7), although functional analysis demonstrates increased flexibility in docking angle and energetic footprint (8, 9). This breadth of potential interactions helps explain the high alloreactivity of naive T cells.

A small population (1–8%) of peripheral T cells in humans and mice express a second TCR capable of pairing with the same TCRβ to form functional secondary TCR heterodimers detectable on the cell surface (10–12). Although determination of the precise number of dual TCR T cells in the periphery is hampered by limited anti-Vα mAbs, these studies clearly demonstrate that a small number of peripheral T cells defy the “one cell, one receptor” maxim. Expression of two TCRα results from continuous rearrangement of both TCRα loci throughout T cell development until positive selection, contrasting with tight allelic exclusion of TCRβ (13–16). Whereas it is estimated that 30% of mature peripheral T cells in humans and mice contain two successfully rearranged TCRα genes, only 1–8% express a secondary TCR at the cell surface (10–12, 17) attributed to preferential pairing between TCR α- and β-chains, leading to a primary dominant TCR heterodimer and a rapidly degraded unpaired TCRα (11, 13, 14, 18). However, both TCRs on the surface of dual TCR T cells are functional and capable of contributing to immune responses (10, 19). We hypothesize that secondary TCRs have a substantial role in broadening the alloreactive T cell repertoire; not only could expression of a second TCR double the chance to recognize allogeneic MHC, but secondary TCRs are not required to undergo thymic selection, giving them a potentially broader repertoire (10, 19–21).

Materials and Methods

Mice

B6 (H-2b), B6.129 (H-2b), BALB/c (H-2d), C57BL/6 (H-2d), BALB/c (H-2d), RIHS/J (H-2j), B10.RIIIS/J (H-2r), SM/J (H-2v), B10.D2 (H-2q), PL/J (H-2t), B6.P (H-2q), and B6.G7 (H-2v) mice were originally purchased from The Jackson Laboratory. Mice heterozygous for TCRα, incapable of expressing two TCR α-chains, were derived by crossing TCRα−/− mice with B6 mice. All mice were bred and housed in specific pathogen-free conditions at the animal facility at the Washington University Medical Center (St. Louis, MO).

Received for publication March 3, 2009. Accepted for publication March 30, 2009.

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1 This work was supported by National Institutes of Health Grant AI-061173 (to P.M.A.) and the W. M. Keck Foundation Fellowship in Molecular Medicine (to G.P.M.).

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Mixed lymphocyte culture

T cells from B6 and B6.Cx<sup>+</sup>−/− mice were purified from collected splenocytes and lymph node cells by magnetic bead separation using anti-CD4 and anti-CD8 microbeads and LS positive selection columns according to manufacturer protocol (Miltenyi Biotec). Purified T cells (1 × 10<sup>6</sup>) were labeled with 5 μM CFSE (Invitrogen) and cultured at a 1:1 ratio with irradiated B6.K and BALB/c splenocytes or with 0.1 μg of plate-bound anti-CD3 (2C11) and anti-CD28 (37.51) mAbs (Biolegend) in 2 ml of RPMI 1640 (Invitrogen) supplemented with 10% FCS (HyClone) for 4 days. Cells were subsequently analyzed by flow cytometry or ELISPOT.

Flow cytometry

Collected cells were labeled with PE-labeled anti-CD8 (53-6.7), Alexa Fluor 647-labeled anti-V<sub>α2</sub> (B20.1) (Biolegend), and Alexa Fluor 750-labeled anti-CD4 (RM4.5; eBioscience), and biotinylated anti-CD8<sub>α</sub>-V<sub>α2</sub> (RR3–16), -V<sub>β8</sub> (KT50), or -V<sub>α11</sub> (RR8–1) in conjunction with streptavidin-PE Cy7 (BD Biosciences). Ly5.1 T cells were labeled with FITC-labeled anti-CD4<sub>δ</sub>-1<sup>+</sup> (A20; Biolegend). All samples were analyzed using a FACSVerse flow cytometer (BD Biosciences) with calculated compensation, and data were analyzed using FlowJo software (Tree Star).

ELISPOT

Alloreactive T cell frequency was assessed using a 48-h MLR performed in replicates of six in 96-well MultiScreen-IP plates (Millipore) coated with purified anti-IFN-γ (RA-6A2; eBioscience), and labeled with biotinylated anti-IFN-γ (XM1G1.2) and streptavidin-conjugated HRP (Southern Biotech). Plates were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich), read on a CTL ImmunoSpot reader, and data analysis was performed using CTL ImmunoSpot 4.0 (Cellular Technology Limited).

Vo<sub>2</sub> 2.102VB TCR T cell hybrids

The secondary Vo<sub>2</sub>. Vo<sub>1</sub> TCR from the 2.102 T cell was cloned from the cDNA library by PCR and the two TCR chains were linked by extension PCR to add cloning sites and a viral P2A cleavage site (22). The polyclonistic TCR construct was cloned into an internal ribosome entry site-linked GFP retroviral expression vector, GFP-RV (gift from Dr. T Kitamura, Limited). The newly constructed construct was cloned into an internal ribosome entry site-linked GFPRetroviral expression vector, GFP-RV (gift from Dr. T Kitamura, Limited). The newly constructed construct was cloned into an internal ribosome entry site-linked GFPRetroviral expression vector, GFP-RV (gift from Dr. T Kitamura, Limited).

IL-2 measurement

IL-2 in culture supernatant was measured by ELISA using 100 μg/well capture mAb (JES6-1A12; Biolegend), 50 μg/well biotinylated-anti-IL-2 detection mAb (JES6-5H4; Biolegend), and 100 μl/well 1/10,000 dilution streptavidin-HRP (Southern Biotech), developed using 100 μl/well 1-step-Ultra tetramethylbenzidine substrate, stopped at 15 min by the addition of 100 μl/well 2 M sulfuric acid, and assessed using a Victor3 plate reader (PerkinElmer). IL-2 concentrations were calculated by linear regression from concurrent IL-2 standard curves.

Graft-vs-host disease (GVHD)<sup>3</sup>

GVHD was induced by i.v. transfer of 2.0 × 10<sup>7</sup> bone marrow cells (BMCs) and 2.5 × 10<sup>5</sup> splenocytes from 6- to 8-wk B6 Ly5.1<sup>+</sup> donor mice into lethally irradiated (10 Gy) B6 or (B6 × CBA)<sub>F</sub> recipients. Disease progression was monitored by daily observation and weekly measurement of mouse weight. Mice were sacrificed at 80% of original weight, and peripheral T cells were collected from spleens and lymph nodes and analyzed by flow cytometry.

Statistics

All data were analyzed nonparametrically by Mann-Whitney U test using Prism 4 software (Graph Pad). Values of p < 0.05 were considered significant.

<sup>3</sup>Abbreviations used in this paper: GVHD, graft-vs-host disease; BMC, bone marrow cell.

**Results and Discussion**

**Dual TCR T cells are involved in in vitro alloreactivity**

To observe involvement of dual TCR T cells in alloreactivity in vitro, we analyzed FACS 4-day MLR cultures using the four existing anti-mouse Vo<sub>2</sub> mAbs. CFSE-labeled T cells from B6 (H-2<sup>b</sup>) mice cultured in a MLR with irradiated splenocytes from either CBA (H-2<sup>k</sup>) or BALB/c (H-2<sup>d</sup>) mice or anti-CD3 and anti-CD28 mAbs for 4 days were analyzed by flow cytometry for the presence of dual TCR T cells. Cells were gated on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and alloreactive T cells were distinguished by gating on cells with diluted CFSE. A, Dual TCR T cells were consistently observed in higher frequency among T cells that had divided in response to allogeneic stimulation as compared with undivided controls. FACS data of dual TCRα T cells from a representative experiment with allogeneic stimulation by B6.K splenocytes show the increased dual TCRα T cells among T cells that had divided in response to allogeneic stimulation as measured by CFSE dilution. B, To compare the frequency of T cells expressing dual TCRα between populations and five independent experiments, T cell numbers were normalized to Vo<sub>2</sub><sup>+</sup> cells, demonstrating a consistent and significant (mean ± SD; *p < 0.05; **p < 0.01) increase in dual TCRα T cells among alloreactive T cells. C, Increased dual TCR T cells were also consistently and significantly (mean ± SD; *p < 0.05; **p < 0.01) observed among alloreactive CD4<sup>+</sup>Vo<sub>2</sub><sup>+</sup> T cells. Stimulation with anti-CD3 and anti-CD28 mAbs did not result in significantly increased dual TCRα T cells (p = 0.18-0.10).

**FIGURE 1.** Dual TCR T cells are increased among alloreactive T cells. CFSE-labeled T cells from B6 (H-2<sup>b</sup>) mice cultured in a MLR with irradiated splenocytes from either CBA (H-2<sup>k</sup>) or BALB/c (H-2<sup>d</sup>) mice or anti-CD3 and anti-CD28 mAbs for 4 days were analyzed by flow cytometry for the presence of dual TCR T cells. Cells were gated on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and alloreactive T cells were distinguished by gating on cells with diluted CFSE. A, Dual TCR T cells were consistently observed in higher frequency among T cells that had divided in response to allogeneic stimulation as compared with undivided controls. FACS data of dual TCRα T cells from a representative experiment with allogeneic stimulation by B6.K splenocytes show the increased dual TCRα T cells among T cells that had divided in response to allogeneic stimulation as measured by CFSE dilution. B, To compare the frequency of T cells expressing dual TCRα between populations and five independent experiments, T cell numbers were normalized to Vo<sub>2</sub><sup>+</sup> cells, demonstrating a consistent and significant (mean ± SD; *p < 0.05; **p < 0.01) increase in dual TCRα T cells among alloreactive T cells. C, Increased dual TCR T cells were also consistently and significantly (mean ± SD; *p < 0.05; **p < 0.01) observed among alloreactive CD4<sup>+</sup>Vo<sub>2</sub><sup>+</sup> T cells. Stimulation with anti-CD3 and anti-CD28 mAbs did not result in significantly increased dual TCRα T cells (p = 0.18-0.10).
FIGURE 2. Secondary TCRα contribute nearly half of the naive alloreactive T cell repertoire. A, Alloreactive T cell frequency of CD4⁺ T cells from B6 mice and from B6.Cα⁺⁻/⁻ mice incapable of expressing secondary TCRα were measured by ELISPOT analysis for IFN-γ production following a 48-h MLR with irradiated B6.K splenocytes. Data from a representative experiment illustrates the consistent 40–60% reduction in alloreactive T cell frequency among CD4⁺ T cells from B6.Cα⁺⁻/⁻ mice. B, Integration of multiple cell concentrations from six independent experiments by normalizing the frequency of CD4⁺ T cell alloreactivity as a percentage of the alloreactivity of CD4⁺ T cells from B6 mice demonstrates the consistent and significant (mean ± SD; p = 0.001) reduction in alloreactivity among CD4⁺ T cells from B6.Cα⁺⁻/⁻ mice. 

Dual TCR T cells contribute disproportionately to the alloreactive T cell repertoire

To assess quantitatively the contribution of secondary TCRα to the alloreactive T cell repertoire, we compared the frequency of alloreactive T cells from mice incapable of expressing two functional TCR α-chains (B6.Cα⁺⁻/⁻, heterozygous for a knockout allele of the gene encoding the constant region of TCRα) with the frequency of alloreactive T cells from B6 mice. Alloreactive CD4⁺ T cell frequency was assessed by ELISPOT for IFN-γ production following MLR against B6.K splenocytes. T cells from B6.Cα⁺⁻/⁻ mice consistently demonstrated a 35–65% decrease in alloreactive frequency (representative experiment shown in Fig. 2A). Integration of multiple responder T cell concentrations from six independent experiments demonstrated a profound and highly statistically significant decrease of nearly 50% of the naive alloreactive T cell frequency in B6.Cα⁺⁻/⁻ mice (Fig. 2B). The dramatic nature of the contribution by a secondary TCRα was unexpected, as several previous reports (10–12) and our own observations (Fig. 1) demonstrated expression of a secondary TCRα on the cell surface on only 1–8% of peripheral T cells.

FIGURE 3. 2.102 secondary TCRα generates a nonthymically selected TCR with specific and distinct alloreactivity. A secondary Vα2, VB1 TCR was identified from the Vα4, VB1-expressing 2.102 T cell clone. A. The Vα2, VB1 secondary TCR is not thymically selected, as RAG⁻⁻/- mice transgenic for this receptor demonstrate thymocytes arrested at the double positive stage of development, and no peripheral αβ T cells (data not shown). B. Double positive thymocytes express the transgenic Vα2. C. The secondary Vα2, VB1 TCR demonstrates specific alloreactivity against H-2d, distinct from the alloreactivity against H-2Ep demonstrated by the primary Vα4, VB1 2.102 TCR. T cell hybrids expressing the secondary Vα2, VB1 TCR were tested for reactivity against a panel of allogeneic irradiated splenocytes, and T cell hybrid reactivity was assessed by the measurement of IL-2 production by ELISA. T cell hybrids expressing the Vα2 secondary TCR were specifically reactive to H-2d APC, but not to H-2p APC, demonstrating a distinct alloreactivity (mean ± SD; p = 0.05).

Demonstration of secondary TCR expanding the alloreactive repertoire

Expansion of the alloreactive T cell repertoire by a secondary TCRα was explored directly at the single cell level by using a T cell clone, 2.102, reactive to Hb/I-Ek and alloreactive to I-Ek (23). We initially identified a Vα2, VB1 TCR from the 2.102 T cell clone and expressed it transgenically in mice. However, T cells from the mouse were not reactive to Hb/I-Ek (24); we subsequently discovered that reactivity to Hb/I-Ek and Ep used a Vα4 TCRα pairing with the identified VB1 TCRβ. The Vα2, VB1 TCR was a secondary TCR expressed on 2.102 T2 cells, allowing us the opportunity to explore the role of a secondary TCR in alloreactivity at the single cell level.

To test the role of this secondary TCRα in positive selection, we crossed the Vα2, VB1 TCR transgenic mouse onto a B6.K RAG⁻⁻/- background. Analysis of thymi from Vα2-, VB1-expressing B6.K mice demonstrated no single positive thymocytes (data not shown). B, Double positive thymocytes express the transgenic Vα2. C. The secondary Vα2, VB1 TCR demonstrates specific alloreactivity against H-2d, distinct from the alloreactivity against H-2Ep demonstrated by the primary Vα4, VB1 2.102 TCR. T cell hybrids expressing the secondary Vα2, VB1 TCR were tested for reactivity against a panel of allogeneic irradiated splenocytes, and T cell hybrid reactivity was assessed by the measurement of IL-2 production by ELISA. T cell hybrids expressing the Vα2 secondary TCR were specifically reactive to H-2d APC, but not to H-2p APC, demonstrating a distinct alloreactivity (mean ± SD; p = 0.05).
splenocytes (H-2b,d,g7,j,k,p,q,r,u,v), with the only alloreactivity observed against H-2d stimulation (data not shown). Remarkably, the Vα2, Vβ1 TCR was specifically alloreactive against H-2d but not against any other allotype, including H-2b (Fig. 3C). Thus, the 2.102 T cell is positively selected by the Vα4, Vβ1 TCR, specific for Hb/E, and alloreactive to I-Eb, whereas the secondary Vα2, Vβ1 TCR is not positively selected in an H-2d thymus but is alloreactive to H-2d. These findings directly demonstrate expansion of the alloreactive repertoire of an individual T cell by expression of a secondary TCRα.

**Dual TCR T cells are dramatically involved in GVHD**

To investigate dual TCR T cells in vivo alloreactivity, we used a parent-into-F1 model of GVHD. Lethally irradiated (B6 × CBA)F1 or B6 mice were reconstituted with 2.0 × 10^7 BMCs and 2.5 × 10^7 splenocytes from B6 Ly5.1^+ mice. Within 4 wk of cell transfer, F1 mice developed clinically significant GVHD manifested as weight loss, hunching, hair loss, and lymphocytic infiltration of the gastrointestinal tract (data not shown). Upon development of clinically significant GVHD, mice were sacrificed and T cells from spleens and lymph nodes were examined by FACS for dual TCRα. Dual TCR T cells were readily identified among transferred Ly5.1^+ T cells in both F1 and control B6 mice, although F1 recipients had consistently more dual TCR T cells (Fig. 4, A and C). Dual TCRα T cells from mice with GVHD were more likely to be activated, as evidenced by down-regulation of TCR on the cell surface (Fig. 4A) and increased cell size (Fig. 4B). F1 recipients demonstrated a range of dual TCR T cells with 6–75% of Vα2^{+}Ly5.1^{+} T cells expressing a second TCRα, contrasting dramatically with only 1–6% of Vα2^{+}Ly5.1^{+} T cells in B6 recipients expressing a second TCRα, similar to the normal frequency of dual TCR T cells in the periphery (Fig. 4C). This increased frequency of dual TCR T cells was consistent in both CD4^+ (Fig. 4D) and CD8^+ T cells (Fig. 4E) from mice with GVHD. The dramatic increase of dual TCR T cells having an activated phenotype specifically in mice with GVHD demonstrates that dual TCR T cells are involved in vivo alloreactivity. Although these findings demonstrate that dual TCRα T cells are significantly involved in GVHD, they do not preclude involvement from conventional single TCR T cells; T cells incapable of expressing dual TCRα still have a high, although reduced, frequency of alloreactive T cells (Fig. 2) expected to contribute to GVHD. The quantitative and qualitative contribution of dual TCRα T cells to GVHD are not clear, although our findings would predict that T cells incapable of expressing dual TCRα may have a reduced capacity to induce GVHD, owing to decreased alloreactive precursor frequency. Observing subtle differences in the development of clinical symptoms of GVHD is problematic, as differences in alloreactivity could be normalized during the 4 wk required for the development of clinical symptoms in our model. However, we predict that examination at earlier time points would reveal the contribution of dual TCR T cells to GVHD development and are currently pursuing the development of early markers of GVHD to facilitate this analysis.

**Concluding Remarks**

Our alloreactivity model suggesting that energetic contributions to binding are required from both germline and nongermline TCR elements does not directly predict the origin of the paradoxically high degree of alloreactivity among naïve T cells. We hypothesize that the high frequency of alloreactive T cells results from multiple sources, including degenerate interactions with allogeneic MHC and polyspecific peptide-MHC interactions (8). The work presented here describes a third contributor to the high frequency of alloreactive naïve T cells, as nonthymically selected secondary TCRs demonstrate a remarkably high frequency of alloreactivity and disproportionately contribute to the alloreactive T cell repertoire. The importance of alloreactivity is emphasized by the important influence of HLA matching and the strong immunosuppression required for the prevention of immunologic rejection in allogeneic transplantation. Advances in defining the molecular basis for alloreactivity and its relation to disease are necessary for the continual improvement of clinical outcomes. Further testing of the mechanistic role of dual TCR T cells in mediating in vivo alloreactivity is warranted, both for improving our fundamental understanding of the nature of disease mediated by alloreactivity as well as for its potential as a target for immune manipulation during transplantation.
Acknowledgments
We thank C. Hsieh, B. Sleckman, E. Unanue, D. Donermeier, C. Hickman, and S. Weber for critical review of the manuscript. T. Mohanakumar for use of the ELISPOT Reader, T. Stappenbeck for assistance in pathology analysis, and D. Kreamalmeyer for maintenance of the mouse colony.

Disclosures
The authors have no financial conflict of interest.

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