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The Ability To Rearrange Dual TCRs Enhances Positive Selection, Leading to Increased Allo- and Autoreactive T Cell Repertoires

Peggy P. Ni,* Benjamin Solomon, † Chyi-Song Hsieh, † Paul M. Allen,* and Gerald P. Morris‡

Thymic selection is designed to ensure TCR reactivity to foreign Ags presented by self-MHC while minimizing reactivity to self-Ags. We hypothesized that the repertoire of T cells with unwanted specificities such as alloreactivity or autoreactivity are a consequence of simultaneous rearrangement of both TCRα loci. We hypothesized that this process helps maximize production of thymocytes capable of successfully completing thymic selection, but results in secondary TCRs that escape stringent selection. In T cells expressing two TCRs, one TCR can mediate positive selection and mask secondary TCR from negative selection. Examination of mice heterozygous for TRAC (TCRα<sup>+</sup>−<sup>−</sup>), capable of only one functional TCRα rearrangement, demonstrated a defect in generating mature T cells attributable to decreased positive selection. Elimination of secondary TCRs did not broadly alter the peripheral T cell compartment, though deep sequencing of TCRα repertoires of dual TCR T cells and TCRα<sup>+</sup>−<sup>−</sup> T cells demonstrated unique TCRs in the presence of secondary rearrangements. The functional impact of secondary TCRs on the naive peripheral repertoire was evidenced by reduced frequencies of T cells responding to autoantigen and alloantigen peptide–MHC tetramers in TCRα<sup>+</sup>−<sup>−</sup> mice. T cell populations with secondary TCRs had significantly increased ability to respond to altered peptide ligands related to their allelogenic ligand as compared with TCRα<sup>+</sup>−<sup>−</sup> cells, suggesting increased breadth in peptide recognition may be a mechanism for their reactivity. Our results imply that the role of secondary TCRs in forming the T cell repertoire is perhaps more significant than what has been assumed. The Journal of Immunology, 2014, 193: 1778–1786.

The TCR is comprised of TCRα and TCRβ-chains generated by gene segment recombination during thymocyte development. Generation of operational TCRs is critical for development of a functional T cell repertoire, as TCRs must specifically and sensitively recognize self- and foreign peptide–MHC (pMHC) ligands to appropriately navigate development and mediate immune responses (1). The TCRβ-chain rearranges in double-negative (DN) thymocytes under tight allelic exclusion and ceases when an in-frame product is made and expressed (2–4). CD4 and CD8 coreceptors are then upregulated, and, in these double-positive (DP) cells, TCRβ-chain rearrangement occurs until halted by positively selecting signals (5–8). Positive selection requires specific recognition of self-pMHC ligands (9–13). This strict requirement results in a majority of thymocytes dying from an inability to undergo positive selection (14, 15). However, presumably in a measure to maximize generation of TCRs capable of mediating positive selection, TCRα gene recombination occurs in DP thymocytes in a simultaneous and iterative fashion on both loci (7, 16). Iterative revision of TCRα, sequential recombination of TRAV and TRAJ segments on the same chromosome, has been demonstrated to be important for efficient positive selection of T cells by enabling multiple opportunities for formation of a successful in-frame TCRα rearrangement (17). However, the impact of simultaneous rearrangement of TCRα loci on both chromosomes for thymocyte selection has not been defined.

Simultaneous rearrangement of both TCRα loci results in a lack of allelic exclusion for TCRα, evidenced by thymocytes and peripheral T cells with two in-frame rearrangements of TCRα (3, 18), and mature T cells with dual TCR expression on the surface (8, 19, 20). In these cells, each TCRα-chain pairs with the same β-chain, giving the cell two distinct pMHC ligand specificities (21, 22). The expression of dual TCRs presents a unique change to the requirements of a thymocyte for successful selection. One TCR can successfully mediate positive selection, enabling the presence of a secondary TCR that does not participate in positive selection (19, 21, 22). Expression of secondary TCRs can also factor importantly during negative selection, masking autoreactive TCRs from deletion (23–25). This masking effect is likely mediated through decreased surface expression of the pathogenic TCR due to TCRα-chain competition for the single TCRβ-chain (26, 27). Thus, the presence of dual TCRs in developing thymocytes provides an unusual lessening of the stringent requirements for thymic selection, which could significantly impact the naive T cell repertoire.

This potential prompted us to examine dual TCR T cell alloractivity as a model of naïve T cell responses. Examination of alloreactive responses in mice genetically lacking dual TCR T cells (TCRα<sup>+</sup>−<sup>−</sup> heterozygous for a mutation in TRAC disrupting formation of a functional TCRα-chain) revealed that secondary

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; APL, altered peptide ligand; B6, C57BL/6; CHO, Chinese hamster ovary; DN, double-negative; DP, double-positive; GVHD, graft-versus-host disease; LCMV, lymphocytic choriomeningitis virus glycoprotein; MOG, myelin oligodendocrye glycoprotein; pMHC, peptide–MHC; SP, single-positive; TFR, transferrin receptor.

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TCRs, which comprise ~10% of the peripheral TCR repertoire in mice, constitute >40% of the response to allogeneic stimuli (22). The impact of secondary TCRs in pathologic alloreactivity is demonstrated in patients developing acute graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell transplantation. In these patients, dual TCR T cells were expanded, activated, and responded preferentially to mismatched alloantigens (28). These data indicate that dual TCR T cells contribute significantly to allogeneic T cell responses. A functional contribution of naturally arising dual TCR T cells to the autoreactive T cell repertoire has been suggested by studies of diabetes in NOD mice (29, 30).

We hypothesized that the potential for unwanted TCR specificities harbored by dual TCR T cells must be balanced by a significant benefit of simultaneous TCRs rearrangement during thymocyte development. We theorized that this benefit likely occurs though improved efficiency of DP thymocyte positive selection. Examination of thymocyte development in TCR expressing two TCR one copy of the TCR gene, were derived by crossing TRAC−/− mice had diminished binding to autopeptide–MHC and allopeptide–MHC tetramers but not to foreign Ag. Population-level analysis of binding to altered peptide ligands (APLs) of an allostimulatory peptide showed that the presence of secondary TCRs enabled recognition of related pMHC ligands, providing a possible mechanism for dual TCR T cell alloreactivity.

Materials and Methods

Mice

C57BL/6 (B6), B6.1y5.1, B6.1y1.1, and B6.K mice were originally purchased from The Jackson Laboratory. TCRα−/− mice, incapable of expressing two TCRα-chains due to a targeted disruption at the 5′ end of one copy of the TRAC gene, were derived by crossing TRAC−/− B6 mice (6) with B6 or B6.1y1.1 mice. Mice were bred and housed in specific pathogen-free conditions at Washington University Medical Center (St. Louis, MO). All use of laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

Flow cytometry

Thymocyte and T cell analyses were performed using anti-CD3 (145-2C11)–PE-Cy7, anti-CD45.2 (104)–PerCP-Cy5.5, anti-CD90.1 (HI1001)–eFluor 450 (eBioscience), anti-CD69 (H1.2F3)–PE-Cy7, anti-CD4 (RM4-5)–AF700, anti-CD8a (53-6.7)–allophycocyanin-Cy7 (Biolegend), and anti-CD45.1 (A20)–allophycocyanin (BD Biosciences). Non–T cells were excluded by labeling with Pacific Blue–labeled anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), and anti-F4/80 (BM8) (BioLegend). TCRα+ and dual TCR T cells were identified among CD3+ CD11b− CD11c+ T/F4/80 splenocytes using anti-TCRα2 (B201)–PE, anti-TCRα3 (RR3-16)–FITC, anti-TCRα8 (KT50)–FITC, and anti-TCRα11 (RR8-1)–FITC (BD Biosciences). Samples were analyzed using LSR II or LSR Fortessa cytometers (BD Biosciences) with calculated compensation, and data were analyzed with FlowJo software (Tree Star). FACSARA II (BD Biosciences) was used for sorting.

Thymocyte culture

Survival was assessed by culturing 10⁷ thymocytes/ml in RPMI 1640 (Life Technologies) supplemented with 10% FBS (PAA Laboratories), 2 mM GlutaMAX, and 50 μg/ml gentamicin for 6 d. Cultures were labeled for CD4, CD8, Annexin V, and 7-aminoactinomycin D (7-AAD; BioLegend) and analyzed by flow cytometry.

Thymocyte transfers

B6.1y5.1 and TCRα−/− Thyl.1 preselection thymocyte populations were bead-enriched for CD53+ cells using anti-CD53 mAb (OX-7; BioLegend) and anti-IgM paramagnetic beads (Miltenyi Biotec). Enriched cell populations were mixed at a 1:1 ratio and injected intrathymically into sublethally irradiated (5 Gy) B6 recipient mice. Thymi from recipient mice were analyzed 7 d postinjection by flow cytometry.

BrdU labeling

Mice were injected i.p. with 1.2 mg BrdU (Sigma-Aldrich), and thymi were harvested at 24-, 48-, and 96-h time points. Thymocytes were labeled for surface markers, fixed with BD CytoFix (BD Biosciences), permeabilized with Permeabilization Wash Buffer (BioLegend), treated with 1 ml DNase I (Sigma-Aldrich) at 50 U/ml, intracellularly labeled with anti-BrdU (BU20A)–FITC (eBioscience), and analyzed by flow cytometry.

FIGURE 1. Thymic generation of mature T cells is deficient in the absence of secondary TCRα rearrangements. (A) Comparison of numbers of thymocytes in 6-wk-old B6 and TCRα−/− mice. Each point represents a single mouse; n = 17, four independent experiments; mean ± SEM, Student t test. (B-E) The effect of secondary TCRα rearrangements on T cell development examined by comparing thymi of B6 and TCRα−/− mice by flow cytometry; n = 10, three independent experiments. (B) Representative plots of CD4 and CD8 labeling of thymocytes from individual mice. (C) Percentages of mature CD4+ or CD8+ SP, CD4+CD8+ DP, or CD4−CD8− DN thymocytes. Each point represents a single thymus; mean ± SEM, Student t test. (D) Representative plots of CD3 and CD69 labeling of thymocytes from individual mice. (E) Percentages of postpositive selection CD3hi/thymocytes. Each point represents a single thymus; mean ± SEM, Student t test.
TCR repertoire analysis

TCRαo^+^ T cells and dual T cell T cells were sorted from TCRαo^+^- and B6 splenocytes by flow cytometry. TCRαo cDNA libraries were generated by PCR (31, 32) and sequenced by 250-cycle paired-end sequencing using an Illumina MiSeq at the Washington University Genome Sequencing Center. TRAV and TRAJ gene segment use was determined by sequence analysis using the International ImMunoGeneTics Information System nucleotide sequence database (33).

Tetramer enrichment

Lymphoproliferative choriomeningitis virus glycoprotein (LCMV)66–77 (DIYKG-YYQFKSV)/I-A^B (LCMV-A^B tetramer)-allophycocyanin, mouse MHC class II Ig Es53–68 (ASFEAQGALANIVDKA)/I-A^A (Es-A^A tetramer)-allophycocyanin, and myelin oligodendrocyte glycoprotein (MOG)52–68 (GWYRSPSRVHIV/A^A (MOG-A^A tetramer)-PE were obtained from the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Murine CD22e54–66 (SGQDLHLSIQKLR)/I-E^a (CD22-E^a tetramer)-PE and murine transferrin receptor (TFR)231–244 (SGKLVHANFGTKKD/I-Ek (TFR-allophycocyanin, and myelin oligodendrocyte glycoprotein (MOG)38–49 (GWYRSPSRVHIV/A^A (MOG-A^A tetramer)-PE were obtained from the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Murine CD22e54–66 (SGQDLHLSIQKLR)/I-E^a (CD22-E^a tetramer)-PE and murine transferrin receptor (TFR)231–244 (SGKLVHANFGTKKD/I-Ek (TFR-E^a tetramer)-PE tetramers were generated using soluble I-E^a produced in Escherichia coli inclusion bodies and refolded with peptide (34). Tetramer enrichment was performed according to published protocols (35). Briefly, cells were incubated with tetramer for 1 h at room temperature, washed, incubated with anti-PE- and/or anti-allophycocyanin-conjugated microbeads for 30 min at 4°C, and passed over magnetized LS columns for positive selection (Miltenyi Biotec). Enriched populations were labeled for surface markers for 20 min at 4°C and analyzed or sorted by flow cytometry.

In vivo alloreactive response

T cells were enriched from B6.Ly5.1 and TCRαo^+^-Thy1.1 spleens using anti-CD4 and anti-CD8 paramagnetic beads and LS columns (Miltenyi Biotec), mixed 1:1, pulsed with 5 mM CFSE (Sigma-Aldrich), and 5–10 × 10^6^ cells were injected i.v. into lethally irradiated (10 Gy) B6.K mice. Splenocytes were harvested at 8 and 24 h after injection, labeled for surface markers, and analyzed by flow cytometry for CFSE dilution.

Statistical analyses

Data were analyzed using Prism 6 software (GraphPad). Mean values of mouse replicate data were analyzed using Student t test. Paired data were analyzed using a paired ratio test. Multicategorical data were analyzed using two-way ANOVA. TCR sequence data were analyzed by linear regression analysis with computation of 99% confidence and predictive intervals and tabular analysis of outliers.
Results
Secondary TCRα rearrangements enable efficient positive selection

The effects of simultaneous TCRα rearrangement on thymocyte development were evaluated by analyzing thymus cellularity and populations in TCRα−/− mice incapable of forming functional secondary TCRα-chains (6). Thymi from B6 and TCRα−/− mice had similar total number of cells (Fig. 1A). There were comparable percentages of immature DN (8.8 ± 1.5 versus 7.6 ± 2.0%), DP (70.1 ± 3.0 versus 76.4 ± 3.6%), and single-positive (SP) cells (14.7 ± 2.2 versus 19.7 ± 2.5%) in TCRα−/− mice compared with B6 (Fig. 1B, 1C). However, closer examination revealed that TCRα−/− mice had significantly reduced percentages of post-selection CD3high thymocytes (8.7 ± 0.6%) compared with B6 (13.3 ± 0.7%) (Fig. 1D, 1E). This specific decrease of postpositive selection thymocytes implies that secondary TCRα rearrangements are important for efficient thymic development.

We hypothesized that positive selection would be the most critical window for the influence of secondary TCRα-chains, as it is estimated that preselection DP thymocytes have an average lifespan of only ~60 h during which they must form a functional TCR to mediate positive selection and continue development (14, 15). To examine positive selection kinetics, we injected B6 and TCRα−/− mice with a pulse dose of BrdU and evaluated subsequent development of labeled thymocytes. There was no difference in the percentage of thymocytes (predominantly DN thymocytes) that incorporated BrdU (Fig. 2A). However, over the following 96 h, pulsed TCRα−/− thymocytes demonstrated a deficient progression to a postselection CD3high phenotype (18.7 ± 2.2%) compared with B6 (27.1 ± 1.8%) (Fig. 2B).

Cotransfer studies of congenically marked B6 and TCRα−/− cells were performed to attribute the deficiency of generating mature SP thymocytes to a cell-intrinsic defect. Preselection CD53+ cells from B6.Ly5.1 and TCRα−/−.Thy1.1 thymocytes were mixed at a 1:1 ratio and intrathymically injected into recipient B6 mice. Transferred cells were analyzed 7 d postinjection by flow cytometry. The ratio of B6.Ly5.1/TCRα−/−.Thy1.1 among total thymocytes was 1.36 ± 0.11, similar to the preinjection ratio. However, examination of postselection CD3high thymocytes revealed a significant skewing toward B6.Ly5.1 cells (1.86 ± 0.15 ratio), suggesting that the inability to perform simultaneous TCRα rearrangements impaired the ability of thymocytes to mature. Comparison of B6.Ly5.1 and TCRα−/−.Thy1.1 thymocyte development within individual thymi underscores this observation (Fig. 2C–E).

To rule out a survival defect as the mechanism for this deficiency, we examined viability in B6 and TCRα−/− thymocytes. Direct ex vivo analysis of Annexin V and 7-AAD labeling of thymocytes did not reveal any differences between B6 and B6.TCRα−/− mice (Fig. 2F). Likewise, B6 and B6.TCRα−/− thymocytes demonstrated similar viability during 6 d of in vitro culture (Fig. 2G), indicating that the competitive advantage of secondary TCR-sufficient thymocytes was not due to a difference in viability.

FIGURE 3. Elimination of secondary TCRα rearrangements does not broadly alter the peripheral T cell compartment. (A) Comparison of numbers of T cells in the spleens of 6-wk-old B6 and TCRα−/− mice. Each point represents a single mouse (n = 6) from three independent experiments; mean ± SEM, Student t test. (B) Comparison of CD4+ and CD8+ T cell composition, measured by flow cytometry of splenocytes. Mean ± SEM of six mice from three independent experiments, Student t test. Comparison of TRAV (C) and TRAJ (D) gene segment use by DNA sequence analysis of splenic T cells from 6-wk-old B6 and TCRα−/− mice (three mice each, 23,877–34,995 TCRα sequence reads/mouse). Mean ± SD for each group; data analyzed by ANOVA.

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Absence of secondary TCRα rearrangements eliminates certain TCR specificities

The contribution of secondary TCRα-chains in mediating thymocyte development suggests that their absence could significantly affect the peripheral T cell repertoire. The lack of secondary TCRα rearrangements did not affect peripheral T cell numbers in the spleen (Fig. 3A) or CD4 or CD8 subsets (Fig. 3B). To examine potential skewing of the TCR repertoire, TRAV and TRAJ gene segment use was analyzed by TCRα cDNA sequence analysis of peripheral T cells from three B6 and three TCRα−/− mice. Analysis of 123,655 B6 and 104,434 TCRα−/− TCRα transcripts revealed similar TRAV (Fig. 3C) and TRAJ (Fig. 3D) gene segment use, indicating that the absence of secondary TCRs does not broadly affect the T cell repertoire.

For a more focused examination, we compared TCRα sequences from TCRα+2 (TRAV14) T cells from TCRα−/− mice with TRAV14 TCRα transcripts from dual TCR T cells (TCRVα2+ and TCRα3/8/11+) from B6 mice. This enabled direct comparison of TCR repertoire composition in the presence or absence of secondary TCRs. From three independent TRAV14+ TCRα transcript libraries for each mouse strain, we analyzed 141,353 B6 TRAV14+ TCRα sequences and 148,228 TCRα−/− TRAV14+ TCRα sequences, yielding 13,646 and 14,002 unique CDR3α sequences, respectively. Of the unique TRAV14+ TRAV3α sequences, 4609 (33.8% of the B6 repertoire) were shared between the two groups (Fig. 4A), indicating a potential for significant differences between the two repertoires. There was no difference between TCRα−/− and dual TCR B6 TRAV14+ TCRα sequences in CDR3 length (Fig. 4B), amino acid composition (Fig. 4C), and TRAJ gene segment use (Fig. 4D). However, our hypothesis proposes that the impact of secondary TCRα rearrangements would be more subtle and reflected in the presence or absence of specific TCRα sequences that might normally be negatively selected against if present as the only TCR. Comparison of the most abundant transcripts revealed considerable overlap (3 out of 10 top-
ranked dual TCR and 4 out of 10 TCRα+/− transcripts were shared) (Fig. 4E). Comparison of all TRAV14 transcript abundance demonstrated a significant correlation between the two populations (r = 0.788) (Fig. 4F). The slope of the correlation suggests that the TCRα repertoire is less diverse in TCRα+/− T cells than B6.

The limits on TCRα diversity implied by regression analysis are more clearly illustrated by examining CDR3 sequences unique to each repertoire. If the absence of a TCRα sequence in one repertoire were due to sampling error or random effect, it would be expected that the frequency of the sequence would fall within the confidence intervals determined by regression analysis. This is true for the majority of TRAV14+TCRα sequences unique to the dual TCR (8430 out of 9037, 93.3%) and TCRα+/− (8884 out of 9393, 94.6%) repertoires, which fall within the 99% confidence intervals. However, the increased frequency of TCRα sequences unique to dual TCR T cells that are outside of expected random sampling error as compared with secondary TCRα-insufficient T cells (p < 0.001, Fisher exact test) suggests that the repertoire lacking secondary TCRs is not a stochastic process, but the result of actively excluding some sequences. Indeed, many TCRα sequences unique to the B6 dual TCR T cells were of relatively high abundance (>0.1% of the TRAV14+ repertoire) as compared with TCRα+/− cells (Fig. 4G). These results illustrate the effect of secondary TCRα rearrangements on the diversity of the peripheral T cell repertoire.

**Secondary TCRs contribute to naive autoreactive and alloreactive repertoires**

Findings from TCRα repertoire analysis suggest that exclusion of distinct sequences in TCRα+/− mice could manifest as a differential ability of the naive T cell repertoire to respond to Ags. We measured the reactivity of naive B6 and TCRα+/− T cells using class II pMHC tetramers to estimate the frequency of Ag-specific T cells (35). We generated I-Ek tetramers loaded with allogeneic peptides CD2254–666 and TFR231–244, previously identified by mass spectrometry of endogenous peptides eluted from I-Ek molecules and known to stimulate alloreactive T cells (36). T cells were labeled with alloantigen pMHC tetramers, enriched by magnetic bead selection, and identified by flow cytometry (Fig. 5A). CD22/I-Ek and TFR/I-Ek tetramer-positive and -negative cells from B6 mice were sorted by flow cytometry and assessed for response to CD22 and TFR peptides. Tetramer-positive populations responded to their peptide, whereas the tetramer-negative cells did not (Fig. 5B), demonstrating the efficiency of our pMHC tetramer labeling. TCRα+/− mice had a consistently decreased frequency of response to individual allogeneic pMHC complexes (Fig. 5C). The average frequencies of TCRα+/− T cells recognizing the TFR/I-Ek and CD22/I-Ek tetramers (8.6 ± 0.9 and 61.0 ± 8.1 cells/10⁵ T cells, respectively) were significantly less than those of B6 T cells (19.9 ± 4.7 and 99.9 ± 14.8 cells/10⁵ T cells). There was no significant difference between B6 and TCRα+/− T cell response to a minor histocompatibility Ag, Ea/I-Aκ, presented by self-MHC I-Aκ (4.3 ± 1.0 versus 3.1 ± 0.9 cells/10⁵ T cells, respectively).

Consistent with our hypothesis proposing that secondary TCRα rearrangements contribute specifically to the recognition of atypical ligands, TCRα+/− mice also had reduced frequencies of naive CD4+ T cells recognizing the autoantigen MOG18–49 presented by I-Aκ (8.6 ± 2.5 cells/10⁵ T cells) as compared with B6 (28.3 ± 9.3 cells/10⁵ T cells) (Fig. 5D). Conversely, recognition of I-Aκ tetramers presenting a foreign Ag, LCMV66–77 was comparable between B6 and TCRα+/− CD4+ T cells (4.7 ± 2.3 versus 5.8 ± 2.5 cells/10⁵ T cells, respectively). These results demonstrate specific contribution of secondary TCRα rearrangements to the alloreactive and autoreactive T cell repertoire.
mice (Fig. 6A). Expansion of transferred T cells was assessed by flow cytometry 24 h after transfer. Although the two cell populations were transferred at equal numbers, recovery of TCRα-β- cells was decreased (ratio TCRα-β-/B6 T cells 0.6 ± 0.1%) (Fig. 6B). Examination of CFSE dilution in the recovered cells demonstrated that TCRα-β-. Thy1.1 T cells were less likely to divide in the first 24 h posttransfer as compared with the secondary TCR-sufficient B6.Ly5.1 cells (Fig. 6C, 6D). The diminished response of TCRα-β- T cells early after allogeneic stimulation in vivo illustrates a functional consequence of the decreased frequency of allogeneic T cells in the absence of secondary TCR rearrangements and underscores the potential importance of dual TCR T cells in driving early pathologic alloreactive responses.

**Elimination of secondary TCRs inhibits recognition of altered allogeneic peptide ligands**

The contribution of secondary TCRs to the allogeneic and autoreactive T cell repertoire has multiple possible mechanistic explanations. One is that elimination of secondary TCR rearrangements results in fewer TCRs, which reduces the probability of recognizing a specific Ag. However, although secondary TCRs comprise <10% of the T cell repertoire, our data indicate that they encompass significantly more of the allogeneic and autoreactive repertoires (Fig. 5C, 5D) (22, 30) but do not affect recognition of conventional foreign Ags. This specific and disproportionate effect suggests that it is not simple stochastic addition of Ag specificities to the repertoire by secondary TCRs, but rather a unique property of secondary TCRs.

To investigate this, we compared functional responses of CD22/I-Ek tetramer binding T cells from B6 and TCRα-β- mice to APLs. Tetramer-positive cells from naive mice were sorted by flow cytometry and stimulated with the wild-type CD22 peptide or CD22 APLs containing single amino acid substitutions at TCR contact sites (P2 and P5). Responses were assessed by measurement of IFN-γ production after 24-h culture. IFN-γ production to either nonspecific stimulation or wild-type CD22 peptide was similar between the two cell types (Fig. 7A). However, I-Ek/CD22-specific B6 T cell populations responded to APLs with mutations at either the P2 or P5 positions, whereas I-Ek/CD22-specific TCRα-β- cells did not (Fig. 7A, 7B). These data suggest that an increased breadth in ligand recognition may underlie the specific atypical ligand recognition by dual TCR T cells at a population level.

![FIGURE 6](http://www.jimmunol.org/DownloadedFrom)

**Discussion**

The existence of a minority of T cells expressing two TCRs in mice and humans has been recognized for some time (19), though the biological significance of these cells has not been well understood. Studies of transgenic TCR systems demonstrated that secondary TCRs recombination may enable TCRs with unwanted reactivities to escape thymic selection and emigrate to the periphery (24, 25). Thus, the potential benefit of simultaneous TCRα-chain development may detrimentally alter the T cell repertoire. In this study, we used mice genetically deficient for secondary TCR rearrangements to define their role in thymocyte development and generation of the T cell repertoire.

Analysis of thymocyte development in TCRα-β- mice demonstrated diminished production of mature postpositive selection CD3high SP cells (Figs. 1, 2), supporting the notion that simultaneous TCRα rearrangements occur to maximize the efficiency of mature T cell production from thymocytes successfully mediating β-selection. Our results demonstrate that secondary TCRα rearrangements are quantitatively important for thymopoiesis, though we cannot ascribe the relative importance of their effects on positive versus negative selection. This will require further investigation in systems where these questions can be separated.

The elimination of secondary TCRα rearrangements did not broadly affect the peripheral T cell repertoire, either in the number of T cells or in the general use of TCRα and TCRβ gene segments (Fig. 3). A focused comparison of TRAV14 TCR transcripts between T cells lacking secondary TCRα rearrangements and T cells with dual TCR expression similarly revealed no differences in the use of TCRβ gene segments or TCR properties such as CDR3 length and amino acid composition. However, dual TCR T cells had numerous high abundance CDR3 sequences that were absent among cells lacking secondary TCRα-chains (Fig. 4). Statistical analysis indicated that the nonappearance of these sequences from TCRα-β- T cells was not simply sampling error but represented a specific absence from their repertoire. The reciprocal was not true, indicating that the presence of these unique TCRα sequences depended on secondary TCRα rearrangements. Although it is not possible to exclude a stochastic process that excluded these TCR sequences from TCRα-β- T cells, we propose that this supports our hypothesis that secondary TCRα rearrangements contribute specificities to the peripheral repertoire that would otherwise be negatively selected in cells expressing a single TCR.

![FIGURE 6](http://www.jimmunol.org/DownloadedFrom)
We propose that secondary TCRs rearrangements increase the T cell repertoire for atypical self- and allogeneic pMHC ligands due to the effects of dual TCR expression on the stringency of thymic selection. Indeed, secondary TCRs contribute significantly and specifically in responses to peptides involved in autoimmunity and alloreactivity, but not cognate ligands (Fig. 5). The decreased response of CD4+ T cells from TCRα−/− mice to MOG/I-Ab tetramers was somewhat unexpected given a previous study reporting no impact on experimental autoimmune encephalomyelitis disease outcome in mice lacking secondary TCRs (29). In that study, the effect of secondary TCRs was measured following immunization with Ag and adjuvant as compared with our interrogation of the naive T cell repertoire. Given that the NOD diabetes model, which does not rely on adjuvants, also indicated a role for dual TCR T cells (29, 30), it seems that secondary TCRs may be important contributors to autoimmunity under physiological conditions. Interestingly, a report indicating that a majority of regulatory T cells in humans express two TCRs (37) underscores the potential for dual TCR T cells to recognize self-pMHC with high affinity. The potential for secondary TCRs to contribute to pathogenic responses has been demonstrated by our previous studies examining dual TCR T cells in mouse models and patients with GVHD (22, 28). We hypothesized that the disproportionate alloreactive responses we observed among dual TCR T cells resulted from increased frequencies of allogeneic-specific T cells among naive dual TCR T cells. This hypothesis is now supported by our data in this study demonstrating decreased numbers of allogeneic precursors and proliferative potential during the early phase of GVHD in the absence of secondary TCRs (Fig. 6). These data highlight the potentially important role for dual TCR T cells in GVHD and autoimmune disease in the earliest phases of disease. We hypothesize that secondary TCRs are important in alloreactivity and autoimmunity due to their uniquely relaxed constraints of thymic selection that may enable T cell cross-reactivity. It has been demonstrated that negative selection is important for eliminating cross-reactive TCRs (38), and thus secondary TCRs masked from negative selection may be more cross-reactive. A potential contribution for secondary TCRs to cross-reactivity is supported by the increased breadth of recognition of allopeptide APLs in wild-type T cells as compared with T lacking secondary TCRs (Fig. 7). Numerous studies have demonstrated that TCR interaction with the peptide is critical for response to allogeneic ligands (36, 39). However, there is uncertainty regarding how much flexibility the TCR has in recognizing multiple ligands and how this relates to alloreactivity (40, 41). Although our data do not question the importance of peptide recognition in alloreactivity, it does indicate a potential mechanism for the increased propensity for dual TCR T cells to respond to specific pMHC ligands. The mechanistic basis for the unusual reactivity of dual TCR T cells warrants further investigation in a system in which the parameters of thymic selection and ligand specificity can be correlated for individual TCRs.

In summary, we show that efficient thymocyte development requires simultaneous rearrangement of both TCRα loci. However, this comes at a cost, with resulting secondary TCRs having an increased ability to respond to self- and allogeneic pMHC. Our results highlight a hitherto underappreciated role of dual TCR-expressing T cells in the development of the T cell repertoire and suggest that therapeutic strategies for combating autoimmune diseases or transplant rejection and GVHD take into account the significant contribution of these uniquely powerful T cells.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


