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BRIEF CONCLUSIVE REPORT

Endogenous co-expression of two T cell receptors promotes lymphopenia-induced proliferation via increased affinity for self-antigen

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Abstract

Approximately 10% of peripheral T cells express 2 functional TCR $\alpha\beta$ heterodimers. Receptor co-expression changes the repertoire of TCRs produced during thymic development, enabling generation of T cells bearing TCRs not capable of mediating positive selection or that would normally be negatively selected. The effect of receptor co-expression on the composition and functionality of the peripheral TCR repertoire is not well defined, though evidence demonstrates dual TCR cells pose an increased risk for unwanted immune responses such as autoimmunity and alloreactivity. Based on our previous finding that dual TCR expression promotes positive selection, we hypothesized that dual TCR expression may enhance T cell homeostasis via increased reactivity against self-peptide:MHC (pMHC) ligands. To examine the effect of dual TCR expression on T cell homeostasis, we performed cotransfer experiments comparing T cells genetically deficient for dual TCR expression (TCR$\alpha^{+/-}$) with wild-type T cells in models of acute and chronic lymphopenia-induced proliferation (LIP). Lack of dual TCR expression resulted in reduced LIP. The effect of dual TCR expression on LIP was most pronounced in acute lymphopenia, which is driven by recognition of low-affinity self-pMHC ligands. Differences in homeostatic proliferation were not attributable to differences in total TCR expression or signaling, but were dependent on interaction with MHC and associated with increased affinity for positively selecting self-pMHC as evidenced by higher expression of CD5 by dual TCR cells from wild-type mice. These results represent an unappreciated novel mechanism driving homeostasis and shaping the T cell repertoire, potentially promoting autoreactive or heterologous immune responses.

KEYWORDS
homeostasis, T cell, TCR

1 | INTRODUCTION

Approximately 10% of peripheral T cells in humans and mice express 2 functional TCRs.[1,2] TCR co-expression arises as a consequence of incomplete allelic exclusion during TCR gene rearrangement.[2–4] Dual TCR expression is predominantly (~90%) the result of allelic inclusion of TCRα loci on both chromosomes producing in-frame rearrangements of *TRAV* and *TRAJ* gene segments. Each in-frame rearrangement can generate TCRα protein capable of pairing with a single TCRβ to form 2 functional TCR$\alpha\beta$ heterodimers.[4,5] Allelic inclusion for TCRα provides an advantage in the ability of double positive CD4$^+$CD8$^+$ thymocytes to generate a TCR capable of the low affinity but highly specific recognition of self-pMHC ligands in the thymic cortex required for positive selection.[6,7] However, co-expression of TCRs decreases the stringency of thymic selection; TCRs incapable of independently supporting positive selection can be passengers on thymocytes bearing a selecting TCR.[8,9] Conversely, TCRs that would normally induce negative selection can be masked by co-expression of a second TCR.[10,11] This results in dual receptor T cells harboring unique TCR clonotypes not present in the absence of dual receptor expression.[7]

Abbreviations: aLIP, acute lymphopenia-induced proliferation; cLIP, chronic lymphopenia-induced proliferation; CM, central memory; EM, effector memory; LIP, lymphopenia-induced proliferation; pMHC, peptide-MHC; Treg, regulatory T cell

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At a clonal level, co-expression of 2 TCRs implicitly increases the potential antigenic reactivity of a given cell. However, the presence of TCR clonotypes that would otherwise not normally be present prompts investigation into the broader effects of dual TCR cells on the composition and antigenic reactivity of the T cell repertoire. Transgenic TCR models have clearly demonstrated that dual receptor expression can enable autoreactive T cells to escape negative selection and contribute to autoimmunity.[8,10–12] However, the effects of dual TCR expression on the antigenic reactivity of naturally occurring polyclonal responses has varied by the antigen and model tested.[13] Using a model of mice genetically deficient in dual TCR co-expression from hemizygous deletion of *TCRAC* (TCR$\alpha^{+/-}$), we have demonstrated that approximately 50% of the alloreactive T cell repertoire and as much as 70% of the repertoire recognizing the myelin oligodendrocyte (MOG) autoantigen $MOG_{38\text{-}49}$:A^b depends on dual TCR co-expression in immunologically naive mice.[7,9] Similar effects were not observed for response to foreign antigen LCMV $GP_{66\text{-}77}$:A^b. These data suggest that dual receptor expression specifically affects the T cell repertoire by promoting recognition of ligands other than typical "cognate" foreign antigen presented by self-MHC as constrained by positive and negative selection.

The composition and reactivity of the peripheral T cell repertoire is not determined solely by thymic selection. In the periphery, the T cell compartment is shaped through a combination of thymopoiesis, encounter with commensal and foreign antigens, and homeostasis. The total size of the T cell compartment is relatively constant, forcing competition between T cell clonotypes for niche space. Several factors, most notably IL-2, IL-7, IL-15, and TCR signaling, act in concert to regulate T cell homeostasis.[14] TCR signaling induced via low-affinity interaction with self-pMHC ligands is a principal requirement for the persistence of T cells in the periphery.[15–18] The ability of a T cell clone to compete for homeostatic survival and proliferation factors is linked to TCR clonotype affinity for self-pMHC; T cells with higher expression of CD5, an indicator of higher-affinity interactions with self-pMHC ligands during positive selection,[19,20] have higher rates of homeostatic proliferation compared to cells with lower expression of CD5.[18,21–23] This provides a selective mechanism in the periphery for regulating the T cell repertoire.

Homeostatic expansion and maintenance is particularly important in setting where thymic contribution to the peripheral repertoire is diminished, such as normal aging[24] or where homeostatic expansion is stimulated such as iatrogenic lymphodepletion in transplantation.[25] In these settings, lymphopenia-induced proliferation (LIP), a form of homeostatic proliferation, is capable of skewing of the T cell repertoire toward clones with increased reactivity for self-antigens and promoting autoreactivity.[26–28] Previous work from our lab and others has demonstrated that dual TCR cells have significantly increased propensity for recognition of self-pMHC, and can participate in autoimmune and transplant-related graft-versus-host-disease pathology.[13,29] The effect of homeostatic expansion on T cells with increased reactivity for self-antigens, as well as the importance of homeostasis in transplantation, prompted us to investigate the effects of dual TCR co-expression on homeostasis.

2 | METHODS

2.1 | Mice

C57BL/6 (B6), B6.Ly5.1, and B6.Thy1.1 mice were originally purchased from Charles River Laboratories (Wilmington, MA, USA). B6.129S2-$Tcra^{tm1Mom}$/J (B6.TCR$\alpha^{-/-}$) mice[30] and MHC II-deficient B6.129S2-$H2^{dlAb1\text{-}Ea}$/J (B6.MHCII$^{-/-}$) mice[31] were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). B6.Thy.1.1.TCR$\alpha^{+/-}$ mice were generated by breading B6.Thy1.1 and TCR$\alpha^{-/-}$ mice. All experimental mice were bred and housed in specific pathogen-free conditions at UCSD. All breeding and experiments were performed according to UCSD IACUC-approved protocols and under the supervision of the UCSD Animal Care Program.

2.2 | Competitive LIP

LIP experiments were performed by adoptive transfer of 1:1 ratio of B6.Ly5.1 and B6.Thy.1.1.TCR$\alpha^{+/-}$ peripheral T cells isolated from spleen by negative selection paramagnetic bead enrichment (EasySep Mouse T Cell Isolation Kit, StemCell Technologies, Vancouver, BC, Canada). A total of 2×10^6 CFSE-labeled peripheral T cells were injected intravenously into sublethally irradiated (600 cGy) B6 or B6.MHCII$^{-/-}$ mice (acute lymphopenia) or unmanipulated B6.TCR$\alpha^{-/-}$ mice (chronic lymphopenia). Recipient mice were kept on water containing 5 mg/ml trimethoprim/sulfamethoxazole and monitored for signs of distress by daily observation. Spleens were recovered at 8 and 28 days after transfer and analyzed by flow cytometry.

2.3 | Flow cytometry

Donor cells were identified using antibodies against congenic markers Ly5.1(A20) and Thy1.1 (OX-7). T cell populations were examined using antibodies against CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), CD3ε (145-2C11), CD5 (53.7.3), CD122 (TM-β1), CD127 (A7R34), TCRVα2 (B20.1), and TCRVα3.2 (RR3-16) (Biolegend, San Diego, CA, USA). Samples were run in batches containing both control and experimental samples and color and fluorescence-minus-one controls. Samples were analyzed using FACSCanto or LSR II instruments with FACSDiva software and data analyzed using FlowJo v10 software (BD Biosciences, San Jose, CA, USA). Proliferation index was calculated as the total number of cell divisions divided by calculated number of precursor cells.

2.4 | Measurement of ERK phosphorylation (pERK)

TCR signaling was examined by flow cytometry measuring pERK after *in vitro* stimulation. Single-cell suspensions of peripheral T cells were prepared from spleens and rested for 1–2 h in RPMI 1640 (HyClone, South Logan, UT, USA) supplemented with 10% FBS (Omega Scientific, Trazana, CA, USA) at 37°C. After resting, cells were stimulated using either 100 ng/ml PMA and 1 μg/ml ionomycin (Sigma, Saint louis, MO, USA) for 15 min or by addition of 10 μg/ml biotinylated anti-CD3ε and 10 μg/ml biotinylated anti-CD28 (37.51, Biolegend) for

7 min, followed by addition of 5 μl streptavidin microbeads (Dynabeads M-280, ThermoFisher Scientific, Waltham, MA, USA) and incubation for 10 min. Stimulated cells were fixed by adding 350 μl 5× Lyse/Fix buffer (BD Biosciences) for 15 min at 37°C, permeabilized by addition of 500 μl ice-cold methanol and incubation on ice for 30 min, and then labeled with antibodies against CD3 and phosphorylated ERK1/2 (pERK) (MILAN8R) (eBioscience, San Diego, CA, USA) for 45 min at room temperature.

2.5 | Statistical analysis

Data were analyzed using Prism 6 software (GraphPad, La Jolla, CA, USA). Data from individual mice was compared nonparametrically using Mann-Whitney test. Proliferation indices and mean fluorescence intensity (MFI) values and ratios were compared using Student's *t* test for mean values and nonparametrically using Wilcoxon's rank-sum test. Two-tailed *P* values ≤0.05 were considered significant.

3 | RESULTS AND DISCUSSSION

3.1 | Absence of dual TCR expression causes disadvantage in LIP

Given our previous demonstrations of increased ability of dual TCR cells to recognize autoantigens, particularly ligands driving positive selection,[7] we hypothesized that dual TCR expression may impart an advantage in homeostatic proliferation via increased recognition of self-pMHC ligands. Because homeostasis depends on multiple factors including niche space, the abundance of homeostatic cytokines, and clonal competition for self-pMHC ligands, we utilized a competitive cotransfer model comparing congenically marked wild-type B6.Ly5.1 T cells and B6.TCR$\alpha^{+/-}$.Thy1.1 T cells to examine the effects of dual TCR expression on LIP. A 1:1 mix of CFSE-labelled B6 and TCR$\alpha^{+/-}$ T cells were injected intravenously into recipient mice; sublethally irradiated (600 cGy) B6 mice ($n = 20$) were used as recipients for acute LIP (aLIP) and T cell-deficient B6.TCR$\alpha^{-/-}$ mice ($n = 23$) were used as recipients for chronic LIP (cLIP) in 3 independent experiments. Recipient mice were sacrificed at day 8 and day 28 post-transfer, and frequencies of B6 and TCR$\alpha^{+/-}$ T cells recovered from spleens were determined by flow cytometry (Fig. 1A).

In the aLIP model, CD4$^+$ and CD8$^+$ TCR$\alpha^{+/-}$ cells were consistently found at significantly lower frequencies than their cotransferred B6 counterparts (Fig. 1B). At day 8 after transfer, TCR$\alpha^{+/-}$ CD4$^+$ cells decreased from their pre-transfer 1.00 ratio of TCR$\alpha^{+/-}$ / B6 cells to a ratio of 0.64 ± 0.05 (mean + SEM, $P = 0.002$) and CD8$^+$ cells at a ratio of 0.62 ± 0.10 ($P = 0.002$). This decreased homeostatic competitiveness for TCR$\alpha^{+/-}$ cells was exacerbated at day 28, with the ratio of TCR$\alpha^{+/-}$ / B6 CD4$^+$ cells decreasing to 0.42 ± 0.07 ($P = 0.002$, compared to day 8) and the ratio of CD8$^+$ cells decreasing slightly to 0.58 ± 0.07 (n.s. compared to day 8). The low numbers of transferred cells recovered at both time points precluded examination of recovered B6 cells for dual TCR expression. Results from the cLIP model were less pronounced than in aLIP (Fig. 1C). At day 8 post-transfer, the ratio of TCR$\alpha^{+/-}$ / B6 CD4$^+$ cells recovered was 0.60 ± 0.08 ($P = 0.003$) and at day 28 the ratio was 0.52 ± 0.10 (n.s. compared to day 8). CD8$^+$ T cell ratios were highly variable, and were not significantly different from the input ratio of 1.00 at either day 8 (0.88 ± 0.23) or day 28 (0.62 ± 0.33) after transfer. These results suggest that dual TCR expression has less effect on cLIP than in aLIP, which is known to be highly affected by competition for self-pMHC ligands.

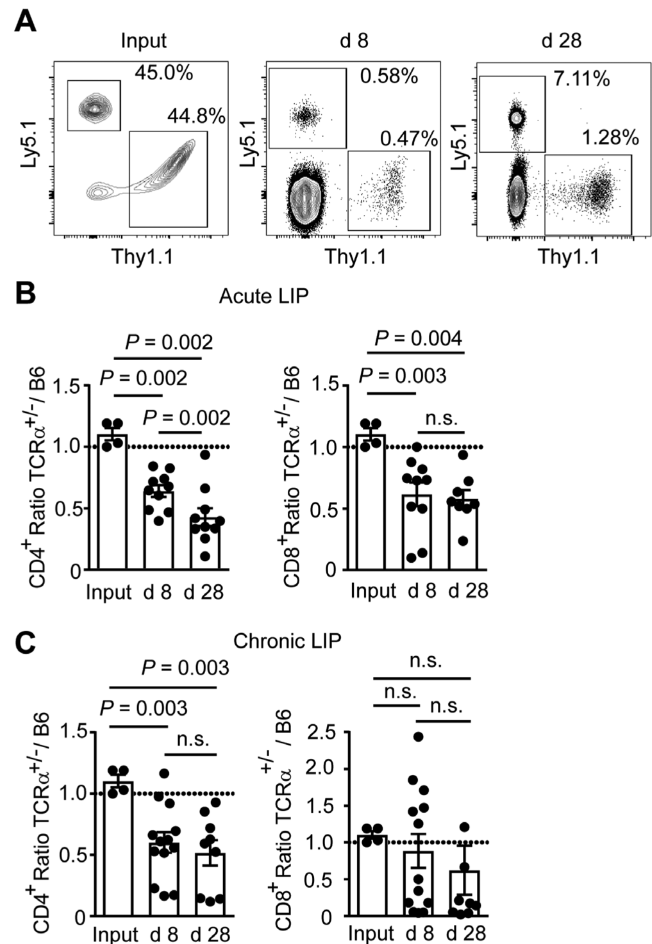

FIGURE 1 Loss of secondary TCR expression reduces homeostatic proliferation in LIP (A) Representative flow cytometry plots showing congenically marked B6.Ly5.1 and B6.TCR$\alpha^{+/-}$. Thy1.1 cells transferred at a 1:1 ratio into sublethally irradiated B6 recipients and recovered at day 8 and day 28 post-transfer. (B) Effects of homeostatic proliferation were assessed by enumerating congenically marked cells by flow cytometry. Ratio of TCR$\alpha^{+/-}$:WT T cells (CD4 or CD8) recovered on day 8 and day 28 post-transfer into sublethally irradiated B6 recipients or (C) chronically T cell-deficient TCR$\alpha^{-/-}$ mice. All data are shown as individual mice from 4 independent experiments. Statistical analyses performed using Mann Whitney rank-sum test

To confirm the role of self-pMHC ligands, we performed competitive aLIP experiments in syngeneic MHC II-deficient mice (B6.MHC$^{-/-}$). The difference in LIP caused by the absence of dual TCR cells was significantly attenuated in MHC$^{-/-}$ recipients (Fig. 2A, ratio TCR$\alpha^{+/-}$/B6 CD4$^+$ T cells 0.88 ± 0.03) compared to wild-type recipients (0.64 ± 0.05, $P < 0.001$). These results indicate that recognition of self-pMHC is the mechanism for differences in LIP attributable to dual TCR expression.

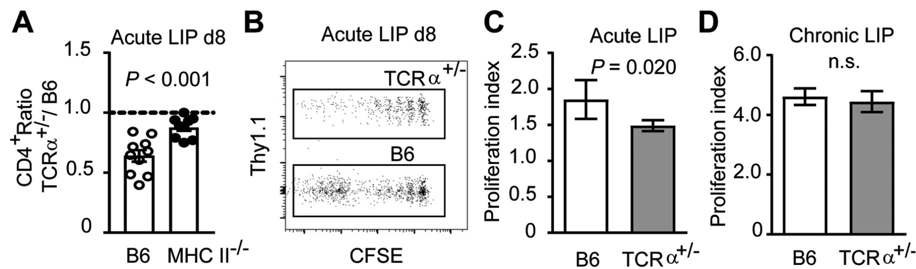

FIGURE 2 Homeostatic advantage for wild-type T cells in lymphopenic conditions is due to increased proliferation in response to endogenous pMHC ligands. (A) Dependence of homeostatic proliferation differences on pMHC was assessed by examining competitive aLIP in sublethally irradiated B6.MHC II$^{-/-}$ recipients. Ratio of TCR$\alpha^{+/-}$:B6 CD4$^+$ T cells from MHC II$^{-/-}$ recipients shown as individual mice from 3 independent experiments. Data from B6 recipient aLIP experiments (as shown in Fig. 1) are presented for comparison. Statistical analyses performed using Mann Whitney rank-sum test. (B) Representative flow cytometry plot of cell proliferation measured by CFSE at day 8 post-transfer into acutely lymphopenic hosts. (C) Difference in LIP is quantified by comparing ratio of TCR$\alpha^{+/-}$ T cell/B6 T cell proliferation index day 8 post-transfer into sublethally irradiated B6 recipients or (D) chronically T cell-deficient TCR$\alpha^{-/-}$ mice. Data are shown as mean ± SEM of TCR$\alpha^{+/-}$ T cell/B6 T cell proliferation index ratio from 10 recipient mice from 3 independent experiments. Statistical analysis performed using Wilcoxon's matched-pairs signed rank test

To confirm that the disadvantage of TCR$\alpha^{+/-}$ T cells in aLIP and cLIP models (Fig. 1) was due to differences in homeostatic proliferation rather than survival, proliferation was assessed by CFSE dilution at day 8 (Fig. 2B). Consistent with the advantage of wild-type T cells in aLIP (Fig. 1B), T cells from B6 mice proliferated significantly more robustly than TCR$\alpha^{+/-}$ T cells (proliferation index 1.85 ± 0.27 divisions/input cell compared to 1.49 ± 0.08 divisions/input cell, $P = 0.020$) in aLIP (Fig. 2C). Conversely, there was no difference in proliferation indices between B6 (4.61 ± 0.28 divisions/input cell) and TCR$\alpha^{+/-}$ T cells (4.44 ± 0.35 divisions/input cell) in cLIP (Fig. 2D), underscoring the limited effect of dual TCR expression in cLIP (Fig. 1C). Unfortunately, the limited number of transferred T cells recoverable combined with technical limitations in identification of dual TCR cells precluded evaluation of whether dual TCR cells among the wild-type B6 T cells proliferated at faster rates or whether they increased in predominance after LIP. Future investigation of this question will require development of novel models for robust and unambiguous differentiation of single and dual TCR T cells and likely require examination of longer time frames for homeostasis to act in shaping the repertoire.

3.2 | Decreased LIP by TCR$\alpha^{+/-}$ cells is not attributable to differences in memory T cell input

We next wanted to affirm that the proliferative capacity was not the result of altered T cell subset composition, such as differences in the frequency of memory T cells which have a reduced total proliferative capacity but may be less dependent on self-pMHC ligand interaction for homeostasis and LIP.[14] To address this, we examined the frequencies of naive (CD44$^-$), effector memory (EM, CD44$^+$CD62L$^-$), and central memory (CM, CD44$^+$CD62L$^+$) CD4$^+$ and CD8$^+$ T cells from the spleens of B6 ($n = 11$) and TCR$\alpha^{+/-}$ ($n = 13$) mice (Fig. 3A). Comparison of B6 and TCR$\alpha^{+/-}$ T cells did not demonstrate significant differences in the frequency of naive CD4$^+$ cells (66.97 ± 8.16% vs. 64.45 ± 8.24%), naive CD8$^+$ cells (80.51 ± 5.28% vs. 78.98 ± 3.68%), EM CD4$^+$ cells (28.79 ± 2.33% vs. 31.84 ± 2.34%), EM CD8$^+$ cells (8.99 ± 1.34% vs. 10.42 ± 1.40%), CM CD4$^+$ cells (4.24 ± 0.47% vs. 3.72 ± 0.45%), or CM CD8$^+$ cells (10.60 ± 1.50%) (Fig. 3B). These results indicate that the decreased LIP of TCR$\alpha^{+/-}$ T cells was not due to characteristics of the transferred population beyond the absence of dual TCR expression.

3.3 | Differences in LIP are not attributable to differences in homeostatic cytokine receptor expression

Amelioration of the difference in aLIP between B6 and TCR$\alpha^{+/-}$ CD4$^+$ T cells in the absence of pMHC II ligands (Fig. 2D) indicates that the ability of TCR to respond to endogenous pMHC ligands is a principle mechanism differentiating homeostatic ability of T cell population lacking dual TCR expression. However, expression of cytokine receptor molecules could also be a cell-intrinsic factor affecting LIP. To examine this, we measured expression of IL-2Rβ (CD122) and IL-7Rα (CD127) by CD4$^+$ and CD8$^+$ T cells from B6 and TCR$\alpha^{+/-}$ mice (Fig. 4). We did not measure any difference in MFI for antibodies labeling CD122 or CD127 on wild-type or TCR$\alpha^{+/-}$ T cells. These data suggest that differential affinity for critical homeostatic cytokines IL-2, IL-7, or IL-15 is not a factor underlying the decreased LIP in T cell populations lacking dual TCR expression.

3.4 | Differences in LIP are not attributable to differences in total TCR expression or TCR signaling

We next wanted to rule out the possibility that variations in the amount of total TCR on the cell surface caused by TCRα heterozygosity could alter the ability to respond to self-pMHC ligands affecting homeostasis and LIP. Measurement of CD3 expression on T cells from B6 or TCR$\alpha^{+/-}$ mice by flow cytometry indicated that T cells from B6 ($n = 9$) or TCR$\alpha^{+/-}$ ($n = 10$) mice express the same amount of total TCR on the cell surface (Fig. 5A). To account for variability between experiments, we calculated the ratio of CD3 MFI for TCR$\alpha^{+/-}$ T cells / B6 T cells for each experiment (Fig. 5B), confirming an absence of

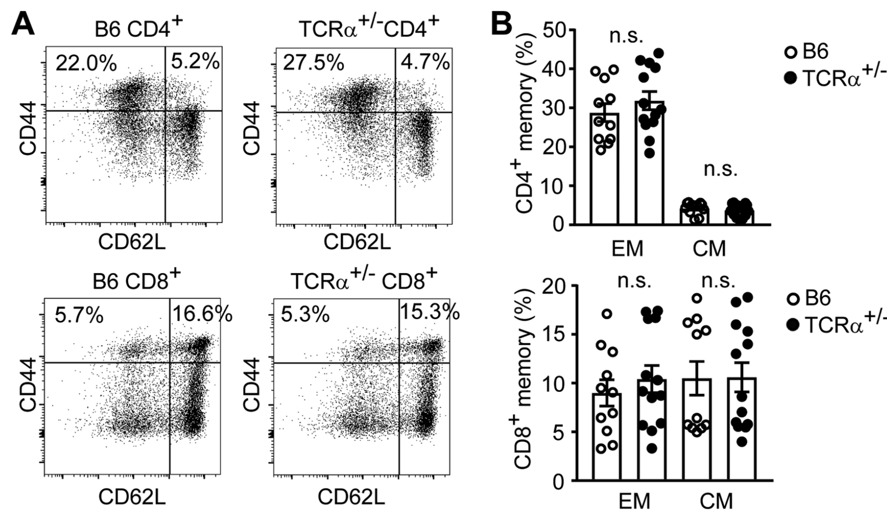

FIGURE 3 **Absence of dual TCR expression does not affect naive or memory T cell populations**. (A) representative flow cytometry plots of naive (CD44⁻), effector memory (EM, CD44⁺CD62L⁻), and central memory (CM, CD44⁺CD62L⁺) CD4⁺ and CD8⁺ T cells from B6 and TCR$\alpha^{+/-}$ mice. (B) Comparison of EM and CM populations from B6 and TCR$\alpha^{+/-}$ mice. Data are shown as individual mice from 3 independent experiments. Statistical analysis performed using Mann Whitney rank-sum test

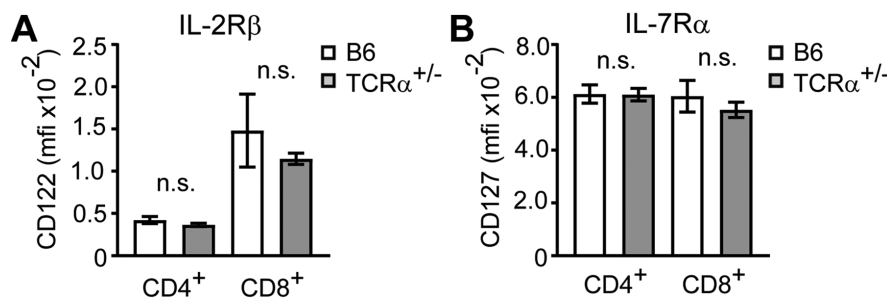

FIGURE 4 **Expression of receptors for homeostatic cytokines does not differ between B6 and TCR$\alpha^{+/-}$ T cells**. Expression of (A) IL-2Rβ (CD122) and (B) IL-7Rα (CD127) was measured on CD4⁺ and CD8⁺ T cells from B6 (n = 6) and TCR$\alpha^{+/-}$ (n = 6) mice in 3 independent experiments. Data shown as mean ± SEM. Groups compared by MFI using Student's t test

effect on total TCR expression by TCRα heterozygosity (1.05 ± 0.07 TCR$\alpha^{+/-}$ / B6 CD3 MFI). Similarly, examination of CD3 expression by TCRVα2⁺Vα3⁺ dual TCR cells (Fig. 5C) from B6 mice did not demonstrate any differences when compared to TCRVα2⁺ or TCRVα3⁺ T cells from B6 mice (ratio CD3 MFI dual TCR/Vα2⁺ cells 0.99 ± 0.06) (Fig. 5D and E).

Next, we examined pERK in B6 and TCR$\alpha^{+/-}$ T cells as a measure of TCR signaling capability. pERK was measured by flow cytometry in T cells from B6 (n = 8) and TCR$\alpha^{+/-}$ (n = 9) mice after *in vitro* stimulation with PMA and ionomycin or anti-CD3 and anti-CD28 attached to microbeads (Fig. 5F). Similar to our previous results using plate-bound anti-CD3/anti-CD28 stimulation for T cell activation,[9] there was no difference in the percentage of B6 or TCR$\alpha^{+/-}$ T cells responding (36.41 ± 1.74% pERK⁺ in B6 vs. 39.88 ± 3.31% in TCR$\alpha^{+/-}$ T cells responding to PMA/ionoycin; 16.20 ± 1.01% pERK+ B6 vs. 14.57 ± 3.8% TCR$\alpha^{+/-}$ T cells responding to anti-CD3/anti-CD28, n.s.) (Fig. 5G). The amount of pERK in each cell was measured by analysis of pERK MFI, to discern whether differences in signal strength existed between B6 and TCR$\alpha^{+/-}$ T cells (Fig. 5H). T cells from B6 and TCR$\alpha^{+/-}$ mice did not demonstrate differences in MFI of pERK⁺ cells when stimulated with either PMA/ionomycin (B6 1357 ± 101.5 MFI vs. TCR$\alpha^{+/-}$ 1348 ± 95.47 MFI, n.s.) or anti-CD3/anti-CD28 (B6 1532 ± 36.51 MFI vs. TCR$\alpha^{+/-}$ 1470 ± 25.74 MFI, n.s.). Together, we interpret these results, in conjunction with our previous examinations of TCR expression and responsiveness to nonspecific stimulation, as indicative that dual TCR T cell reactivity is not attributable to increased amounts of TCR present on the cell surface or an inherent hyperresponsiveness to stimulation. Additionally, the specific deficits observed in LIP by TCR$\alpha^{+/-}$ T cells cannot be attributed to decreased TCR availability for stimulation or general hyporesponsiveness to stimulation.

3.5 | Dual TCR cells have increased CD5 expression consistent with increased affinity for self-pMHC

The lack of an observable in TCR expression or signaling, cytokine receptor expression, or cellular subset composition implies that receptor specificity for the endogenous self-pMHC ligands driving aLIP is the most likely mechanistic explanation for decreased LIP in the absence of dual TCR cells (Figs. 1 and 2). As a measure of differences in affinity for self-pMHC ligands, we examined expression of CD5 on CD4⁺ and CD8⁺ T cells from B6 and TCR$\alpha^{+/-}$ mice (Fig. 6A). The expression level of CD5 is determined during positive selection based on TCR affinity for selecting self-pMHC,[19,20] and high

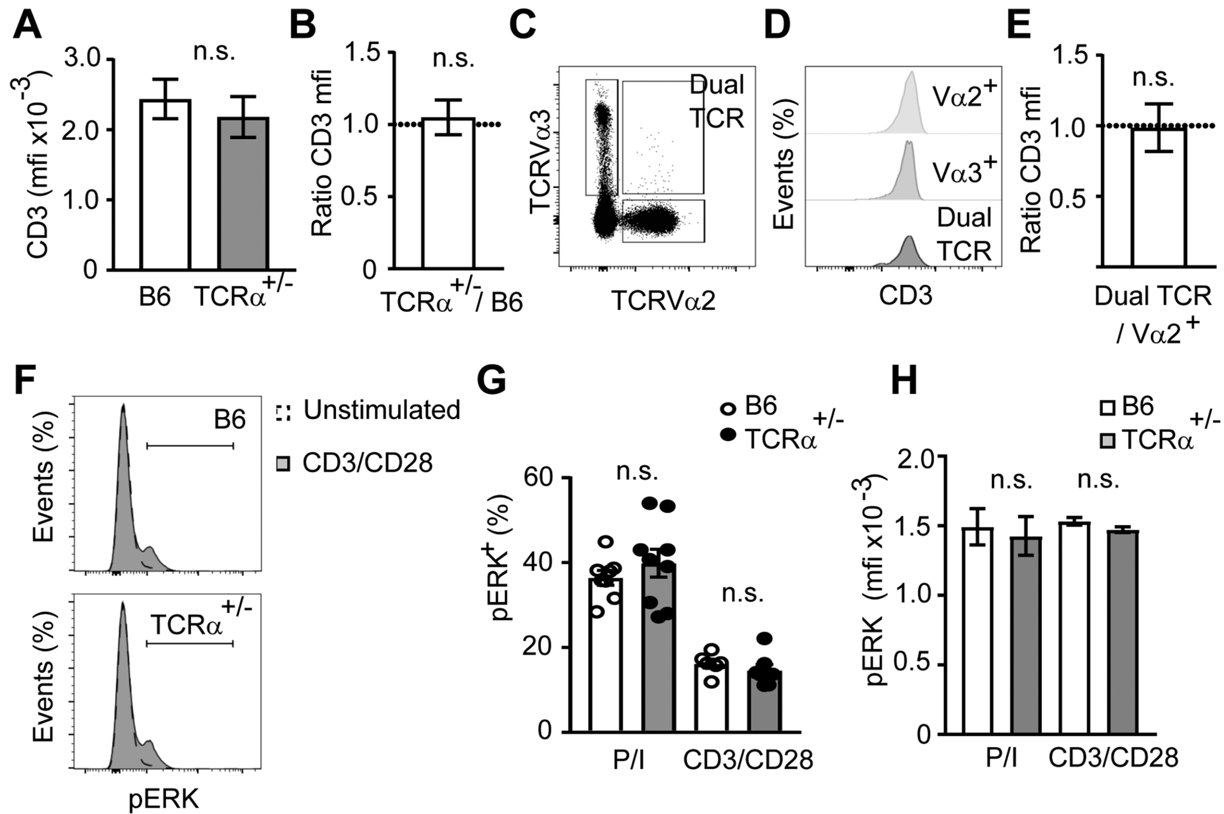

FIGURE 5 **Neither elimination of secondary TCRs nor co-expression of dual TCRs alters total TCR expression**. (A) Quantification of CD3 expression by mean fluorescence intensity (MFI) on B6 ($n = 10$) or TCR$\alpha^{+/-}$ ($n = 10$) T cells. Data are mean ± SEM from 3 independent experiments. Statistical analysis performed using Student's *t* test. (B) CD3 expression across three independent experiments ($n = 10$ mice) was compared by analysis of ratio CD3 MFI for TCR$\alpha^{+/-}$/B6 T cells for each experiment. Data shown as mean ± SEM. Statistical analysis performed using Wilcoxon's matched-pairs signed rank test. (C) Representative flow cytometry plot showing identification of TCRVα2$^+$, TCRVα3$^+$, and TCRVα2$^+$Vα3$^+$ dual TCR cells from B6 T splenocytes. (D) Representative flow cytometry histogram comparing expression of CD3 as a measure of total TCR complex in TCRVα2$^+$, TCRVα3$^+$, and TCRVα2$^+$Vα3$^+$ dual TCR cells (E) CD3 expression across three independent experiments ($n = 9$ mice) was compared by analysis of ratio CD3 MFI for TCRVα2$^+$Vα3$^+$/TCRVα2$^+$ T cells for individual mice. Data shown as mean ± SEM. Statistical analysis performed using Wilcoxon's matched-pairs signed rank test. (F) TCR signaling capacity was assessed by measuring ERK1/2 phosphorylation 15 min after stimulation with PMA and ionomycin or 10 min after stimulation with anti-CD3 and anti-CD28 bound to microsspheres. Representative flow cytometry histogram comparing phosphorylation of ERK1/2 in B6 or TCR$\alpha^{+/-}$ T cells. (G) Percentage of B6 and TCR$\alpha^{+/-}$ T cells expressing pERK. Data are individual mice from representative experiment of 3 independent experiments for each condition. Statistical analysis performed using Mann Whitney rank-sum test. (H) Quantification of ERK phosphorylation by mean fluorescence intensity (MFI) on B6 or TCR$\alpha^{+/-}$ T cells. Data are mean ± SEM from all experiments ($n = 9$–10 mice/group). Statistical analysis performed using Student's *t* test

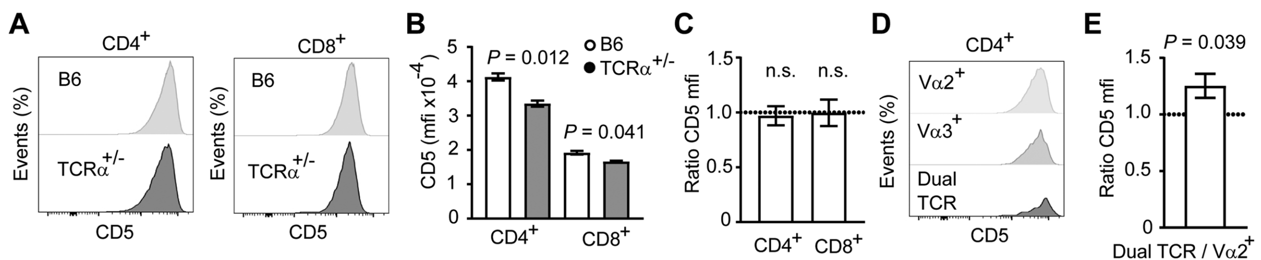

FIGURE 6 **Dual TCR cells have higher expression of CD5, indicating increased affinity for self-antigens**. (A) Representative flow cytometry histogram comparing CD5 expression by CD4$^+$ and CD8$^+$ T cells from B6 or TCR$\alpha^{+/-}$ mice. (B) Quantification of CD5 expression by mean fluorescence intensity (MFI) on B6 and TCR$\alpha^{+/-}$ T cells. Data are individual mice from representative experiment of 3 independent experiments. Statistical analysis performed using Student's *t* test. (C) CD5 expression across three independent experiments ($n = 10$ mice) was compared by analysis of ratio CD5 MFI for TCR$\alpha^{+/-}$/B6 T cells for each experiment. Data shown as mean ± SEM. Statistical analysis performed using Wilcoxon's matched-pairs signed rank test. (D) Representative flow cytometry histogram comparing expression of CD5 as a measure of total TCR complex in TCRVα2$^+$, TCRVα3$^+$, and TCRVα2$^+$Vα3$^+$ dual TCR cells (E) CD5 expression across three independent experiments ($n = 11$ mice) was compared by analysis of ratio CD5 MFI for TCRVα2$^+$Vα3$^+$/TCRVα2$^+$ T cells for individual mice. Data shown as mean ± SEM. Statistical analysis performed using Wilcoxon's matched-pairs signed rank test

CD5 expression is correlated with increased potential for homeostatic proliferation.[18,21–23] Within individual experiments, CD4$^+$ and CD8$^+$ T cells from TCR$\alpha^{+/-}$ mice (n = 11) demonstrated a trend of decreased CD5 expression compared to wild-type B6 mice (n = 10) mice (Fig. 6B). However, significant differences in CD5 expression by CD4$^+$ and CD8$^+$ cells were not observed across experiments (0.97 ± 0.05 TCR$\alpha^{+/-}$ / B6 CD4$^+$ CD5 MFI, 1.00 ± 0.07 TCR$\alpha^{+/-}$ / B6 CD8$^+$ CD5 MFI) (Fig. 6C).

The absence of a significant measurable difference in CD5 expression across the entire T cell population does not preclude a difference specific to dual TCR cells; the relatively low frequency (~10%) of dual TCR cells may obfuscate differences specific to that population when measured as part of the whole population. Hence, we examined CD5 expression on TCRVα2$^+$Vα3$^+$ dual TCR CD4$^+$ cells as compared to TCRVα2$^+$ or TCRVα3$^+$ CD4$^+$ T cells from B6 mice (n = 11) (Fig. 6D). The lower numbers of CD8$^+$ T cells as compared to CD4$^+$ cells precluded robust examination of CD5 expression by dual TCR CD8$^+$ cells. Dual TCR T cells demonstrated increased expression of CD5 across experiments (ratio CD5 MFI dual TCR/Vα2$^+$ cells 1.25 ± 0.11, P = 0.039) (Fig. 6E). Although increased expression of CD5 does not definitively demonstrate increased affinity for self-pMHC ligands, this data in conjunction with our previous demonstration that dual TCR expression promotes interaction with similar low-affinity self-pMHC ligands driving positive selection,[7] supports our hypothesis for the mechanism responsible for increased aLIP in the presence of dual TCR cells.

Previous examinations of mice genetically deficient for dual TCR expression have not identified overt T cell compartment defects,[7,9,32,33] indicating that dual TCR expression is not absolutely required for T cell development, homeostasis, or function. However, this does not preclude important effects of dual TCR expression on the development, composition, or function of the T cell repertoire. The data presented here identify a novel mechanism, preferential LIP, through which dual TCR cells can shape the peripheral T cell repertoire. Promotion of aLIP by dual TCR expression is consistent with our previous demonstration of the increased ability of dual TCR cells to respond to endogenous self-pMHC ligands.[7] The effect of dual TCR expression on was less expected, as though cLIP is also dependent upon pMHC interaction, it has been attributed to TCR recognition of endogenous commensal bacterial antigens rather than self-antigens,[18,34,35] and previous work from our lab has not observed differences in the ability to recognize foreign antigen between dual TCR cells and T cells lacking dual TCR expression.[7,35] However, we have observed increased cross-reactivity of dual TCR cells for presented peptide antigens, which may explain this effect.[7] Studies examining the role of dual TCR cells in commensal antigens, particularly given the importance of endogenous commensal organism antigens in establishing and maintaining immunologic tolerance[36] in the gut may be important for understanding the loss of tolerance in disease states. In both aLIP and cLIP, the driving force promoting LIP by dual TCR cells may promote unwanted heterologous or autoreactive responses. This may be particularly important in settings where homeostatic proliferation is a primary force shaping the T cell repertoire, such as in aging or iatrogenic lymphopenia in transplantation.

AUTHORSHIP

A.B. was responsible for the experimental design, performed experiments, analyzed data and wrote this paper. B.J. performed experiments and analyzed data. G.P.M. was responsible for the experimental design, analyzed data, and wrote this paper.

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DISCLOSURES

The authors declare conflicts of interests.

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