UNIVERSITY OF CALIFORNIA SAN DIEGO

The role of dual receptor T cells in lymphocyte reconstitution and development of chronic graft versus host disease post-hematopoietic stem cell transplant.

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requirements for the degree Doctor of Philosophy

in

Biology

by

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DEDICATION

For my father, the wind beneath my wings.

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LIST OF ABBREVIATIONS

TCR T cell receptor

MHC Major histocompatibility complex

HSCT Hematopoietic stem cell transplant

aGVHD acute graft versus host disease

cGVHD chronic graft versus host disease

DN double negative

DP double positive

RAG recombinase activating gene

NOD non-obese diabetic

MOG myelin oligodendrocyte glycoprotein

LCMV lymphocytic choriomeningitis virus

MBP myelin basic protein

CDR3 complementarity determining region 3

NP nucleoprotein

Treg T regulatory cell

TCRα T cell receptor alpha

TCRβ T cell receptor beta

BMT bone marrow transplant

aLIP acute lymphopenia induced proliferation

cLIP chronic lymphopenia induced proliferation

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ABSTRACT OF THE DISSERTATION

The role of dual receptor T cells in lymphocyte reconstitution and development of chronic graft versus host disease post-hematopoietic stem cell transplant

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T cells that express 2 T cell receptors (TCRs), generated by productive rearrangement of both TCRα loci which pair with a single TCRβ, comprise ~10 percent of peripheral T cells in mice and humans. However, these dual receptor cells contribute 30-50% of the auto- and

alloreactive T cell repertoires in naive mice and to acute graft versus host disease in patients post-transplant. The disproportionate contribution of dual TCR T cells to these processes suggests that they may contain a repertoire of TCRs with atypical reactivities. Our laboratory focuses on dual TCR T cells as a naturally-existing model to investigate thymic selection and T cell repertoire reactivity. In this work we investigate the highly alloreactive nature of dual TCR T cells by studying chronic graft versus host disease as a model linking thymic development to peripheral reactivity and disease pathology. We show that increased thymic production of dual TCR cells due to ineffective thymic selection results in an altered peripheral repertoire comprised of cells that are reactive to self. Patients with chronic graft versus host disease have a significantly increased and activated population of dual TCR cells that display a proinflammatory phenotype, as determined by targeted RNA sequencing. Using a mouse model of bone marrow transplant we were able to determine that defective negative selection due to thymic damage inflicted by pre-transplant conditioning was the cause of the increased dual TCR production. Lastly, we demonstrate a competitive advantage for dual TCR cells in lymphopenic conditions driven by their high affinity for self-peptide:MHC.

Chapter 1: The highly alloreactive nature of dual receptor T cells.

1.1 Introduction

T lymphocytes are essential mediators of adaptive immunity. The T cell receptor (TCR) controls T cell activation through recognition of specific linear peptides presented by MHC molecules [1]. It is understood that TCR function is constrained by three fundamental rules of T cell biology; that each lymphocyte bears a single receptor that imparts antigen specificity [2], that a TCR recognizes a specific linear peptide presented by MHC [3], and that TCRs are restricted to recognizing antigens presented by self-MHC as a consequence of thymic selection [4,5]. However, studies over the past several decades have revealed that T cells do not always adhere to dogma. Alloreactivity, the ability of T cells to respond to allogeneic MHC, is a well-established phenomenon with clinical implications for transplantation [6]. There is significant evidence for T cell cross-reactivity [7]. And the existence of T cells expressing two TCRs with different specificities has been known for over two decades [8].

This review will examine work investigating the development and function of dual TCR T cells. Recent studies have provided evidence for the necessity as well as deleterious consequences of dual TCR expression. The specific predisposition of dual TCR T cells for alloreactivity, and the resulting contribution to pathology, specifically graft versus host disease (GVHD) following allogeneic hematopoietic stem cell transplantation (HSCT) will be reviewed. This review will highlight the unique opportunities presented for examining post-transplant immune responses and understanding fundamental T cell biology through study of these cells.

1.2 Discovery of T cells expressing two receptors

Defining the rules of T cell antigen recognition provided a perspective that the identity of a T cell is inextricably tied to the antigen it recognizes. This view was complicated by description of dual specificity T cell clones responding to allogeneic cells *in vitro* as well as to the immunizing antigen presented by autologous cells [9,10]. This heterologous immunity suggested these cells were either cross-reactive or expressed a second receptor imparting a secondary specificity. One such clone, A10, did indeed have two in-frame rearrangements of TCR□, along with a single in-frame TCRα rearrangement [11]. Both TCRα proteins were detected in the cell, providing potential for expression of two αβTCR heterodimers. However, only a single αβTCR enabled response to ovalbumin:I-A^k and I-A^s by transfected TCR-deficient T cell hybrids, indicating that a singular cross-reactive TCR was responsible for both antigenic specificities of the clone. The presence of in-frame rearrangement of both TCRα loci was similarly observed in approximately one-third of peripheral T cells in other examinations of antigen-specific mouse T cell clones [12-14]. Similar estimates have recently been provided using large-scale DNA sequencing approaches to examine human TCR repertoires [15,16]. These data provide evidence of the potential for co-expression of two TCRs by a single T cell, and suggest that allelic inclusion of TCRα is a physiologically normal process.

The first evidence for the functionality of naturally co-expressed TCRs came from examination of TCRVα expression on human peripheral blood lymphocytes [8]. Approximately 10^{-3}–10^{-4} peripheral blood T cells exhibited coincident labeling with two anti-TCRVα (TCRVα2, TCRVα12, or TCRVα24) monoclonal antibodies (mAbs). Based upon the frequencies of labeling with each of the TCRVα mAbs individually, it could be estimated that between 1-10% of human peripheral T cells expressed two TCRs on their surface. Critically, the functionality of

both receptors on dual TCR clones was demonstrated by response to stimulation via mAbs against either TCR. Subsequent investigations in mice found similar frequencies of T cells expressing two TCRαβ chains on the cell surface [17,18], and revealed the existence of T cells expressing two TCRα chains, though at much lower frequencies (< 1%) [19-21]. Together, these data provide evidence that the one cell, one receptor rule is not absolute, but instead that a small population of T cells expressing two functional TCRs exists under normal physiological conditions.

1.3 Dual TCR T cells arise as a consequence of normal thymopoiesis

T cell development is a multistep process whereby hematopoietic progenitor cells respond to instructive cues directing their differentiation in the thymus [22]. The principle task for developing thymocytes is to generate a functional TCR that will enable them to be positively selected, avoid negative selection, and egress to the periphery [1]. This process (Figure 1.1) is begun in $CD4^-CD8^-$ double-negative (DN) thymocytes, where the RAG genes act to recombine germline-encoded TCRβ variable (V), diversity (D), and joining (J) gene segments to form a functional TCRβ protein. Typically, only one TCRβ locus is in an open chromatin state to enable recombination, ensuring allelic exclusion [23-25]. After functionality of the TCRβ chain is ensured via β-selection, the thymocyte proliferates and progresses to the $CD4^+CD8^+$ double-positive (DP) stage. DP thymocytes recombine the TCRα V and J segments to produce a TCRα protein.

Functionality of the αβTCR heterodimer is tested in DP thymocytes by the process of positive selection, which requires recognition of specific self-peptide:MHC (pMHC) ligands for promotion to $CD4^+$ or $CD8^+$ T cells. The kinetics of this process are not entirely defined, though it is known that a majority of DP thymocytes do not receive a positively selecting signal and die

over a period of days [26]. Presumably to improve the efficiency of this process, TCRα gene rearrangement is not subject to the allelic exclusion observed for TCRβ [27]. Both TCRα chromosomal loci are in an open, accessible state in DP thymocytes [28], and RAG gene expression continues until the thymocyte receives a positively selecting signal [29] enabling iterative recombination [30,31]. These mechanisms provide multiple chances for producing a TCRα capable of paring with the TCRβ and mediating positive selection.

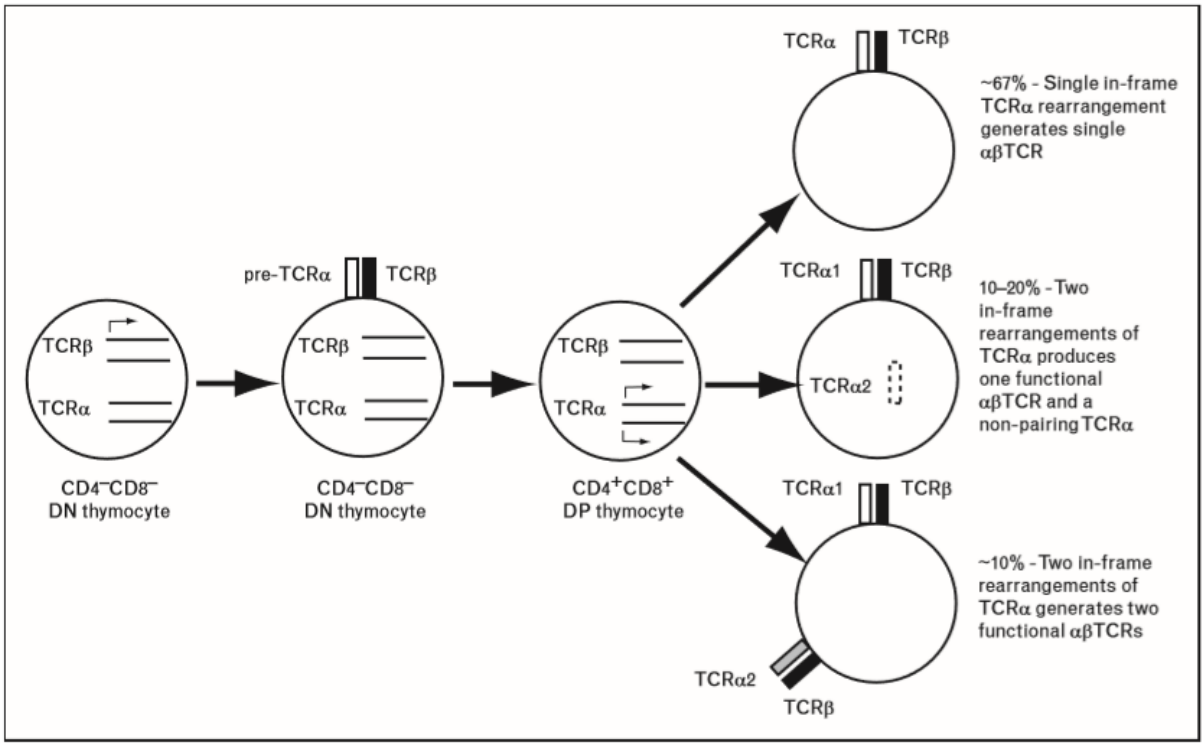

Figure 1.1 Dual TCR T cells arise as a consequence of normal thymopoiesis. Developing thymocytes generate αβTCRs by rearrangement of germline-encoded V, D, and J gene segments. DN thymocytes typically rearrange TCRβ genes on only one chromosome, ensuring strict allelic exclusion and generation of a single TCRβ protein. Conversely, DP thymocytes rearrange both TCRα loci simultaneously and iteratively to maximize generation of an αβTCR capable of mediating thymic selection. TCRα allelic inclusion results in a majority of mature T cells expressing a single TCR and a small fraction expressing two functional TCRs.

Consequences of TCR alleleic inclusion were first evidenced in transgenic TCR systems. While the presence of a transgenic TCRβ results in few endogenous TCRβ gene rearrangements, endogenous TCRα rearrangements are relatively common [23,27,32], highlighting differences between TCRα and TCRβ regulation. In the setting of transgenic TCRs and non-selecting MHC, endogenous TCR rearrangements are observed at increased frequencies and required for development of mature T cells [30,32-34], demonstrating the importance of allelic inclusion. This also affects normal thymopoiesis, as thymocytes hemizygous for TCRα ($TCR\alpha^{+/-}$) are developmentally impaired [35]. $TCR\alpha^{+/-}$ mice have normal numbers of DN and total DP thymocytes, but exhibit a 41% decrease in post-positive selection $CD3^{high}$ DP thymocytes. This decrease is attributable to decreased positive selection, as $TCR\alpha^{+/-}$ DP thymocytes progressed to $CD3^{high}$ at approximately half the rate of wild-type thymocytes in pulse-chase and competitive intra-thymic transfer assays.

While TCRα allelic inclusion is beneficial for improving the efficiency of positive selection, it also presents a hazard by enabling escape of thymocytes bearing autoreactive TCRs from negative selection. Studies of transgenic autoreactive TCRs have demonstrated that the co-expression of endogenous secondary TCRα chains can result in down-regulation of the transgenic TCR sufficient for escape from negative selection, but retaining functional autoreactivity in the periphery [33,36-38]. Presumably this occurs due to competition for the fixed amount of CD3 components of the TCR complex [8]. A similar phenomenon was observed with incomplete TCRα allelic exclusion for the KRN transgenic TCR [39].

Effects of secondary TCRs on selection of the overall TCR repertoire have been more difficult to define. $TCR\alpha^{+/-}$ mice retain capability to develop autoimmunity in models of experimental autoimmune encephalitis, lupus [40] and collagen-induced arthritis [41], indicating

that dual TCR T cells do not contain the entirety of the autoreactive repertoire. In contrast, the same study found that in NOD mice challenged with cyclophosphamide, insulitis and diabetes were reduced by 74% and 100% respectively in the absence of dual TCR T cells. This difference is noteworthy, as this model did not require administration of adjuvant, and suggests that the effect may relate to the underlying autoreactive T cell repertoire. Recent examination of the naive dual TCR T cell repertoire demonstrated that the absence of secondary TCRs in TCR$\alpha^{+/-}$ mice reduced the frequency of T cells recognizing the autoantigen MOG_{38-49}:I-A^b by 70% while not affecting response to a foreign antigen, $LCMV_{66-77}$:I-A^b [35]. The autoimmune potential of secondary TCRs is underscored by a recent examination of a model of aerosolized ovalbumin-induced airway inflammation where the transgenic DO11.10 TCR mediated thymic selection and responded to the immunogen, but endogenous TCRs were required for autoimmunity [42]. These results highlight the capability of naturally-arising dual TCR T cells to contain unwanted reactivity that would otherwise be removed by negative selection (Figure 1.2).

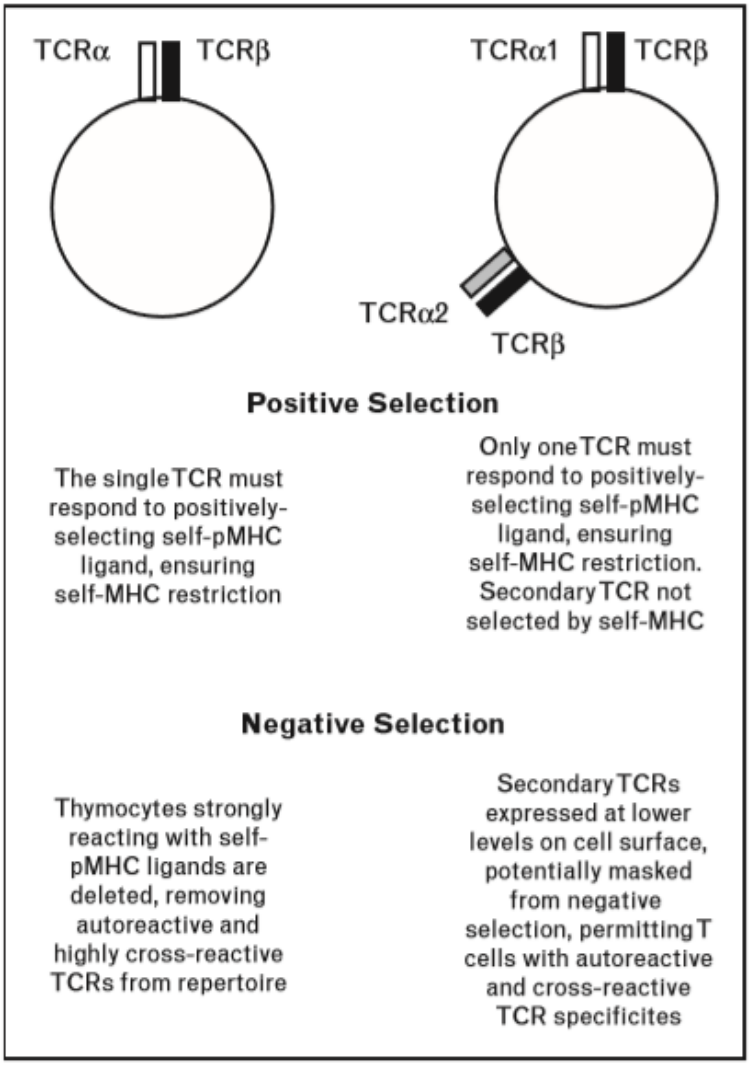

Figure 1.2 Effects of secondary TCRs on thymic selection and repertoire formation. T cells expressing a single TCR rely on that TCR to mediate thymic selection. Positive selection ensures self-MHC restriction, while negative selection eliminates thymocytes bearing TCRs with unwanted autoreactive or highly cross-reactive specificities. Secondary TCRs can rely on the function of the primary TCR and are thus not subject to stringent thymic selection. This may have an important relationship with the atypical reactivity of dual TCR T cells in the periphery.

1.4 Secondary TCRs expand the antigenic repertoire of peripheral T cells

The most obvious evidence for secondary TCRs expanding the antigenic repertoire comes from transgenic TCR systems, where immunization with foreign antigens generates T cell clones bearing the transgenic TCR responding to its antigen, as well as an endogenous TCR responding to the immunogen [33,43,44]. The pathogenic potential for heterologous immunity by dual TCR T cells was shown in system where the transgenic TCR recognizing MBP_{79-87}:H2-K^k was only capable of mediating encephalitis during viral infection after activation mediated by endogenous secondary TCRs responding to viral antigens [38]. At the repertoire level, the effects of dual TCR expression on antigenic recognition are more difficult to observe. The absence of secondary TCR rearrangements in TCR$\alpha^{+/-}$ mice does not affect use of V or J gene segments or general composition of the CDR3 [35]. However, high-throughput TCRα sequencing revealed the presence of high-frequency TCRα clonotypes among dual TCR T cells that were conspicuously absent in the TCR$\alpha^{+/-}$ population. This suggests that dual TCR T cells may contain unique antigenic specificities. Assessment of antigen recognition in naive mice using fluorophore-labeled peptide:MHC tetramers did not demonstrate differences between wild-type and TCR$\alpha^{+/-}$ T cells in recognition of a foreign antigen presented by self-MHC ($LCMV_{66-77}$:I-A^b), but TCR$\alpha^{+/-}$ T cells had a 70% reduction in recognition of autoantigen (MOG_{38-49}:I-A^b) and 49% and 57% reductions in response to MHC-mismatched alloantigens ($CD22_{654-666}$:I-E^k and $TFR_{231-244}$:I-E^k respectively). These differences in antigen-reactive T cell frequency could affect pathogenesis of autoimmune and alloreactive responses.

1.5 Dual TCR T cells in alloreactivity

A contribution of secondary TCRs to pathologic alloreactivity was first evidenced by examining rejection of allogeneic skin grafts in F5 TCR transgenic mice (influenza $NP_{366-374}$:H2-

D^b) [45]. T cells isolated from rejected grafts expressed endogenous TCRα and TCRβ, which were necessary for rejection, as F5.RAG1$^{-/-}$ T cells were not alloreactive. Expansion of the alloreactive repertoire in a naturally-occurring dual TCR T cell was evidenced by the 2.102 T cell clone [46]. The 2.102 clone contains two TCRα chains (Vα2 and Vα4) and a single TCRβ (Vβ1). The Vα4Vβ1 TCR mediates thymic selection, is reactive to a foreign protein hemoglobin$_{64\text{-}76}$:I-E^k and alloreactive to I-E^p, while the secondary Vα2Vβ1 TCR does not mediate positive selection but enables alloreactivity to H-2^d [47].

Increased alloreactivity at the clonal level from expression of a secondary TCR is intuitive, as expression of a second receptor doubles the chances for antigen recognition. Since dual TCR T cells comprise only a small portion of the peripheral T cell repertoire, it could be expected that they may only have a minor contribution to polyclonal alloreactive responses. However, genetic elimination of secondary TCRs reduced *in vitro* alloreactive responses to MHC-mismatched cells by over 40% [47]. This disproportionate effect from the loss of the relatively small numbers of TCRs suggests that dual TCR T cells have an inherent predisposition for responding to allogeneic MHC. Indeed, the same study demonstrated a 2-fold preferential expansion of dual TCR T cells by *in vitro* allogeneic stimulation as compared to non-specific stimulation via anti-CD3 and anti-CD28 mAbs, and a similar effect was observed in human dual TCR T cells [48]. This selective effect of secondary TCRs on alloreactivity is underscored by subsequent studies demonstrating their disproportionate contribution to the naive T cell repertoire recognizing specific allogeneic MHC ligands, but not foreign peptides presented by self-MHC [35]. The effect of secondary TCRs on recognition of minor histocompatibility antigens (miHA) is less clear, as mixed results have been observed in mice and humans. Genetic elimination of secondary TCRs had no effect on the naive T cell response to miHA Eα$_{52\text{-}68}$:I-A^b

[35], while human dual TCR T cells were found to respond to the miHA HA-$1^{H}_{137\text{-}145}$:HLA-A*02:01 at frequencies 10-fold higher than unsorted peripheral blood T cells [48]. The reasons for the differences between these findings are unclear.

Importantly, alloreactivity imparted by secondary TCRs translates to a disproportionate contribution of dual TCR T cells to *in vivo* pathologic alloreactive responses. Using a parent-to-F_1 MHC-mismatched model of transplantation, dual TCR T cells were calculated to comprise as much as 75% of peripheral T cells in mice with severe acute GVHD, compared with 1-6% of T cells in control mice 4 weeks following syngeneic transplantation [47]. Expansion of dual TCR T cells begins with the earliest phases of GVHD, as wild-type T cells exhibit 170% of the proliferative capacity of $TCR\alpha^{+/-}$ T cells in the first 24 hours after transfer to a MHC-mismatched recipient [35]. Investigations in human HSCT patients demonstrated similar results. Dual TCR T cells were present in patients with symptomatic acute GVHD at frequencies 5.3-fold higher than in healthy controls, comprising 58.6$\pm$6.4% of peripheral T cells [48]. In these patients, dual TCR T cells were activated and produced significantly more IFN-γ and IL-17a than single TCR T cells, demonstrating their pathogenic capability. This effect was dependent on donor T cells, and was more pronounced in patients receiving HLA-mismatched grafts. Dual TCR T cell expansion and activation was not observed in allogeneic HSCT patients not developing acute GVHD. Together these data demonstrate the pathologic potential for dual TCR T cells to selectively and disproportionately influence pathologic alloreactivity.

1.6 Conclusions

The potential for dual TCR T cells to contain unwanted reactivity has been theorized since their discovery. Recent demonstration of the importance of TCRα allelic inclusion for efficient thymic selection indicates secondary TCRα generation is a normal physiologic process.

However, identification of the autoimmune and alloimmune potential of dual TCR T cells indicates a cost. Knowing this, the questions now become what is the underlying mechanistic basis for the reactivity of dual TCR T cells, how do secondary TCRs affect the antigenic repertoire, and what are the consequences or opportunities related to clinical transplantation?

The most simplistic explanation for increased autoreactivity and alloreactivity by dual TCR T cells is that a second TCR provides a second opportunity for antigen recognition at the clonal level. Two important points of evidence suggest that this is not a sufficient explanation. First, dual TCR T cells are selectively over-represented in alloreactive responses, a phenomenon not observed in responses to other antigens. Secondly, genetic elimination of secondary TCRα rearrangements in TCRα$^{+/-}$ mice eliminates only a small fraction of the TCR repertoire, but results in dramatic and disproportionate reductions in alloreactivity without affecting responses to foreign antigens. It is likely that the ability of secondary TCRs to avoid negative selection, as evidenced in transgenic TCR systems, results in dual TCR T cell clones containing autoreactive or highly cross-reactive TCRs that would otherwise be eliminated. While the relationship between thymic selection and TCR reactivity in dual TCR T cells is undefined, preliminary efforts have demonstrated that secondary TCRs may contribute to flexibility in the recognition of allogeneic ligands; alloantigen-specific T cells from TCRα$^{+/-}$ mice had no ability to recognize closely-related altered peptide ligands *in vitro*, unlike wild-type alloantigen-specific T cells [35]. This result is in line with evidence of the importance of negative selection in eliminating cross-reactive TCRs [49], though more work is required to define this relationship.

Given the effects of secondary TCR expression in thymic selection, it is logical to question their effect on the peripheral T cell repertoire. Instructive signals given to developing thymocytes through the TCR have important effects including CD4/CD8 lineage commitment,

regulatory T cell (Treg) differentiation, and homeostatic capability [50]. Effects of secondary TCRs on these processes are not known, though it has been reported that human Tregs contain a 3-fold higher proportion of dual TCR T cells [51]. It is not known whether these were thymically-derived Tregs or cells peripherally converted to the Treg phenotype. However, this suggests that the increased reactivity of dual TCR T cells may make them effective regulators of immune responses.

While the effects of dual TCR T cells in transplantation observed thus far have been deleterious, it is conceivable that their selective alloreactivity could be harnessed for development of novel therapies. The autoreactive and alloreactive potential of these cells may make them excellent candidates for cellular immunotherapies to enhance graft-versus-leukemia effects in HSCT. Conversely, evidence of dual TCR T cells as Tregs suggests that they may be amenable to strategies promoting allograft tolerance. Further study of dual TCR T cells will continue to define their role in normal physiology, revealing novel aspects of T cell biology, and possibly provide new avenues for development of novel therapeutic approaches.

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Chapter 2: Pro-inflammatory dual receptor T cells in chronic graft versus host disease.

2.1 Abstract

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

2.2 Introduction

Allogeneic HSCT presents a medically-relevant example of the double-edged nature of T cell function. Post-allogeneic HSCT mortality is driven by three main causes, malignancy relapse, graft versus host disease (GVHD), and opportunistic infection [1,2]. Opportunistic infection and malignant disease relapse represent, in part, a failure of T cells to mediate protective immunity [3,4]. Conversely, GVHD is unwanted or misdirected T cell function that results in multi-organ immune-mediated damage [5,6]. Acute GVHD (aGVHD) is caused by a

robust response of donor T cells in the hematopoietic stem cell (HSC) graft against recipient alloantigens [6], while increasing evidence indicates that chronic GVHD (cGVHD) represents a failure of effective self-tolerance by the newly-developed immune system [5-12]. It is a goal in HSCT to differentiate T cells capable of mediating effective post-transplant protective immunity or those promoting GVHD, with the aim of identifying specific mechanisms driving pathology.

T cell activity is primarily determined by specific recognition of peptide antigens presented by MHC (pMHC) through the T cell receptor [13]. Conventional αβ T cells express a single αβTCR heterodimer generated by recombination of germline-encoded variable (V), diversity (D), and joining (J) gene segments. This process enables formation of a diverse TCR repertoire capable of highly specific responses to a wide range of potential antigenic stimuli. Functionality of the non-genetically-encoded TCR proteins is ensured via positive and negative selection during development in the thymus, which ensures T cells bear a TCR capable of recognizing foreign antigens presented by self-MHC while minimizing T cells with unwanted reactivity by inducing apoptosis of thymocytes bearing crossreactive or autoreactive TCRs [14-19]. In HSCT, the thymus is subject to extensive insult by pre-transplant irradiation and chemotherapy conditioning regimens, which combined with thymic GVHD and normal thymic involution in older patients, limit the efficacy of thymopoiesis in HSCT patients [20]. This results in not only decreased T cell production, but also impaired elimination of autoreactive/alloreactive cells which contribute to GVHD [7-20].

We have identified the ~10% of T cells naturally expressing 2 TCRs [21] as a subset uniquely predisposed to mediating alloreactive responses, including aGVHD in mouse models and human HSCT patients [22]. Dual TCR cells are generated in normal physiology from simultaneous rearrangement of both TCRα loci in $CD4^+CD8^+$ double positive (DP) thymocytes

as a mechanism to maximize positive selection efficiency [23]. However, promotion of positive selection comes with the cost of reduced stringency of thymic selection requirements for dual receptor thymocytes; only one of the TCRs on a dual receptor cell is required to mediate positive selection [24.25] and expression of a second receptor can mask TCRs that would otherwise be eliminated by negative selection [26-28]. This results in emergence of populations of dual TCR cells containing unique TCRα clonotypes that would not be present under more stringent selection [23]. The presence of these unique TCRs does not appear to affect responses to foreign antigens, but is associated with increased frequency of cells reactive against alloantigens and autoantigens [23.29]. These reactivities underlie the potential for dual TCR T cells to initiate pathologic alloreactive and autoimmune responses observed in patients and animal models [25,28,30,31].

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

2.3 Materials and Methods

2.3.1 Human subjects

Peripheral blood leukocytes were collected from consenting healthy adult volunteers during apheresis platelet donation at the San Diego Blood Bank. WBCs were recovered from apheresis filters. Adult patients receiving allogeneic HSCT at UCSD Moores Cancer Center were recruited for participation and provided informed consent. Patients received standard of care therapy, including pre-transplant conditioning and granulocyte colony-stimulating factor mobilized HSCs or bone marrow. Peripheral blood samples from HSCT patients (10-30 ml) were collected by peripheral venipuncture at routine follow-up visit or at admission for treatment of symptomatic cGVHD. Chronic GVHD was assessed using NIH diagnosing and staging criteria [32]. All samples and data from healthy donors and HSCT patients were de-identified and assigned a study-unique identifier linking data and samples. Leukocytes were collected by density gradient centrifugation (Lymphoprep, Stem Cell Technologies) and stored at -80^{o}C until analysis. All collection, storage, and analysis of samples and patient data were performed under the approval of the UCSD Human Studies Institutional Review Board.

2.3.2 Flow cytometry

Human peripheral blood T cells were labeled with LIVE/DEAD Yellow viability dye (Thermo-Fisher Scientific) prior to labeling with TruStain Fc block, anti-CD3 (HIT3a)-PE-Cy5, anti-CD4 (OKT4)-APC-Cy7, anti-CD8 (HIT8a)-PerCP-Cy5.5, anti-CD45RA (HI100)-BV421, anti-CD45RO (UCHL1)-AF700, anti-TCRVα7.2 (3C10)-BV605 (Biolegend), anti-TCRVα24 (C15)-PE (Beckman Coulter), anti-TCRVα12.1 (6D6.6)-FITC, anti-TCRVα2 (F1) (Thermo-Fisher Scientific) labeled with AF647 (Molecular Probes), anti-TCRVα4 (5B2) and anti-TCRVα9

(2B2) [31] labeled with Pacific Orange and AF594 respectively (Molecular Probes). Samples were analyzed on a FACSCanto or LSR II instruments (BD Biosciences) with FACSDiva software. Samples were run in batches containing both control and experimental samples. Cutoffs for defining positive labeling were determined using fluorescence minus-one controls for surface labeling and isotype controls for intracellular labeling. Data were analyzed using FlowJo v10 (Tree Star).

2.3.3 Single-cell TCR clonotype and transcription factor analysis

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

2.3.4 Statistical analysis

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

2.4 Results

2.4.1 Flow cytometry identified increased frequencies of dual TCR cells in patients with cGVHD

Given the ability of dual TCR T cells to selectively mediate pathologic alloreactive and autoimmune responses in patients and animal models [25,28,30,31], we examined T cells in peripheral blood samples from patients developing cGVHD 19 – 48 months after allogeneic HSCT (n = 9), allogeneic HSCT patients not developing cGVHD within a comparable time period post-transplant (n = 4), and age-matched healthy controls (n = 5). We utilized our previously described approach of flow cytometry analysis for pair-wise TCRVα expression [31]. Using antibodies recognizing TCRVα2, TCRVα4, TCRVα7, TCRVα9, TCRVα12, and TCRVα24 enables examination of approximately 15% of the TCRα repertoire (Figure 2.1A) and identification of 15 pair-wise combinations of TCRVα expression (Figure 2.1B). Using this flow cytometry approach, dual TCR cell frequency was evaluated both as absolute number of dual TCR cells among all cells labeled with anti-TCRVα mAbs and as a percentage of dual TCR cells potentially identifiable (using the most consistently abundant TCRVα examined, TCRVα12, to normalize between samples) (Figure 2.1C). These analyses enabled normalization for differential use of TCRVα gene segments between individuals. While increased frequencies of T cells expressing the 6 TCRVα segments that we are capable of examining by flow cytometry increased the absolute number of dual TCR cells identifiable, there was no bias in the frequency of identifiable dual TCR cells caused by these differential TCRVα use (Figure 2.1D). Blinded independent analysis of dual TCR T cell frequencies by two different investigators demonstrated excellent correlation (Figure 2.1E), indicating the robustness of this approach. While the frequency of dual TCR cells identifiable using the method is low, examination of large numbers

of T cells (~1 x 10^6/sample) and stringent gating strategies enables robust and consistent enumeration of dual TCR cells.

TCRVα use was similar between allogeneic HSCT patients and healthy controls (Figure 2.2A). Dual TCR cells were readily identifiable in both healthy controls and HSCT patients (Figure 2.2B). Peripheral blood samples from healthy donors demonstrated 8.8 ± 0.8 dual TCR cells/10^3 TCRVα$^+$ T cells (mean ± s.e.m.), with an estimated frequency of 17.9 ± 6.8% of peripheral T cells (Figure 2.2C). These frequencies are consistent with previous investigations of dual TCR T cells from our group as well as others. HSCT patients with symptomatic cGVHD had a 3-fold increase in dual TCR cells (26.5 ± 5.6 cells/10^3 TCRVα$^+$ T cells, P = 0.012; 58.1 ± 12.4% P = 0.028). HSCT patients without symptomatic cGVHD did not evidence a similar increase in dual TCR cells (9.1 ± 1.6 cells/10^3 TCRVα$^+$ T cells, 34.6 ± 15.1%). Dual TCR frequency was not significantly different between cGVHD disease severity groups (NIH mild/moderate cGVHD, n = 5, 30.5 ± 9.6 dual TCR cells/10^3 TCRVα$^+$ T cells vs. severe cGVHD, n = 4, 21.4 ± 4.5 dual TCR cells/10^3 TCRVα$^+$ T cells, P = 0.397). Dual TCR cell frequency in patients with cGVHD was not affected by pre-transplant conditioning regimen (reduced-intensity conditioning, n = 6, 24.4 ± 7.9 dual TCR cells/10^3 TCRVα$^+$ T cells vs. myeloablative conditioning, n = 3, 30.6 ± 7.4 dual TCR cells/10^3 TCRVα$^+$ T cells, P = 0.562).

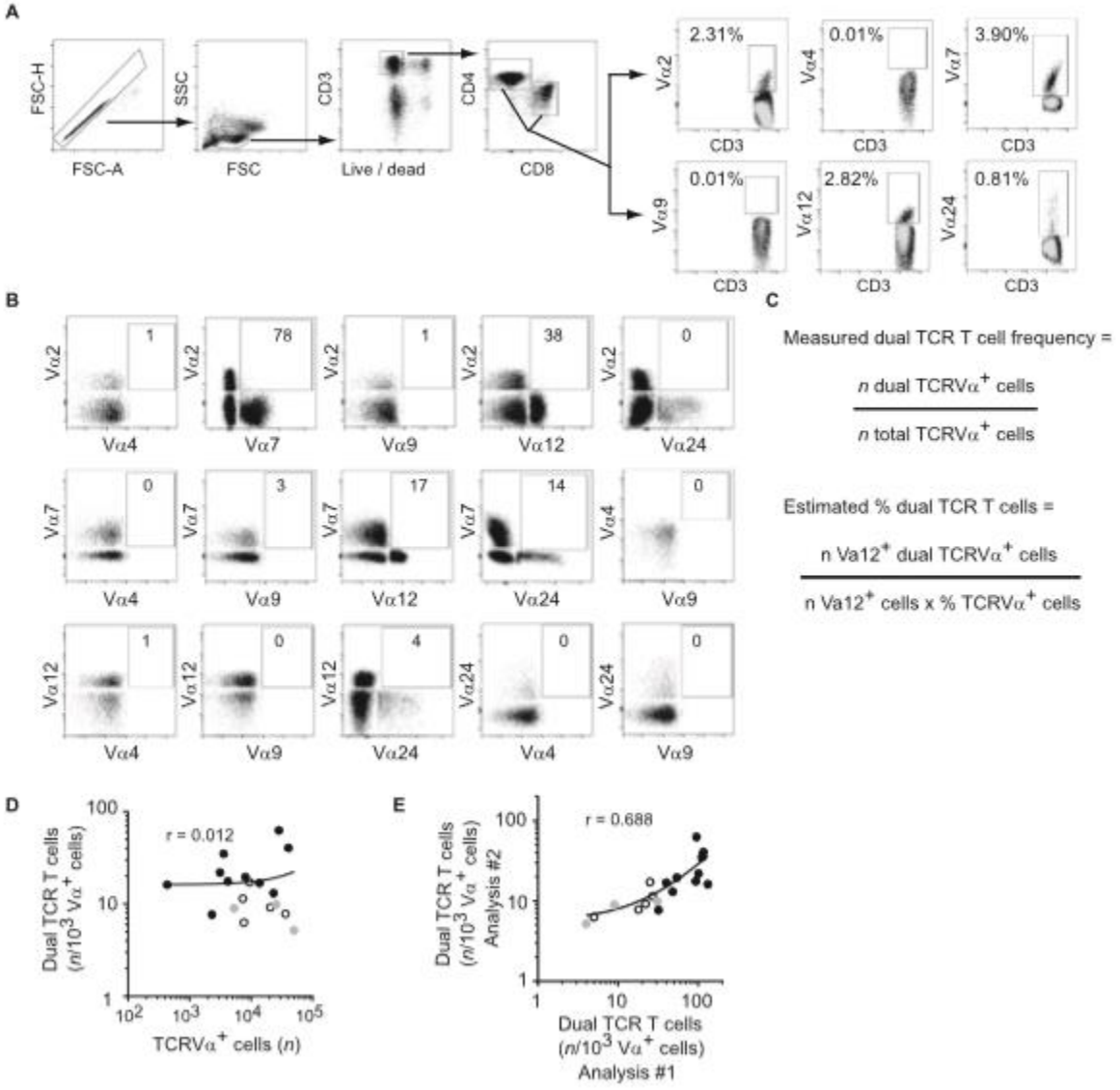

Figure 2.1 Identification of dual TCR cells by flow cytometry. Dual TCR cells were identified in peripheral blood samples by pair-wise labeling with antibodies recognizing TCRVα2, TCRVα4, TCRVα7, TCRVα9, TCRVα12, and TCRVα24. (A) Representative sample demonstrating gating strategy. Singlet live $CD3^+CD4^+$ or $CD8^+$ cells were analyzed for TCR expression. TCRVα gating was determined using FMO controls (gray). Percentage of T cells expressing specified TCRVα indicated. (B) Dual TCR cells were enumerated from total cells labeled with TCRVα mAbs using 15 pair-wise TCRVα combinations. (C) Dual TCR T cell frequency was enumerated as the total number of dual TCR cells identified from the total number of $TCRV\alpha^+$ cells. Total dual TCR T cell frequency was estimated as the number of dual TCR cells compared to the potential number of dual TCR T cells based on TCRVα labeling frequency using TCRVα12 to normalize between samples. (D) Correlation between total $TCRV\alpha^+$ cells and measured dual TCR cells. Data shown are individual samples; healthy donors in open circles, allogeneic HSCT patients without cGVHD in grey circles, cGVHD patients in black circles. Correlation calculated by nonlinear regression. (E) Correlation between 2 independent readings of human peripheral blood flow cytometry data. Data shown are individual samples; healthy donors in open circles, allogeneic HSCT patients without cGVHD in grey circles, cGVHD patients in black circles. Correlation calculated by nonlinear regression.

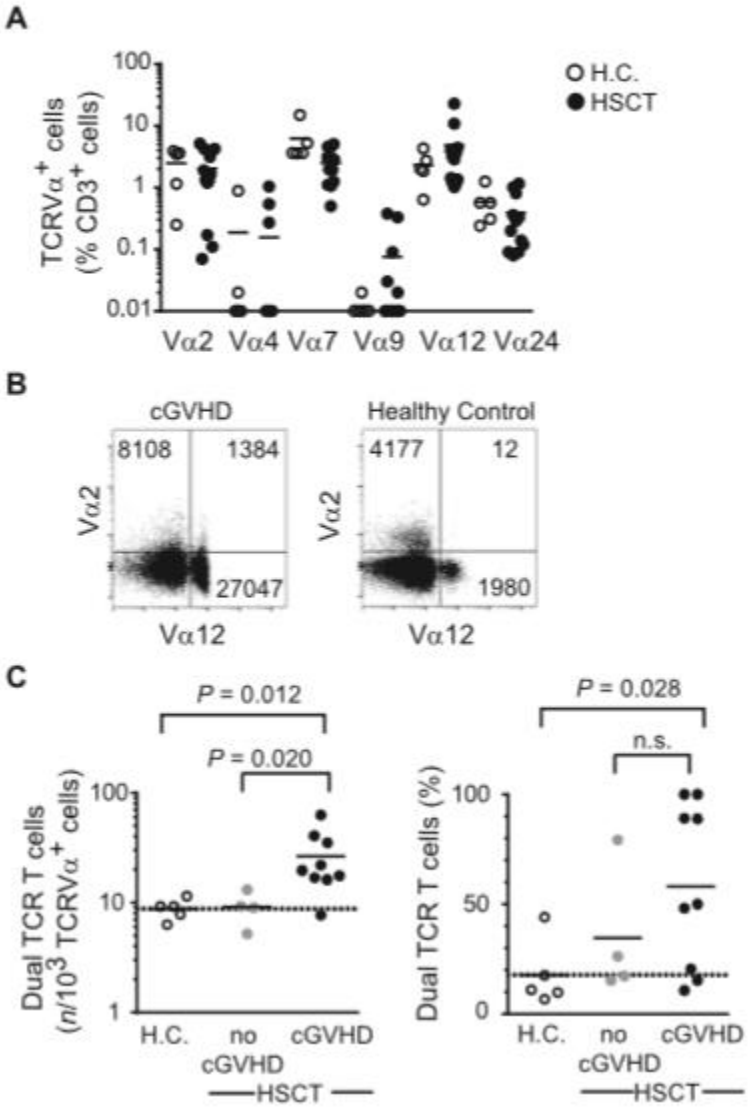

Figure 2.2 Dual TCR cells are increased in frequency and activated in patients with cGVHD. Dual TCR T cells were identified in peripheral blood samples from healthy donors, allogeneic HSCT patients without cGVHD, and allogeneic HSCT patients with symptomatic cGVHD using pair-wise labeling for TCRVα. (A) Frequency of peripheral blood T cells labeled with indicated antibodies recognizing indicated TCRVα. Data shown are individual patients. (B) Representative example of pair-wise labeling for the most frequent TCRVα pairs, TCRVα2 and TCRVα12 from healthy donor and patient with cGVHD. (C) Enumeration of dual TCR T cells identified by flow cytometry, shown as dual TCR cell frequency in individual patients, compared by Mann-Whitney test. Absolute dual TCR cell frequency estimated using equation described in Figure 1C. Data shown are in individual patients compared by Mann-Whitney test.

2.4.2 Dual TCR cells are selectively activated in cGVHD

The use of multi-parameter (13 color) flow cytometry enables phenotypic examination of dual TCR cells and comparison of their phenotype with that of other cells in the same sample. Dual TCR cells did not evidence any difference in total TCR expression as measured by CD3 expression (Figure 2.3AB). Dual TCR cells were also equally likely to have a naive ($CD45RA^+$) phenotype as the T cell population in general (41.1 $\pm$ 7.1%and 38.8 $\pm$ 5.9% respectively, P = 0.311) (Figure 2.3CD). However, dual receptor cells were much more likely to be activated as compared to all TCRVα mAb^+ cells in patients with cGVHD, as evidenced by expression of CD69 (24.3 $\pm$ 10.0% of dual TCR cells compared to 8.1 $\pm$ 4.6% al TCRVα^+ cells, P = 0.004) (Figure 2.3EF).

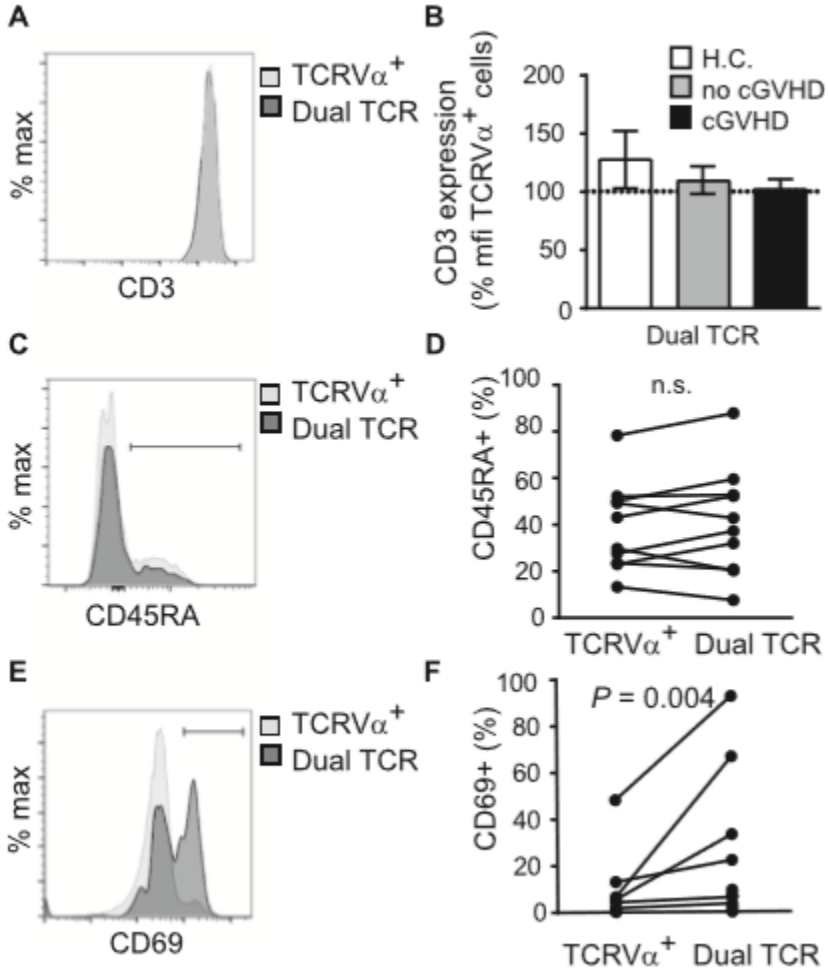

Figure 2.3 Phenotypic examination of dual TCR cells in patients with cGVHD. Phenotype of dual TCR T cells identified by pair-wise TCRVα mAb labeling was compared with all TCRVα$^+$ cells in samples. (A) Representative example of CD3 expression by all TCRVα$^+$ cells and dual TCR cells. (B) Comparison of CD3 expression by all TCRVα$^+$ cells and dual TCR cells in samples from healthy donors, allogeneic HSCT patients without cGVHD, and patients with symptomatic cGVHD. Data shown are mean $\pm$ s.e.m. of percentage of CD3 mean fluorescence intensity (mfi) of dual TCR cells compared to CD3 mfi of all TCRVα$^+$ cells. (C) Representative example of CD45RA expression by all TCRVα$^+$ cells and dual TCR cells. (D) Comparison of CD45RA expression by all TCRVα$^+$ cells and dual TCR cells in samples from patients with symptomatic cGVHD. Data shown are individual samples with groups linked by line. Data compared using ratio paired *t* test. (E) Representative example of CD69 expression by all TCRVα$^+$ cells and dual TCR cells identified by pair-wise labeling. (F) Comparison of CD69 expression by all TCRVα$^+$ cells and dual TCR cells in patients with symptomatic cGVHD. Data shown are individual samples with groups linked by line. Data compared using ratio paired *t* test.

2.4.3 Single-cell RNA sequencing confirms increased dual TCR cells in cGVHD

Identification of dual TCR T cells by flow cytometry is critically restricted by a paucity of available reagents. This significantly limits our ability to examine the entire potential dual TCR repertoire. Furthermore, the flow cytometry approach does not unambiguously identify T cells expressing a single TCR, as it would be expected that a number of cells express secondary TCRs that cannot be measured by available reagents. To overcome this, we adopted a single-cell barcoded PCR strategy that enables identification of TCRαβ clonotypes combined with selected transcription factor/effector gene expression by multiplexed next-generation DNA sequencing [33] and MiTCR gene rearrangement analysis software [34]. Individual T cells (176/sample) from healthy donors (n = 4) and HSCT patients with symptomatic severe cGVHD (n = 4, Patients 6, 14, 15, and 18) were isolated by flow cytometry into 96-well plates for single-cell analysis. A stringent cutoff of 10^3 TCR sequence reads was applied to minimize the possibility for cross-contamination resulting in false-positive TCR sequences for a given well. Using this cutoff, we identified in-frame TCRβ rearrangements in 75.0% and TCRα in 70.0% of sorted wells (Figure 2.4A). Further refinement of the data was performed by eliminating cells with 2 in-frame TCRβ transcripts to avoid the possibility for having sorted 2 cells into a single well as well as eliminating TCRα transcripts paired with more than 1 TCRβ from a given patient to minimize the possibility for cross-contamination resulting in false-positive identification of dual TCR cells. Wells with 2 TCRα transcripts demonstrated a range of ratios between the transcripts (Figure 2.4B). Together, these analyses confidently identified TCRαβ clonotypes in 49.6% of wells tested.

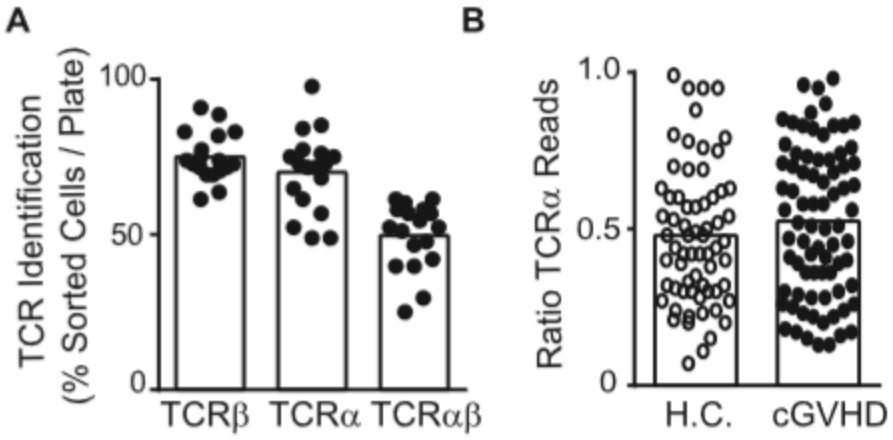

Figure 2.4 Identification of TCR clonotypes by single-cell RNA sequencing. Peripheral blood T cells from allogeneic HSCT patients with symptomatic cGVHD and age-matched healthy controls were analyzed by single-cell barcoded sequencing for TCRα and TCRβ. (A) Efficiency of identifying TCRβ, TCRα, and paired TCRαβ clonotypes using analysis criteria. Data are individual patient samples with group means shown. (B) Comparison of ratio of TCRα read counts for dual TCR cells from healthy donors and cGVHD patients. Data shown are individual dual TCR cells from 4 samples in each group, with group means indicated.

Using the single-cell sequencing strategy, we identified TCRαβ clonotypes from 429 T cells from healthy donors and 422 T cells from patients with cGVHD (Figure 2.5A). Two in-frame TCRα transcripts were identified in 68 (18.8%) of cells from healthy donors, consistent with other reports as well as the estimate from our flow cytometry data (Figure 2.2C). Single-cell TCR sequencing of peripheral blood T cells from 4 patients with severe cGVHD (NIH grading criteria) demonstrated a significantly higher percentage of cells with 2 in-frame TCRα transcripts (23.4%, $P = 0.019$) as compared to healthy controls. The frequency of dual TCR cells identified in these patients was comparable to that estimated by flow cytometry for these 4 samples (21.9%). There was no difference in the relative read counts of paired TCRα transcripts in dual receptor cells from patients with cGVHD (0.52 ± 0.03, mean + s.e.m.) compared to dual receptor cells from healthy controls (0.48 ± 0.03, $P = 0.248$) (Figure 2.4B). These data validate the data from the flow cytometry approach and enable specific examination of the TCR repertoires of dual receptor cells.

We have previously used deep sequencing of mouse TCRα repertoires to demonstrate that dual receptor T cells contain a unique repertoire of TCRs that are not present among T cells with a single receptor [23]. The TCR repertoire of dual receptor cells from patients with cGVHD demonstrated broad use of *TRBV*, and *TRAV* segments with clonal distribution frequencies comparable to dual TCR cells from healthy donors (Figure 2.5B). Dual TCR T cells from both healthy donors and patients with cGVHD had decreased relative breadth of clonal distribution as compared to single TCR T cells, though this is possibly attributable to the relatively limited number of dual TCR clones identified. TCRs from dual receptor cells did not demonstrate structural abnormalities in the CDR3α or CDR3β, with CDR3 lengths comparable to single TCR cells from cGVHD patients as well as single or dual TCR cells from healthy donors (Figure

2.5C). Similarly, TCRs from dual receptor cells did not demonstrate preferential use of basic (R, H, K), acidic (D, E), small (G, A), nucleophilic (S, T, C), hydrophobic (V, L, I, M, P), aromatic (F, Y, W), or amide (N, Q) amino acids in the CDR3α or CDR3β regions (Figure 2.5D). The single-cell sequence data provide additional confidence in our observation of increased dual TCR T cell frequencies in HSCT patients with cGVHD by flow cytometry and unambiguously identify TCRαβ clonotypes from dual receptor cells for examination of their activity in cGVHD.

A

Patients	Dual TCR Frequency	
	Flow Cytometry	Single-cell TCR Sequencing
Healthy Controls (n = 4)	17.5%	68/361 (18.8%)
Severe cGVHD (n = 4)	23.4%	80/342 (23.4%) P = 0.019

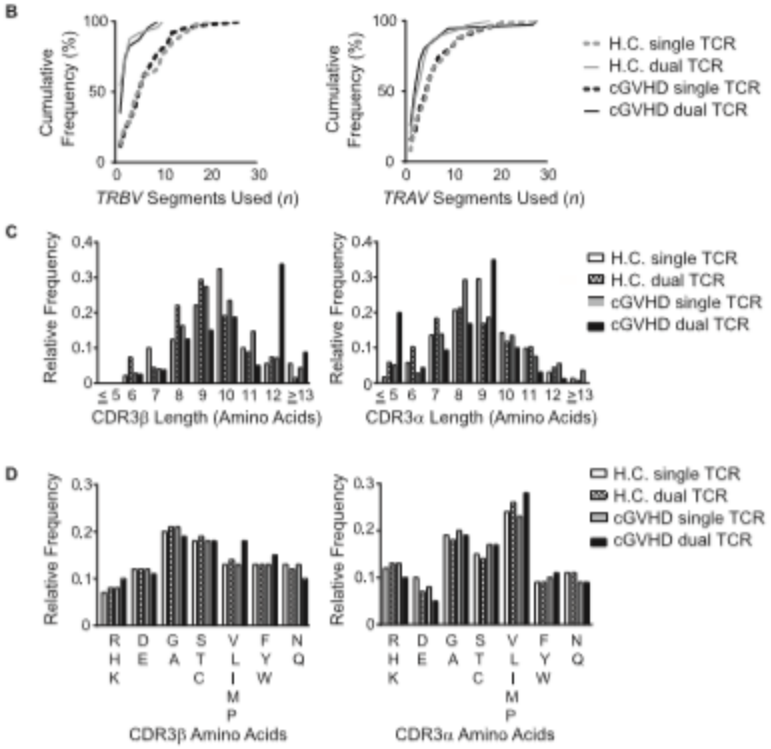

Figure 2.5 Single-cell sequencing identifies increased dual TCR cells in patients with cGVHD. Peripheral blood T cells from allogeneic HSCT patients with symptomatic cGVHD and age-matched healthy controls were analyzed by single-cell barcoded sequencing for TCRα and TCRβ. (A) Single-cell sequencing identified increased frequencies of peripheral blood T cells expressing 2 TCRs in patients with cGVHD as compared to healthy donors. Data shown as the number and percentage of T cells with 2 TCRs among all cells with identifiable TCRαβ clonotypes. Data are compared by Fisher's exact test. Dual TCR cell frequencies for both groups were comparable to estimates from flow cytometry analyses. (B) Comparison of *TRBV* and *TRAV* gene segment use by single and dual TCR cells from healthy donors and cGVHD patients. Data are shown as cumulative distribution of gene segment use by TCR clonotypes within each group. (C) CDR3β and CDR3α amino acid length by single and dual TCR cells from healthy donors and cGVHD patients. Data are shown as relative frequency for CDR3 amino acid length by TCR clonotypes within each group. (D) CDR3β and CDR3α amino acid use by single and dual TCR cells from healthy donors and cGVHD patients. Data are shown as relative of amino acids present in CDR3 regions of TCR clonotypes within each group.

2.4.4 Dual TCR cells in cGVHD are pro-inflammatory

A key benefit of the multiplexed single-cell sequencing approach is the ability to link TCR clonotypes with phenotype. Using the described multiplex phenotype PCR primers [33] we examined expression of 14 key transcription factors and effector molecules. However, T cells were not re-stimulated *ex vivo,* which limited information from cytokine and effector molecule analysis. Therefore, we focused phenotypic analysis on transcription factors capable of differentiating T cell functional subsets including *RUNX1*, *RUNX3*, *TBX21*, *RORC*, *GATA3*, *BCL6*, and *FOXP3* [35]. The nested PCR approach used for single-cell library construction provides qualitative but not quantitative analysis of gene expression. Read count cutoff values for positive gene expression were determined by analysis of read count distribution. $CD4^+$ ($RUNX1^+$) and $CD8^+$ ($RUNX1^-$) dual TCR T cells from patients with cGVHD (Figure 2.6A) demonstrated frequent expression of *TBX21* (Tbet), a promoting factor for pro-inflammatory Th1 $CD4^+$ and cytotoxic $CD8^+$ T cell function [36]. *TBX21*-expressing cells were significantly more common in both single TCR (59.8% $CD4^+$ cells, 17.6% $CD8^+$ cells) and dual TCR cells (70.0% $CD4^+$ cells, 18.8% $CD8^+$ cells) from patients with cGVHD as compared to healthy donors (50.4% $CD4^+$ cells, $P = 0.009$, 8.5% $CD8^+$ cells, $P = 0.054$), indicating involvement of these cells in cGVHD (Figure 2.6B). *GATA3* and *BCL6* were also commonly expressed by dual TCR cells. No difference in expression of these factors was observed between T cells from patients with cGVHD and healthy controls. Few $FOXP3^+$ $CD4^+$ regulatory T cells (Tregs) were observed in patients with cGVHD (1.4%), though, the number of cells identified was not statistically different from healthy donors (3.8%, $P = 0.261$).

Comparison of transcription factor expression by single TCR and dual TCR cells did not identify specific variances suggesting differences in function associated with cGVHD. However,

dual TCR T cells in patients with active cGVHD were significantly more likely to express at least one of the effector function-driving *TBX21*, *GATA3*, or *BCL6* as compared to single TCR cells (92.5% of dual TCR $CD4^+$ cells compared to 76.6% of single TCR $CD4^+$ cells, $P = 0.034$; 81.2% of dual TCR $CD8^+$ cells vs. 67.6% of single TCR $CD8^+$ cells, $P = 0.371$). A similar difference was not observed when comparing dual TCR and single TCR cells from healthy donors (76.0% of dual TCR $CD4^+$ cells compared to 74.6% of single TCR $CD4^+$ cells, 62.5% of dual TCR $CD8^+$ cells vs. 78.2% of single TCR $CD8^+$ cells). These results are in-line with our observation of selective activation of dual TCR T cells as evidenced by CD69 expression (Figure 2.3E) and suggest that dual TCR T cells are selective mediators of disease.

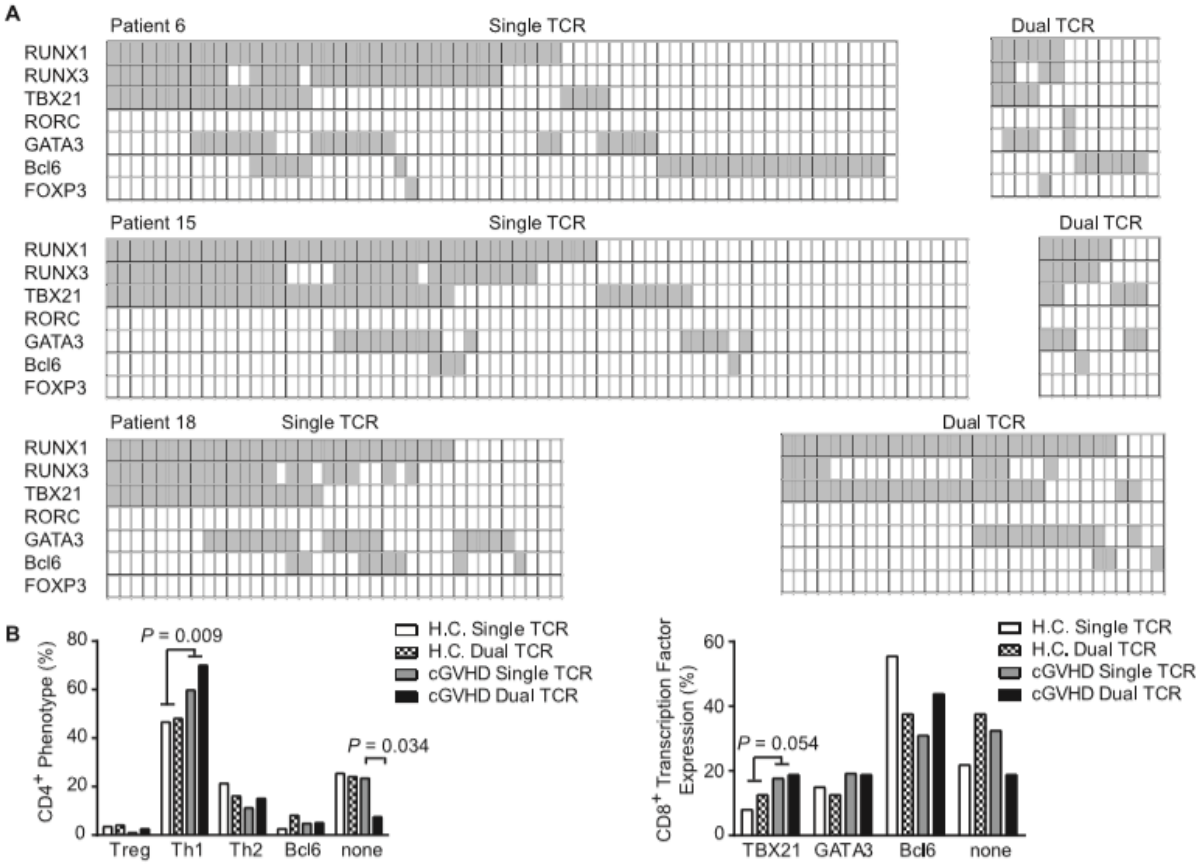

Figure 2.6 Dual TCR cells in cGVHD are preferentially activated with a pro-inflammatory phenotype. Expression of lineage-directing transcription factors and effector molecules by peripheral blood T cells from allogeneic HSCT patients with symptomatic cGVHD and age-matched healthy controls were linked to TCRαβ clonotypes, including dual TCR cells, by barcoded sequence analysis. (A) Expression of lineage-directing transcription factors by single and dual TCR cells identified from single-cell sequencing. Red denotes detected expression above threshold. (B) Comparison of gene expression by single TCR and dual TCR cells from healthy donors and cGVHD patients. Data indicate percentage of *RUNX1*$^{+}$ ($CD4^{+}$) and *RUNX1*$^{-}$ ($CD8^{+}$) cells expressing indicated gene. Data between groups compared by Fisher's exact test.

2.5 Discussion

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

Single-cell sequencing enabled focused examination of suspected pathogenic cells, permitting separation of population-specific signal from background bulk population noise. Our analysis of transcription factors associated with driving T cell effector phenotypes demonstrated a predominance of $CD4^{+}$Th1 and $CD8^{+}Tbet^{+}$ cells. This is consistent with other observations of cGVHD driven by pro-inflammatory T cells [37]. We also observed frequent expression of

GATA3 and *BCL6*, though the relationship to function is not directly evident, as they have multiple effects on T cell function. *GATA3* is associated with driving $CD4^+$Th2 responses, but is also involved in T cell homeostasis and Th9 differentiation [38]. *BCL6* is associated with differentiation of follicular helper T cells (Tfh), which have recently been identified as having a role in antibody-mediated cGVHD [39,40], but is also associated with multiple other effects including $CD8^+$ T cell proliferative burst and promoting memory T cell development [41-43]. The pleiotropic functions of *BCL6* likely obfuscate any differences in Tfh cells between single and dual TCR cells in our patients. Further study with additional phenotypic or gene expression markers would possibly identify these differences. Regardless, it is likely that multiple T cell effector subsets contribute both independently and synergistically to multimodal cGVHD pathology. An overarching feature may be defective post-transplant Treg production and function which enables immune dysregulation and subsequent cGVHD pathogenesis [44,45]. To this end, we observed decreased frequencies of $CD4^+$*FOXP3*$^+$ cells in patients with cGVHD as compared to healthy controls, though the low numbers of cells precluded statistical confidence in the data. Future focused examinations of post-transplant Treg TCR repertoires using the single-cell approach could be insightful in evaluating changes in the Treg TCR repertoire associated with cGVHD.

Increases in dual TCR cell frequency associated with symptomatic cGVHD observed in allogeneic HSCT patients must result from either expansion of donor cells transferred as part of the HSC allograft or from T cells derived from post-transplant thymopoiesis. Our previous examinations indicated that allogeneic HSCT patients did not evidence expansion of donor dual TCR cells peri-transplant (90 days) in the absence of GVHD [31]. Here, patients developing cGVHD were 9- 67 months post-transplant, a period where thymic production of new T cells

would be expected [20]. These data suggest that the dual TCR cells associated with cGVHD may have arose from post-transplant thymopoiesis rather than expansion of donor cells, though it is impossible to definitively determine the origin of these cells. It has become increasingly evident that cGVHD is a consequence of defects in thymic regeneration of the T cell repertoire after transplantation. We propose that dual TCR T cell production could be a mechanism linking qualitatively defective thymopoiesis and cGVHD. Dual TCR thymocytes have an advantage during positive selection in the thymus [23], which could combine with defective post-transplant negative selection to generate a specific subset of cells with high risk for the types of immune responses driving cGVHD [7-12]. A direct link between thymic dysfunction and cGVHD pathogenesis in human subjects has proven elusive due to the substantial heterogeneity of the T cell compartment. Longitudinal analyses of post-HSCT T cell repertoire formation will be essential to truly understand the origin and natural progression of pathogenic cells in cGVHD. Our data presented here indicate dual TCR T cells may be a specific and useful marker to examine effects of HSCT on the generation of T cells with increased pathogenic potential.

2.7 Acknowledgements

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Chapter 3: Radiation induced thymic damage increases dual TCR production due to defective thymic selection

3.1 Abstract

Defective post-transplant thymopoiesis is associated with chronic graft versus host disease (cGVHD), a multi-organ pathology affecting up to 80% of patients following allogeneic hematopoietic stem cell transplantation (HSCT). Previous work demonstrated that the subset of T cells expressing two T cell receptors (TCRs) is predisposed to alloreactivity, driving selective and disproportionate activity in acute and chronic GVHD in both mouse models and HSCT patients. Here we investigate dual TCR cells in post-transplant thymic restoration of the T cell compartment. Increased dual receptor thymocyte development in a mouse model was enabled by defective negative selection caused by radiation injury to thymic stroma.

3.2 Introduction

T cell function is primarily determined by specific recognition of peptide antigens presented by MHC (pMHC) through the T cell receptor (TCR) (*1*). Conventional αβ T cells express αβTCR heterodimers generated from recombination of germline-encoded variable (V), diversity (D), and joining (J) gene segments. This process enables generation of a highly diverse TCR repertoire capable of highly specific responses to a wide range of potential antigenic stimuli. Functionality of the non-genetically-encoded TCR proteins is ensured via positive and negative selection during development in the thymus (*2*). Positive selection requires thymocytes to express a TCR capable of low-affinity interactions with a restricted set of self-pMHC antigens in the thymic cortex, ensuring the potential for interaction with foreign antigens presented by

self-MHC (*3*). Limits to the reactivity of the T cell repertoire are imposed by negative selection, which eliminates thymocytes bearing autoreactive or crossreactive TCRs (*4-6*). Under normal physiologic circumstances, positive and negative selection act together to generate a T cell repertoire capable of mediating protective immunity and maintaining self-tolerance (*7*).

Allogeneic hematopoietic stem cell transplantation (HSCT) presents a medically-relevant example of the dichotomous nature of T cell function. Post-allogeneic HSCT mortality is driven by three main causes, malignancy relapse, graft versus host disease (GVHD), and opportunistic infection (*8, 9*). Opportunistic infection and malignant disease relapse represent, in part, a failure of T cells to mediate protective immunity (*10, 11*). Conversely, GVHD is unwanted or misdirected T cell function that results in multi-organ immune-mediated damage (*12, 13*). Acute GVHD (aGVHD) is caused by a robust response of donor T cells in the hematopoietic stem cell (HSC) graft against recipient alloantigens (*13*), while increasing evidence indicates that chronic GVHD (cGVHD) represents a failure of effective self-tolerance by the newly-developed immune system (*12, 14-19*). Thus, it is a goal in HSCT to differentiate T cells capable of mediating effective post-transplant protective immunity or those promoting GVHD, with the aim of facilitating development of the former while limiting the latter.

We have identified the ~10% of T cells naturally expressing 2 TCRs (*20*) as a subset uniquely predisposed to mediating alloreactive responses, including aGVHD in mouse models and human HSCT patients (*21*)**.** Dual TCR cells are generated in normal physiology from simultaneous rearrangement of both TCRα loci in $CD4^+CD8^+$ double positive (DP) thymocytes as a mechanism to maximize chances for positive selection (*22*). However, promotion of positive selection comes with the cost of reducing the stringency of thymic selection requirements for dual TCR T cells; only one of the TCRs on a dual receptor cell is required to mediate positive

selection (*23, 24*) and expression of a second receptor can mask TCRs that would otherwise be eliminated by negative selection (*25-27*). This results in emergence of populations of dual TCR T cells containing unique TCRα clonotypes that would not be present under more stringent selection (*22*). The presence of these unique TCRs does not appear to affect the response of the naive T cell repertoire to foreign antigens, but is associated with increased frequency of cells reactive against alloantigens and autoantigens (*22, 28*). These reactivities underlie the potential for dual TCR T cells to initiate pathologic alloreactive and autoimmune responses that have been observed in patients and animal models (*24, 27, 29, 30*). The alloreactive and autoimmune potential of dual TCR T cells suggests that they could be important contributors to cGVHD.

While normal conditions of thymopoiesis limit production of autoreactive or highly crossreactive T cells, the thymus is subject to extensive insult in HSCT by pre-transplant irradiation and chemotherapy conditioning regimens. These treatments, combined with thymic GVHD and normal thymic involution in older patients, limit the efficacy of thymopoiesis in HSCT patients (*31*). Depletion of cortical and medullary thymic epithelial cells (cTECs and mTECs) by pre-transplant conditioning results in not only decreased T cell production, but also impaired elimination of autoreactive/alloreactive cells which contribute to GVHD (*14-19*). We hypothesized that generation of dual TCR T cells, which are normally capable of avoiding stringent thymic selection requirements, could be significantly increased in thymi damaged by pre-transplant conditioning and thus be a source of potentially pathogenic T cells in cGVHD.

To test this hypothesis, we examined development of dual receptor T cells in a syngeneic HSCT model (avoiding complication by thymic GVHD). Dual TCR T cells were generated at increased frequency after transplantation of T cell-depleted syngeneic bone marrow into lethally-irradiated mice. This increase was associated with depletion of mTECs and a resulting specific

impairment in negative selection. The potential impact of post-transplant generation of dual TCR T cells is demonstrated by the increased numbers of dual TCR T cells in patients with cGVHD. Using a single cell sequencing approach, we determined that dual TCR T cells in cGVHD patients are disproportionately activated with a pro-inflammatory phenotype. Our data provides compelling evidence that dual TCR T cells are a potentially pathogenic subset of T cells that are specifically generated as a consequence of thymic damage due to pre-transplant conditioning.

3.3 Materials and methods

3.3.1 Mice

C57BL/6 (B6) and B6.Ly5.1 mice were originally purchased from Charles River Laboratories. Mice were bred and housed in specific pathogen-free conditions at UCSD. Transplantation experiments were performed by intravenous injection of 10^7 T cell-depleted syngeneic bone marrow cells (depleted using magnetic bead selection to < 1% of transferred cells) collected from femur and tibia of 6-8 wk old mice 1 d after 10 Gy irradiation via Cs^{137} irradiator. Transplanted mice were kept on water containing 5 mg/ml trimethoprim/sulfamethoxazole and monitored after transplantation for signs of distress by daily observation and weekly weight measurement. Spleen, thymus, and lymph nodes (popliteal, inguinal, and axillary) were recovered from mice at 2, 4, 8, 16, and 20 weeks after BMT and age-matched control mice. All animal breeding and experiments were performed in accordance with and under the approval of the UCSD Animal Care Program.

3.3.2 Flow cytometry

Mouse thymocytes and T cells from spleen and lymph nodes were isolated by mechanical disruption. Red blood cells were removed by hypotonic lysis with ACK lysis buffer. TECs were isolated by enzymatic digestion in RPMI 1640 (Mediatech) containing 0.125% collagenase and 0.1% DNAse or 0.125% collagenase/dispase and 0.1% DNAse (Roche Diagnostics) at 37ºC according to established protocol (*47*). TECs were labeled with UEA-1-FITC (Vector Labs), anti-CD45 (30-F11)-PerCP-Cy5.5, anti-Ly51 (6C3)-PE, anti-CD326 (EpCAM) (G8.8)-APC, and anti-MHC II (M5/114.15.2)-PE-Cy7 (Biolegend). Thymocytes and peripheral T cells were examined for dual TCR expression by labeling with anti-CD4 (GK1.5)-APC-Cy7, anti-CD8 (53-6.7)-PerCP-Cy5.5, anti-TCRV□2 (B20.1)-APC, anti-TCRV□3.2 (RR3-16)-PE, anti-TCRV□8.3 (B21.14)-FITC (Biolegend). Positive selection of thymocytes was examined using anti-CD4-APC-Cy7, anti-CD8-PerCP-Cy5.5, anti-CD3□ (145-2C11)-FITC, and anti-CD69 (H1.2F3)-PE-Cy7 (Biolegend). Negative selection was examined using anti-CD4-APC-Cy7, anti-CD8-PerCP-Cy5.5, anti-CD69-PE-Cy7, anti-CD279 (PD-1) (29F.1A12)-PE (Biolegend), and intracellular labeling with anti-activated caspase 3(Cell Signaling) and donkey anti-rabbit IgG-AF647 using True-Nuclear fix/perm buffer (Biolegend). Samples were analyzed on a FACSCanto or LSR II instruments (BD Biosciences) with FACSDiva software. Samples were run in batches containing both control and experimental samples. Cutoffs for defining positive labeling were determined using fluorescence minus-one controls for surface labeling and isotype controls for intracellular labeling. Data were analyzed using FlowJo v10 (Tree Star).

3.3.3 Thymus histology

Thymi were recovered at 2, 4, 8, and 16 weeks after BMT or from age-matched control mice. One lobe was used for flow cytometry analysis of thymocyte and TEC populations and the second lobe was used for histologic analysis. Tissue sections were cut from blocks of formalin-fixed paraffin embedded mouse thymus tissue. Four micron tissue sections were stained with an antibodies to mouse cytokeratin 8 (TROMA-1, Univ of Iowa) and mouse cytokeratin 5 (Biolegend). Slides were stained on a Ventana Discovery Ultra (Ventana). Antigen retrieval was performed using CC1 for 40 minutes at 95^{o}C. Primary antibodies were incubated on the sections at 1:6000 (Ck-5) or 1:300 (Ck-8) for 32 minutes at 37^{o}C followed by UltraMap (Ventana) and DAB detection. Slides were rinsed, dehydrated through alcohol and xylene and coverslipped. Histology was examined using an Axio Imager2 (Zeiss).

3.3.4 Statistical analysis

Data were analyzed using Prism 6 software (GraphPad). Nonparametric analyses were performed in longitudinal experiments using 2-way ANOVA with multiple comparisons. Comparisons of individual groups were performed nonparametrically using Mann-Whitney test. Intrasample comparisons of phenotype for single and dual TCR cells were performed using ratio paired *t* test. Frequencies of categorical data were compared using Fisher's exact test.

3.4 Results

3.4.1 Impaired negative selection results in increased generation of dual TCR T cells by post-transplant thymopoiesis

To test if dual TCR T cell development was affected by thymic effects of pre-transplant conditioning, we utilized a model of syngeneic transplantation of T cell-depleted (TCD) B6 bone marrow cells into lethally-irradiated B6 mice. Total body irradiation (TBI) is a common component of myeloablative pre-transplant conditioning regimens with well-described detrimental effects on the thymus (*32*). Use of a syngeneic TCD bone marrow transplant system enabled separation of the effects of post-transplant thymopoiesis from alloreactivity. Dual TCR T cell generation was assessed by flow cytometry for $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes co-expressing TCRVα2, TCRVα3.2, or TCRVα8.3 (Fig. 3.1A). Thymi from transplanted mice and age-matched controls were examined 2, 4, 8, 16, and 20 weeks post-transplant (n = 4 - 11 mice per group at each time point). As expected, persistently decreased numbers of thymocytes were recovered in transplanted mice compared to controls (P = 0.001) (Fig 3.1B). Among thymocytes developing after transplantation, $CD4^+$ and $CD8^+$ SP thymocytes expressing dual TCRs were consistently present with increased frequency (20.1 $\pm$ $1.1/10^4$ $TCV\alpha^+$ SP thymocytes, mean $\pm$ s.e.m.) compared to age-matched controls (17.1 $\pm$ 0.9 dual TCR thymocytes/10^4 TCRVα mAb^+ cells, P = 0.008) (Fig. 3.1C). This increase in dual TCR thymocytes in transplanted mice indicates a defect in post-transplant regeneration of the T cell repertoire with potential risk from a subset of T cells known to harbor alloreactive and autoreactive receptor specificities.

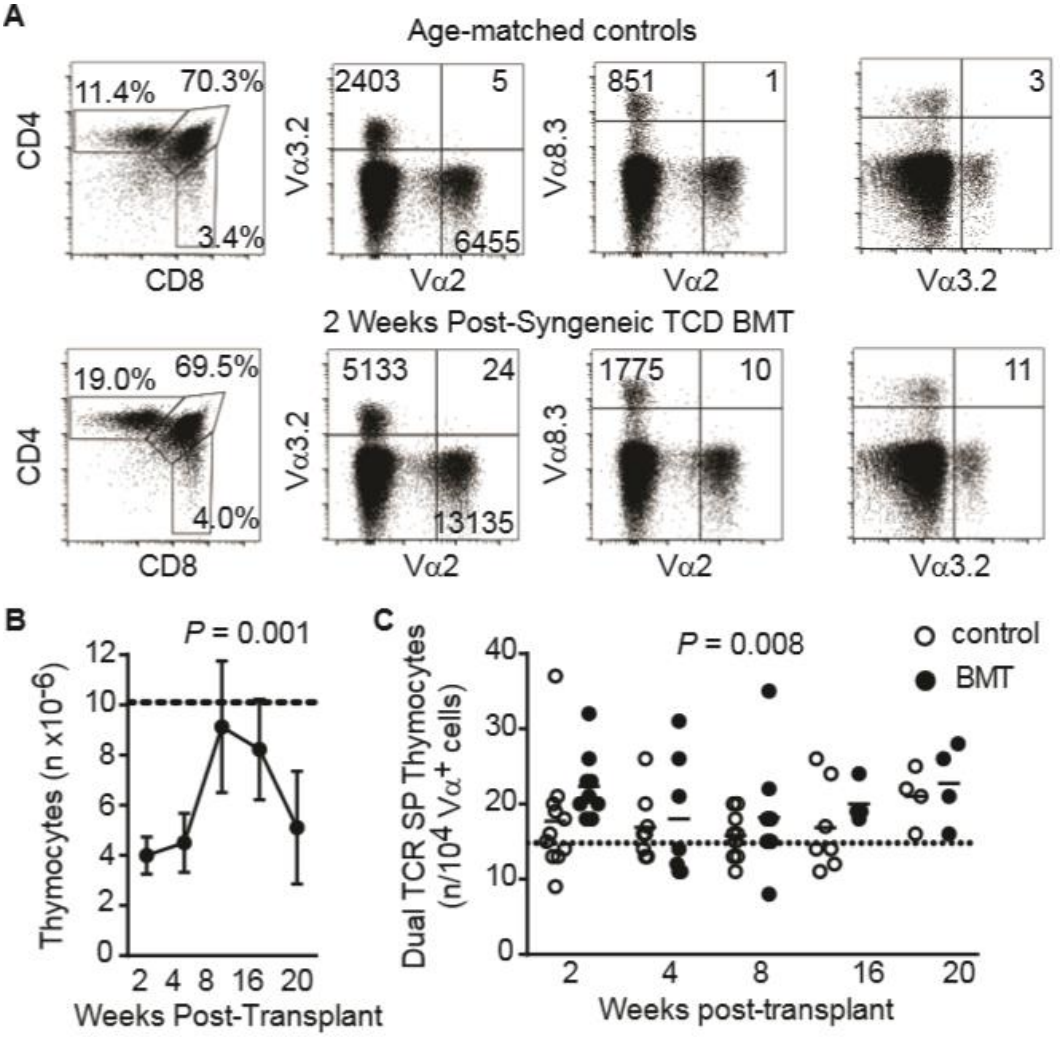

Figure 3.1 Increased dual TCR T cells after transplant result from increased thymic production. Thymic production of $CD4^+$ and $CD8^+$ dual TCR thymocytes was assessed in TCD BMT mice at 2, 4, 8, 16, and 20 weeks after transplantation by flow cytometry using pair-wise labeling with antibodies recognizing Vα2, Vα3.2, and Vα8.3. (A) Representative flow cytometry plot of $CD4^+$ and $CD8^+$ SP thymocytes from a mouse 2 week post-BMT and an age-matched control. (B) Post-transplant thymic cellularity was assessed by manual counting of viable thymocytes from 1 thymic lobe. Data shown as mean $\pm$ s.e.m. of 4 – 9 mice per group per time point, compared to average of age-matched healthy control mice (n = 32). Thymocyte numbers compared by 2-way ANOVA with multiple comparisons. (C) Dual TCR cells quantified as the number of $V\alpha2^+V\alpha3.2^+$, $V\alpha2^+V\alpha8.3^+$, or $V\alpha3.2^+V\alpha8.3^+$ cells among all $V\alpha2^+$, $V\alpha3.2^+$, or $V\alpha8.3^+$ SP thymocytes. Data shown are individual mice from 3 independent experiments. Dual TCR cell frequencies compared by 2-way ANOVA with multiple comparisons.

Given the demonstration of qualitative change in thymopoiesis evidenced by increased production of dual TCR thymocytes, we next sought to identify whether positive selection, negative selection, or both processes were impaired in the post-transplant thymus. To do this, we examined measures of positive selection and negative selection in mice transplanted with syngeneic TCD bone marrow 4 weeks after transplantation (n = 9). Positive selection was examined by flow cytometry to identify $CD3^{high}$ $CD4^{+}CD8^{+}$ double positive (DP) thymocytes (Fig 3.2A). Transplanted mice did not demonstrate significant changes in the ability of DP thymocytes to find a positively selecting signal and mature to the $CD3^{high}$ stage (1.0 $\pm$ 0.2%, mean $\pm$ s.e.m) compared to age-matched controls (n = 8, 1.2 $\pm$ 0.1%, P = 0.337) (Fig. 3.2B). Likewise, transplanted mice did not demonstrate impaired progression of DP thymocytes to the SP stage (SP/DP ratio 0.27 $\pm$ 0.09) compared to control mice (SP/DP ratio 0.26 $\pm$ 0.05, P = 0.622) (Fig. 3.2C).

We examined negative selection in transplanted mice by flow cytometry for detection of activated caspase-3, a marker of thymocyte apoptosis (*33*) (Fig. 3.2D). Activation of caspase-3 was detectable in both DP and SP thymocytes, indicative of apoptosis. Transplanted mice had a significantly reduced frequency in of apoptosis in SP thymocytes (0.02 $\pm$ 0.01%, mean $\pm$ s.e.m. vs. 0.08 $\pm$ 0.03% in controls, P = 0.004) (Fig. 3.2E). DP thymocytes did not show a statistically significant difference, though transplanted mice trended toward reduced frequency of apoptosis (0.02 $\pm$ 0.01% vs. 0.04 $\pm$ 0.01%, P = 0.007).

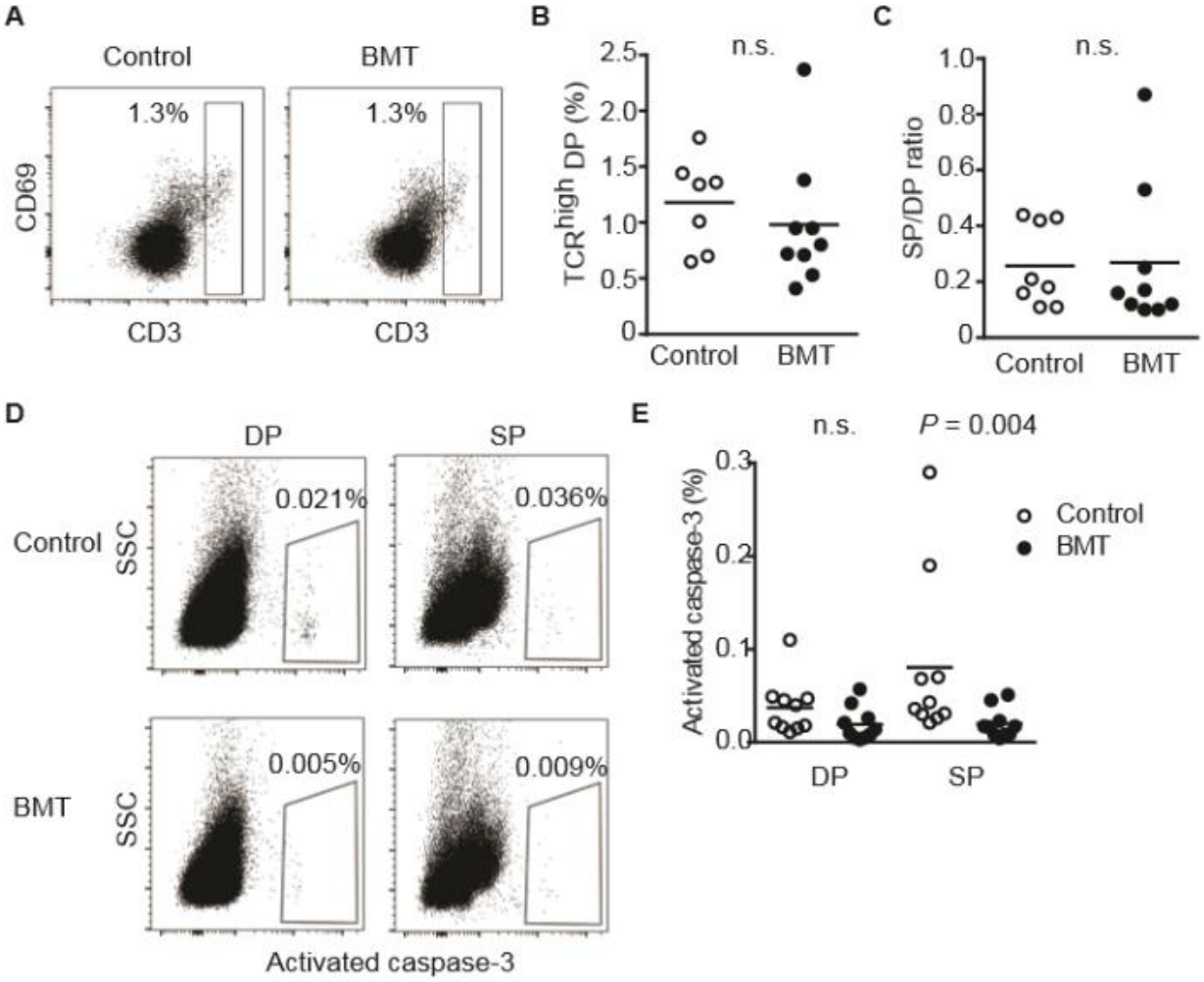

Figure 3.2 Negative selection is specifically impaired in post-transplant mice.Thymic selection was assessed in TCD BMT mice 4 weeks after transplantation by flow cytometry. (A) Representative data of evaluation of positive selection by quantifying $CD3^{high}$ DP thymocytes. (B) Comparison of $CD3^{high}$ DP thymocyte frequency from TCD BMT and age-matched control mice from 3 independent experiments. (C) Comparison of SP thymocyte / DP thymocyte ratio (gated as Fig.3.1A) for thymocytes from TCD BMT and age-matched control mice from 3 independent experiments. (D) Representative data of negative selection analysis by intracellular labeling for cleaved caspase-3. (E) Comparison of cleaved caspase-3^{+} DP and SP thymocyte frequency from TCD BMT and age-matched control mice from 3 independent experiments. All data from TCD BMT and control mice compared using Mann-Whitney.

The difference in effect on apoptosis between SP and DP thymocytes suggested that irradiation-induced changes in the post-transplant thymus may differentially affect the cortical and medullary compartments. We examined the effects of pre-transplant irradiation in our model by histologic and flow cytometry analyses of thymic stromal cells from mice after TCD bone marrow transplantation (n = 3 – 9/group per time point). Histological analysis of the thymus demonstrated peri-transplant hypocellularity, particularly of cytokeratin $K5^+$ medullary epithelial cells (mTECs) and disruption of the cortico-medullary architecture (Fig. 3.3A). Hypocellularity persisted throughout the 20 weeks following transplant, though the cortico-medullary architecture began to be restored as early was 4 weeks after transplant. While the absolute number of thymic epithelial cells (TECs) was reduced after pre-transplant irradiation, quantitative analysis of TEC populations by flow cytometry (Fig. 3.3B) identified a disproportionate loss of $EpCAM^+CD45^-UEA\text{-}1^+MHC\text{-}II^+$ mTECs in transplanted mice (13.8 $\pm$ 2.1%, mean + s.e.m.) as compared to age-matched controls (16.9 $\pm$ 1.0%, $P = 0.035$) (Fig. 3.3C). $EpCAM^+CD45^-Ly51^+MHC\text{-}II^+$ cortical TECs (cTECs) were present in transplanted mice at frequencies similar to control mice (26.2 $\pm$ 2.8% vs. 28.4 $\pm$ 2.7%, $P = 0.740$). This selective loss of mTECs explains the specific defect in negative selection of SP thymocytes observed in mice after transplantation (Fig. 3.2F), and suggests that defective negative selection may facilitate dual TCR T cells evasion of negative selection.

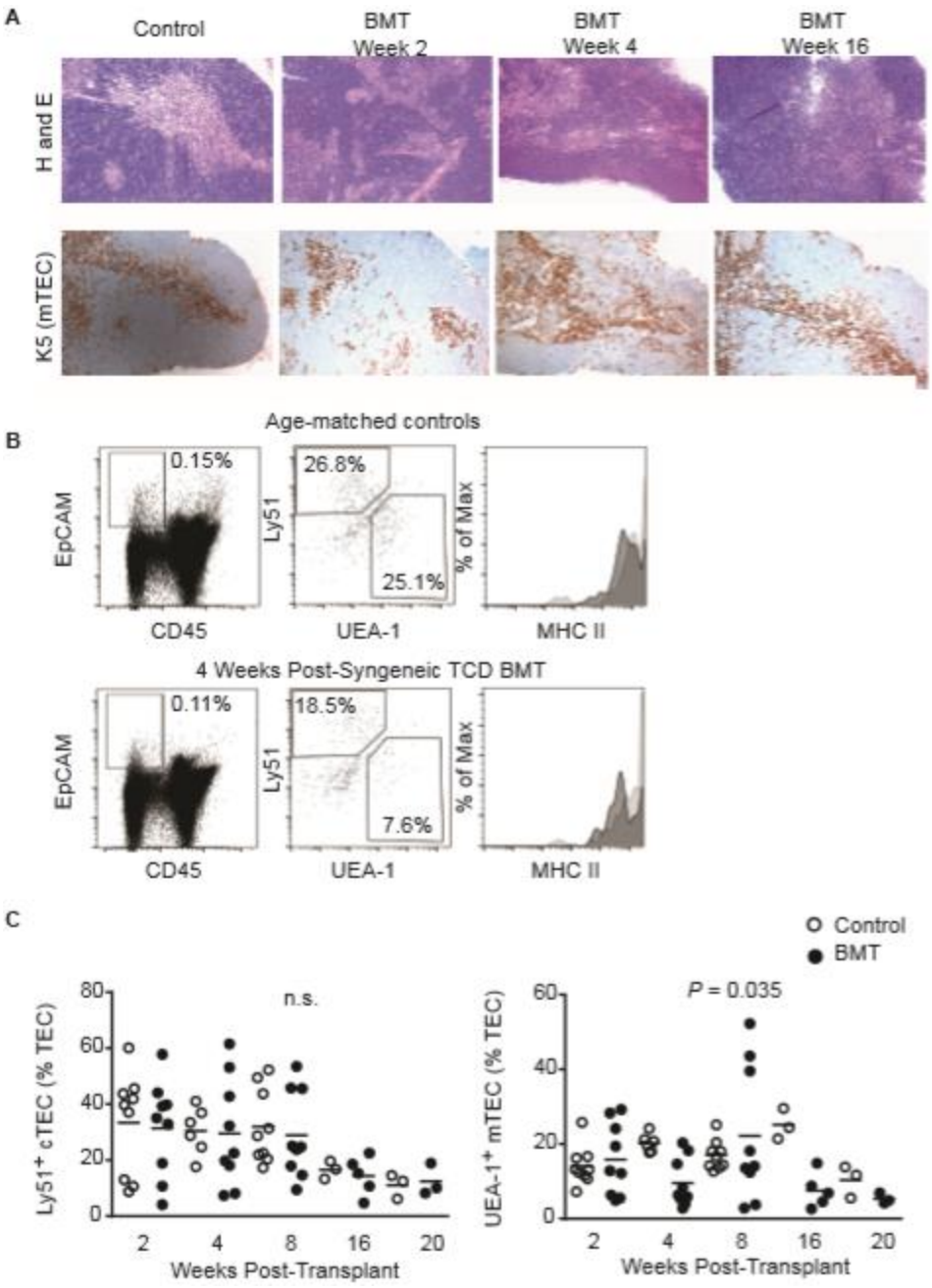

Figure 3.3 Pre-transplant irradiation specifically affects mTEC compartment. Effects of pre-transplant irradiation on thymic stroma were assessed by histological and flow cytometry analyses at 2, 4, 8, 16, and 20 weeks after transplantation. (A) Thymic architecture was assessed by H & E staining, and mTEC cellularity was assessed by immunohistochemical analysis. Representative examples of 1 TCD-BMT and 1 age-matched control mouse shown per time point. (B) $EpCAM^{+}CD45^{-}MHC\text{-}II^{+}Ly51^{+}$ cTECs and $EpCAM^{+}CD45^{-}MHC\text{-}II^{+}UEA\text{-}1^{+}$ mTECs were quantified by flow cytometry. Representative examples shown. (C) Quantification of cTECs and mTECs by flow cytometry. Data shown are individual mice from 3 independent experiments compared by 2-way ANOVA with multiple comparisons.

3.4.2 Increased thymic production of dual TCR T cells affects the post-transplant peripheral T cell repertoire

Increased thymic generation of dual TCR T cells (Fig. 3.1) presents the possibility of significant risk for changes to the post-transplant T cell repertoire that could contribute to cGVHD. We assessed the emergence of dual TCR T cells during reconstitution of the peripheral T cell compartment 2, 4, 8, 16, and 20 weeks after TCD bone marrow transplantation (n = 4 - 11 mice per group at each time point) by flow cytometry for $CD4^+$ and $CD8^+$ splenocytes co-expressing TCRVα2, TCRVα3.2, or TCRVα8.3 (Fig. 3.4A). Peripheral $CD4^+$ and $CD8^+$ T cells derived from post-transplant thymopoiesis were measurable by 2 weeks post-transplant, and continued to increase in number over the 20 week study period (Fig. 3.4B). Dual TCR T cells were detected at 17.6% higher frequencies in transplanted mice ($18.0 \pm 1.1/10^4$ $V\alpha^+$ T cells, mean $\pm$ s.e.m.) after transplant as compared to age-matched control mice ($15.3 \pm 0.8/10^4$, $P = 0.012$) (Fig. 3.4C). These data demonstrate that the increased production of dual TCR T cells associated with the post-transplant impairment in negative selection results in an identifiable and measurable change in the peripheral T cell repertoire associated with increased risk for pathogenic reactivity.

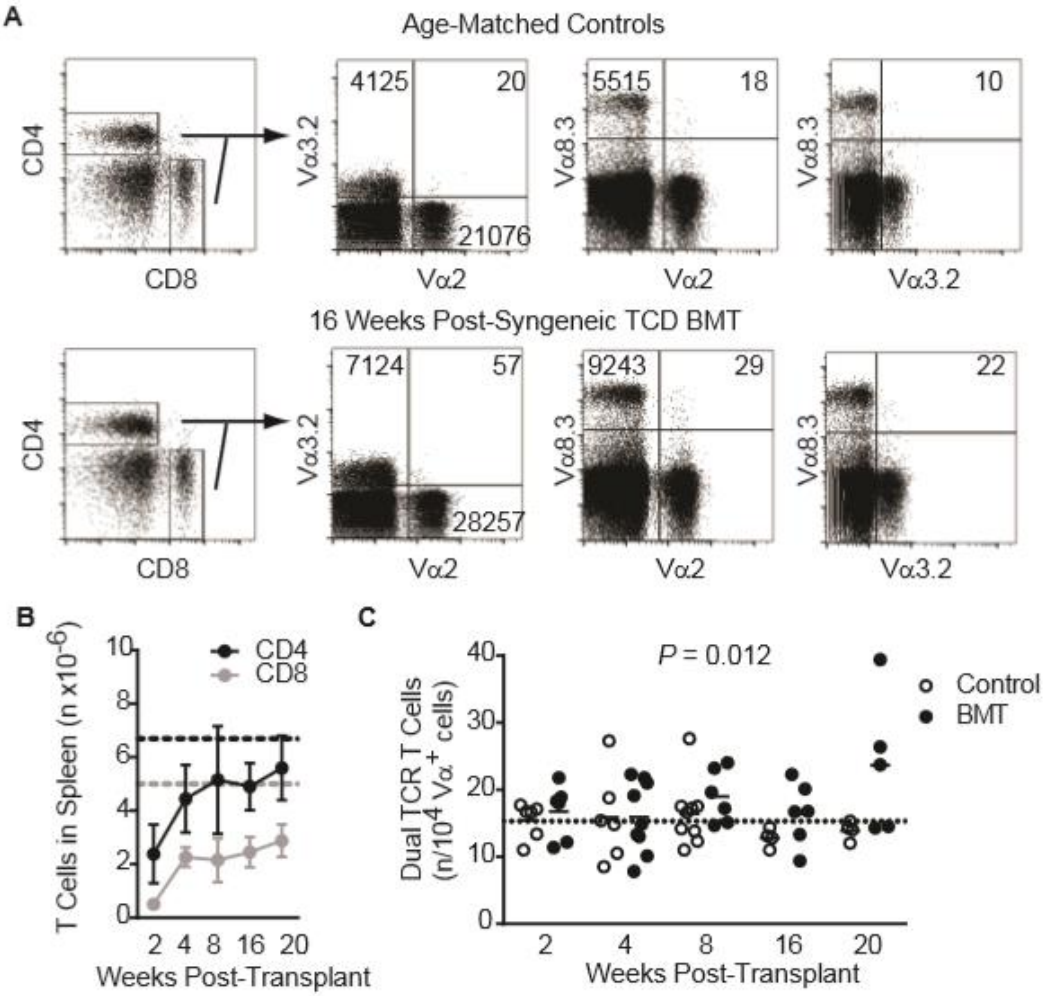

Figure 3.4 Dual receptor cells are increased among peripheral T cells derived after transplantation. Peripheral T cell repopulation in TCD-BMT mice was assessed by examining splenocytes at 2, 4, 8, 16, and 20 weeks after transplantation. (A) Dual TCR T cells were identified among $CD4^+$ and $CD8^+$ splenocytes by flow cytometry using pair-wise labeling with antibodies recognizing Vα2, Vα3.2, and Vα8.3. Representative flow cytometry plot of $CD4^+$ and $CD8^+$ T cells from a mouse 16 weeks post-transplantation and an age-matched control. (B) Enumeration of CD4 and CD8 T cells in spleen. Data shown as mean $\pm$ s.e.m. for BMT mice at each time point (n = 5-9) from 3 independent experiments. Line represents mean of age-matched control mice. (C) Dual TCR cells were quantified as the number of $V\alpha2^+V\alpha3.2^+$, $V\alpha2^+V\alpha8.3^+$, or $V\alpha3.2^+V\alpha8.3^+$ cells among all $V\alpha2^+$, $V\alpha3.2^+$, or $V\alpha8.3^+$ cells. Data shown are individual mice from 3 independent experiments. Dual TCR cell frequencies were compared by 2-way ANOVA with multiple comparisons.

3.5 Discussion

The pathogenic link between incomplete negative selection and development of systemic inflammatory disease characterizing cGVHD led us to investigate the potential involvement of the subset of T cells naturally co-expressing 2 TCRs. Dual receptor T cells contain a unique repertoire of TCR clonotypes (*22*) that likely originate from the reduced stringency for thymic selection parameters in these cells; only 1 of the 2 TCRs is required to mediate positive selection (*23, 24*) and TCR co-expression can mask autoreactive receptors from negative selection (*25-27*).These unique TCRs selectively contain high frequencies of reactivity against alloantigens and autoantigens (*22, 28*) which imparts potential to drive pathologic alloreactive and autoimmune responses in animal models of autoimmunity (*27, 29*) and aGVHD (*22, 24*). Thus, we hypothesized that altered thymic selection resulting from thymic stroma damage resulting from pre-HSCT conditioning could result in generation of this specific subset of cells with high risk for the types of immune responses driving cGVHD.

Our data demonstrate that thymic production of dual TCR cells is persistently increased following lethal irradiation and subsequent transplantation of TCD-bone marrow cells in a syngeneic mouse model (Fig. 3.1). This model enables attribution of changes in thymic selection to the effects of pre-transplant irradiation and avoids complication by alloimmune damage to thymic stroma. Increased post-transplant dual TCR cell production is associated with a significant defect in negative selection of SP thymocytes (Fig. 3.2) attributable to persistent selective loss of mTECs in the post-transplant thymus (Fig. 3.3). The loss of mTECs and subsequent defect in negative selection in our model was predicted by previous demonstrations of the specific effects of radiation on mTECs (*32*). We propose that differences in post-transplant death of DP and SP thymocytes reflects the differential effects of pre-transplant radiation on

cTECs and mTECs, though it may also indicate differences in the biology of these cells. Apoptosis of SP thymocytes results from strong interaction with self-pMHC ligands primarily in the medulla while DP thymocytes can undergo apoptosis as a consequence of an inability to find a positively-selecting ligand or by negative selection (*2*). We attempted to differentiate between these possibilities in activated caspase-3^+ DP thymocytes by examining PD-1 expression as a marker of apoptosis induced through active TCR signaling (*6*). We consistently observed PD-1 expression by activated caspase-3^+ SP thymocytes (data not shown), but were unable to consistently do so in DP thymocytes, precluding dissection of death by neglect from negative selection of DP cells. These different possibilities may have implications for the effects on the TCR repertoire, as negative selection of DP thymocytes has been demonstrated to primarily affect cells with highly cross-reactive TCRs (*6*) while negative selection of SP thymocytes by mTECs primarily eliminates cells bearing TCRs with strong reactivity to peripheral tissue antigens (*38*). Nonetheless, the effect on dual TCR thymocyte selection provides a specific marker to measure generation and integration into the peripheral repertoire of cells with described pathogenic potential. It has become increasingly evident that cGVHD is a consequence of defects in thymic regeneration of the T cell repertoire after transplantation. Animal models have demonstrated that thymopoiesis is impaired after transplantation through a variety of mechanisms including decreased seeding of the thymus with lymphocyte precursors, quantitatively impaired production of mature T cells causing restriction of the T cell repertoire, reduced efficacy of negative selection enabling emergence of potentially autoreactive T cells, and impaired generation of regulatory T cells. It is likely that all of these mechanisms contribute to development of pathogenic cellular and humoral immune-mediated multi-organ dysfunction. However, a direct link between thymic dysfunction and cGVHD pathogenesis in human subjects

has proven elusive due to the substantial heterogeneity of the T cell compartment. Our data presented here indicate dual TCR T cells as a marker to examine effects of HSCT on the generation of T cells with increased pathogenic potential. We propose that identification of dual TCR cells as a specific and measurable subset associated with cGVHD provides an opportunity to directly examine differential effects of pre-transplant conditioning, both in human patients and animal models, on restoration of a protective and self-tolerant T cell repertoire necessary for optimal long-term clinical outcomes.

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Chapter 4: Endogenous co-expression of two T cell receptors promotes lymphopenia-induced proliferation via increased affinity for self-antigen

4.1 Abstract

Approximately 10% of peripheral T cells express 2 functional T cell receptor (TCR) αβ 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 co-transfer 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.

4.2 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 (3). Dual TCR expression is predominantly (~90%) the result of allelic inclusion of both TCRα loci in double-positive $CD4^+CD8^+$ (DP) thymocytes prior to positive selection (2, 4). This can produce in-frame rearrangements of *TRAV* and *TRAJ* gene segments on both chromosomes, each producing TCRα protein capable of pairing with the thymocyte's single TCRβ to form 2 functional TCRαβ heterodimers (5). The allelic inclusion of TCRα provides an advantage in the ability of DP 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). These effects result in dual receptor T cells harboring unique TCR clonotypes that cannot be and are not present in the absence of dual receptor expression (7).

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 peripheral 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 (reviewed in (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_{38-49}: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_{66-77}:A^b. These data suggest that dual receptor expression specifically affects the T cell repertoire by promoting recognition of ligands other than the typical "cognate" foreign antigen presented by self-MHC, as constrained via 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, 15). TCR signaling induced via low-affinity interaction with self-pMHC ligands is a principal requirement for the persistence of T cells in the periphery (16, 17). These TCR:self-pMHC interactions deliver TCR pathway signals that combine with cytokine receptor signals to rescue cells from apoptosis and promote proliferation (18, 19). 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 (20, 21), have stronger responses to homeostatic cytokine signals and have higher rates

of homeostatic proliferation compared to cells with lower expression of CD5 (19, 22-24). 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 (25) or where homeostatic expansion is stimulated such as iatrogenic lymphodepletion in transplantation (26). 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 (27-29). 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 (reviewed (13)). The effect of homeostatic expansion on T cells with increased reactivity for self-antigens, as well as the importance of homeostasis in transplantation, led us to investigate the effects of dual TCR co-expression on homeostasis.

4.3 Material and Methods

4.3.1 Mice

C57BL/6 (B6), B6.Ly5.1, and B6.Thy1.1 mice were originally purchased from Charles River Laboratories. B6.129S2-Tcra^{tm1Mom}/J (30) (B6.TCR$\alpha^{-/-}$) MHC II-deficient B6.129S2-H2$^{dlAb1\text{-}Ea}$/J (B6.MHCII$^{-/-}$) (31) mice were originally purchased from the Jackson Laboratory. 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.

4.3.2 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). Donor T cell populations were examined by flow cytometry to ensure 1:1 ratio. A total of 2 x 10^6 carboxyfluorescein succinimidyl ester (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 from mice at 8 and 28 days after transfer and analyzed by flow cytometry.

4.3.3 Flow cytometry

Peripheral T cells were isolated from spleens by mechanical disruption. Red blood cells were removed by hypotonic lysis with ACK lysis buffer. Prior to transfer, donor cells were labeled with 5 μM CFSE. Donor peripheral T cells were identified using antibodies against congenic markers Ly5.1(A20)-phycoerythrin (PE)-Cy7 and Thy1.1 (OX-7)-allophycocyanin (APC). T cell populations were examined using antibodies against CD4 (GK1.5)-APC-Cy7, CD8 (53-6.7)-PerCP-Cy 5.5, CD44 (IM7)-APC, CD62L (MEL-14)-PE, CD3ε (145-2C11)-fluoroscein isothiocyanate (FITC), CD5 (53.7.3)-PE-Cy7, TCRVα2 (B20.1)-APC, and TCRVα3.2 (RR3-16)-PE (Biolegend, San Diego, CA). All samples were run in batches containing both control

and experimental samples as well as color and fluorescence-minus-one controls. Samples were analyzed using FACSCanto or LSR II instruments with FACSDiva software and data was analyzed using FlowJo v10 software (BD Biosciences, San Jose, CA). Proliferation of transferred T cells was measured by flow cytometry analysis of CFSE dilution. Proliferation index was calculated as the total number of cell divisions divided by the calculated number of precursor cells.

4.3.4 Measurement of ERK phosphorylation

TCR signaling was examined by flow cytometry measuring ERK phosphorylation after *in vitro* stimulation. Single-cell suspensions of peripheral T cells were prepared from spleens and rested for 1-2 hours in RPMI 1640 (HyClone) supplemented with 10% Fetal Bovine Serum (Omega Scientific) at 37^{o}C. After resting, cell were stimulated using either Phorbol myristate acetate (PMA) and ionomyclin or anti-CD3 and anti-CD28. PMA/ionomycin stimulation was performed by addition of PMA (100 ng/ml) and ionomycin (1 μg/ml) Sigma, Saint Louis, MO) and incubation for 15 minutes. CD3/CD28 stimulation was performed by addition of 10 μg/ml biotinylated anti-CD3ε and 10 μg/ml biotinylated anti-CD28 (37.51, Biolegend) for 7 minutes, followed by addition of 5 μl streptavidin microbeads (Dynabeads M-280, ThermoFisher Scientific, Waltham, MA) and incubation for 10 minutes. Stimulated cells were fixed by adding 350 μl 5x Lyse/Fix buffer (BD Biosciences) for 15 minutes at 37 C. Fixed cells were permeabilized by addition of 500 μl ice-cold methanol, mixed by vigorous vortexing, and incubation on ice for 30 minutes. Fixed and permeabilized cells were labeled with antibodies against CD3 (145-2C11)-FITC and phosphorylated ERK1/2 (pERK) (MILAN8R)-APC (eBioscience, San Diego, CA) for 45 minutes at room temperature in the dark.

4.3.5 Statistical analysis

Data were analyzed using Prism 6 software (GraphPad, La Jolla, CA). Data from individual mice was compared nonparametrically using Mann-Whitney test. Proliferation indices and mean fluorescence intensity (MFI) values and ratios were compared non-parametrically using Wilcoxan rank-sum test. Two-tailed *P* values ≤ 0.05 were considered significant.

4.4 Results

4.4.1 Absence of dual TCR expression causes disadvantage in LIP

Given our previous demonstrations of increased ability of dual TCR cells to recognize autoantigens (7), we hypothesized that dual TCR expression may impart an advantage in homeostatic proliferation resulting from increased recognition of endogenous self-pMHC ligands. Since homeostasis depends on multiple factors including niche space, the abundance of homeostatic cytokines, and clonal competition for self-pMHC ligands, we utilized a competitive co-transfer 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 d 8 and d 28 post-transfer, and frequencies of B6 and TCR$\alpha^{+/-}$ T cells recovered from the spleen were determined by flow cytometry (Fig. 4.1A).

In the aLIP model, $CD4^+$ and $CD8^+$ $TCR\alpha^{+/-}$ cells were consistently found at significantly lower frequencies than their co-transferred B6 counterparts (Fig. 4.1B). At d 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 $\pm$ 0.05 (mean + s.e.m., $P = 0.002$) and $CD8^+$ cells at a ratio of 0.62 $\pm$ 0.10 ($P = 0.002$). This decreased homeostatic competitiveness for $TCR\alpha^{+/-}$ cells was further exacerbated at d 28, with the ratio of $TCR\alpha^{+/-}$ / B6 $CD4^+$ cells decreasing to 0.42 $\pm$ 0.07 ($P = 0.002$, compared to d 8) and the ratio of $CD8^+$ cells decreasing slightly to 0.58 $\pm$ 0.07 (n.s. compared to d 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. 4.1C). At d 8 post transfer, the ratio of $TCR\alpha^{+/-}$ / B6 $CD4^+$ cells recovered was 0.60 $\pm$ 0.08 ($P = 0.003$) and at day 28 the ratio was 0.52 $\pm$ 0.10 (n.s. compared to d 8). $CD8^+$ T cell ratios were highly variable, and were not significantly different from the input ratio of 1.00 at either d 8 (0.88 $\pm$ 0.23) or d 28 (0.62 $\pm$ 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.

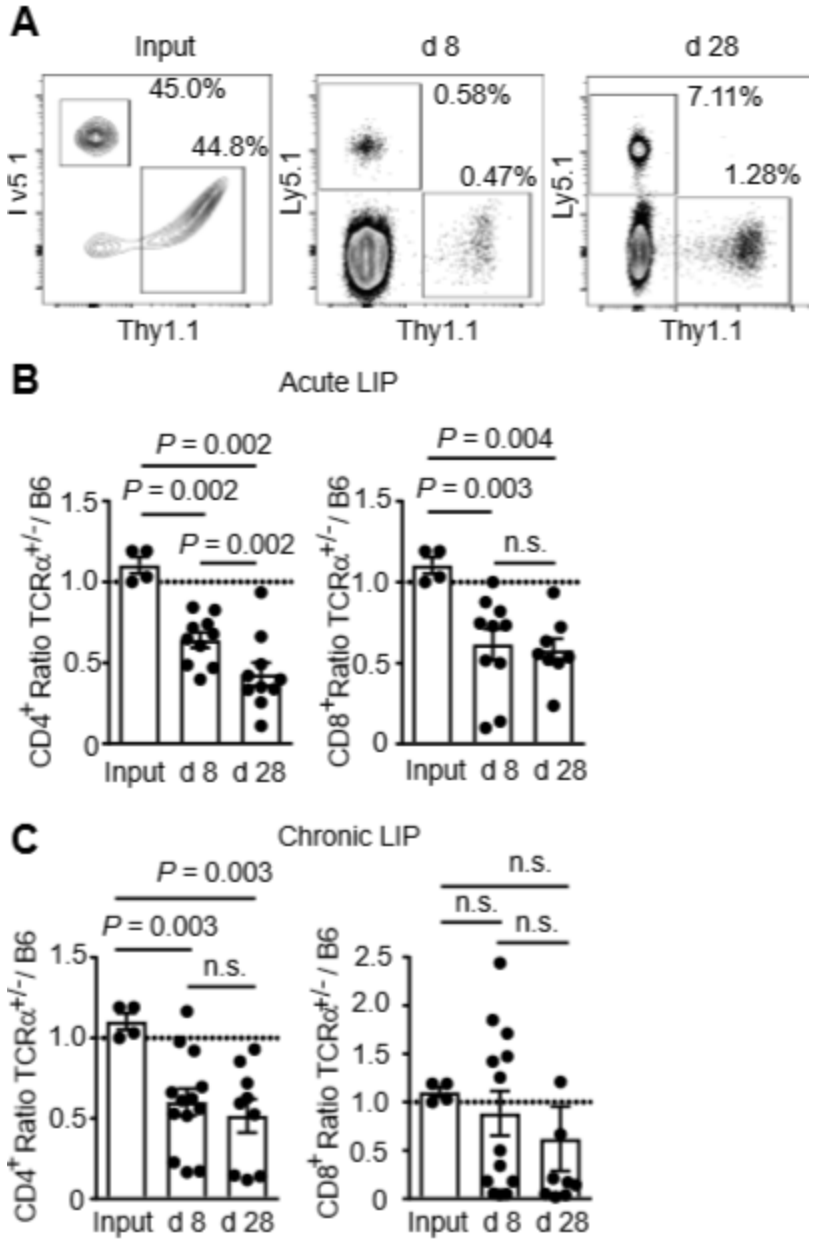

Figure 4.1 Loss of secondary TCR expression reduces homeostatic proliferation in LIP (A) Representative flow cytometry plots showing congenically marked B6.Ly5.1 and B6.TCR$\alpha^{+/-}$.Thy1.1 cells transferred at a 1:1 ratio into sublethally irradiated B6 recipients and recovered at d 8 and d 28 post-transfer. (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 d 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 that the disadvantage of TCR$\alpha^{+/-}$ T cells in aLIP and cLIP models (Fig 4.1) was due to decreased proliferation, proliferation was assessed by CFSE dilution at d 8 (Fig. 4.2A). Proliferation was quantified by calculating the proliferation index for transferred cells. Consistent with the advantage of wild-type T cells in aLIP (Fig. 4.1B), T cells from B6 mice proliferated significantly more robustly than TCR$\alpha^{+/-}$ T cells (proliferation index 1.85 $\pm$ 0.27 divisions/input cell compared to 1.49 $\pm$ 0.08 divisions/input cell, $P = 0.020$) in aLIP (Fig. 4.2B). Conversely, there was no difference in proliferation indices between B6 (4.61 $\pm$ 0.28 divisions/input cell) and TCR$\alpha^{+/-}$ T cells (4.44 $\pm$ 0.35 divisions/input cell) in cLIP (Fig. 4.2C), underscoring the limited effect of dual TCR expression in cLIP (Fig. 4.1C). Together, we interpreted these results demonstrating deficient homeostasis in TCR$\alpha^{+/-}$ T cells as indicating that the presence of dual TCR expressing T cells enhanced the proliferative response in aLIP.

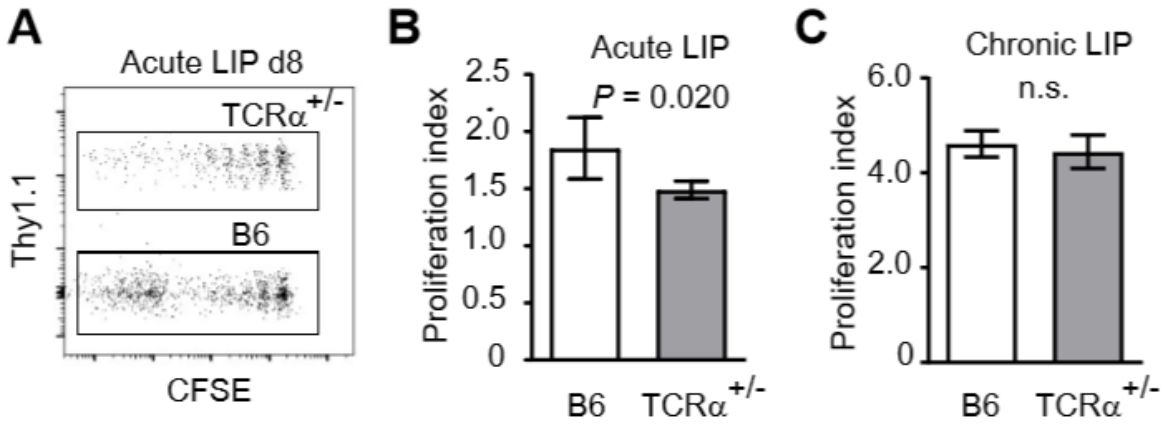

Figure 4.2 Homeostatic advantage for wild-type T cells in lymphopenic conditions is due to increased proliferation (A) Representative flow cytometry plot of cell proliferation measured by CFSE at d 8 post-transfer into acutely lymphopenic hosts. (B) Difference in LIP is quantified by comparing ratio of TCR$\alpha^{+/-}$ T cell/B6 T cell proliferation index d 8 post-transfer into sublethally irradiated B6 recipients or (C) chronically T cell-deficient TCR$\alpha^{-/-}$ mice. Data are shown as mean $\pm$ s.e.m. of TCR$\alpha^{+/-}$ T cell/B6 T cell proliferation index ratio from 10 recipient mice from 3 independent experiments. Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

4.4.2 Decreased LIP in the absence of dual TCR expression is not attributable to differences in memory T cell compartments

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. 4.3A). Comparison of B6 and $TCR\alpha^{+/-}$ T cells did not demonstrate significant differences in the frequency of naive $CD4^+$ cells (66.97 $\pm$ 8.16% vs. 64.45 $\pm$ 8.24%), naive $CD8^+$ cells (80.51 $\pm$ 5.28% vs. 78.98 $\pm$ 3.68%), EM $CD4^+$ cells (28.79 $\pm$ 2.33% vs. 31.84 $\pm$ 2.34%), EM $CD8^+$ cells (8.99 $\pm$ 1.34% vs. 10.42 $\pm$ 1.40%), CM $CD4^+$ cells (4.24 $\pm$ 0.47% vs. 3.72 $\pm$ 0.45%), or CM $CD8^+$ cells (10.60 $\pm$ 1.50%) (Fig. 4.3B). 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.

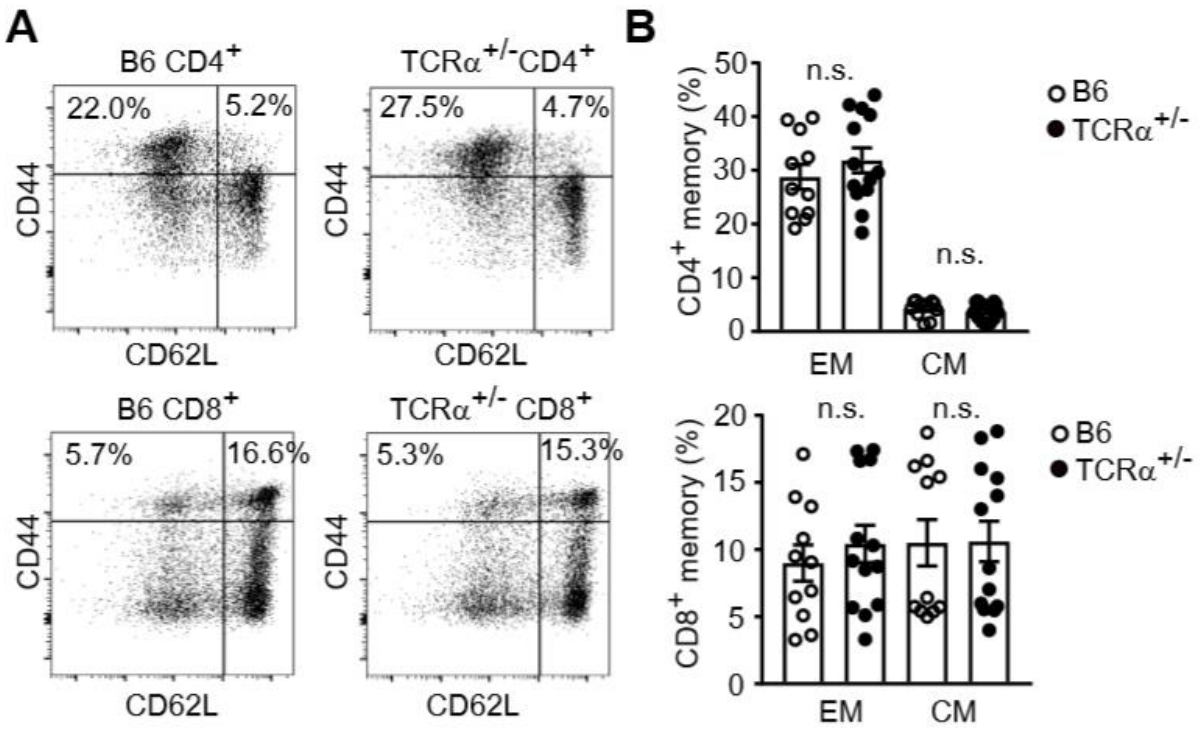

Figure 4.3 Absence of dual TCR expression does not affect naive or memory T cell populations. (A) representative flow cytometry plots of naive ($CD44^-$), effector memory (EM, $CD44^+CD62L^-$), and central memory (CM, $CD44^+CD62L^+$) $CD4^+$ and $CD8^+$ T cells from B6 and $TCR\alpha^{+/-}$ mice. (B) 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.

4.4.3 Elimination of dual TCR expression does not alter total TCR expression or TCR signaling

Co-expression of dual TCRs results in competition of TCRαβ heterodimer clonotypes for shared components of the TCR signaling complex (32). We have previously shown that naturally-arising dual TCR cells do not have increased total TCR expression or general responsiveness through the TCR (7, 33, 34). We have similarly shown that genetic elimination of dual TCR expression using TCR$\alpha^{+/-}$ mice does not affect non-specific activation, proliferation, or functional response through the TCR via anti-CD3 antibody (7, 9). However, we wanted to rule out the possibility that variations in the amount of total TCR on the cell surface caused by TCRα heterozygosity could possibly alter TCR signaling affecting homeostasis and LIP. To accomplish this, we analyzed CD3 expression on the surface of T cells from B6 or TCR$\alpha^{+/-}$ mice (Fig. 4.4A). Analysis of the mean fluorescent intensity (MFI) of CD3 labeling by flow cytometry showed that T cells from B6 (n = 9) or TCR$\alpha^{+/-}$ (n = 10) mice express the same amount of total TCR (Fig. 4.4B). 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. 4.4C), which illustrates the consistent lack of difference in total TCR expression by TCR$\alpha^{+/-}$ T cells (1.05 $\pm$ 0.07 TCR$\alpha^{+/-}$ / B6 CD3 MFI). Similarly, examination of CD3 expression by TCRV$\alpha2^{+}$V$\alpha3^{+}$ dual TCR cells (Fig. 4.4D) from B6 mice did not demonstrate any differences when compared to TCRV$\alpha2^{+}$ or TCRV$\alpha3^{+}$ T cells from B6 mice (ratio CD3 MFI dual TCR/V$\alpha2^{+}$ cells 0.99 $\pm$ 0.06) (Figs. 4.4E,F).

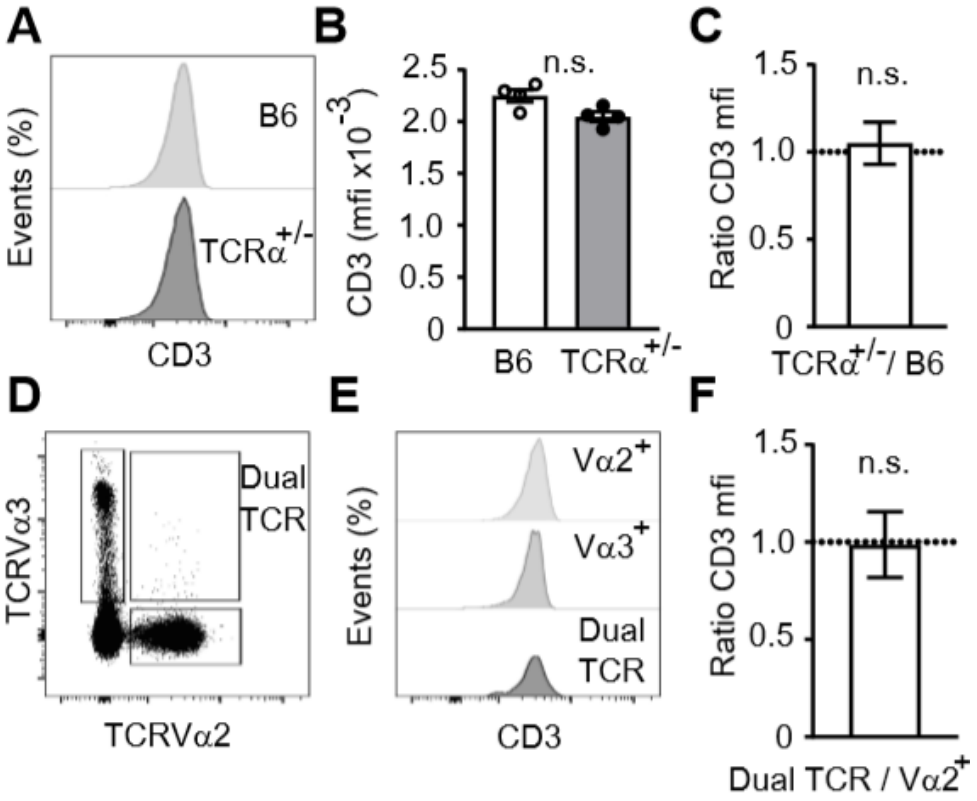

Figure 4.4 Neither elimination of secondary TCRs nor co-expression of dual TCRs alters total TCR expression. (A) Representative flow cytometry histogram comparing expression of CD3 as a measure of total TCR complex in B6 or TCR$\alpha^{+/-}$ T cells. (B) Quantification of CD3 expression by mean fluorescence intensity (MFI) on B6 or TCR$\alpha^{+/-}$ T cells. Data are individual mice from representative experiment of 3 independent experiments. Statistical analysis performed using Mann Whitney rank-sum test. (C) 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 $\pm$ s.e.m. Statistical analysis performed using Wilcoxon matched-pairs signed rank test. (D) Representative flow cytometry plot showing identification of TCRV$\alpha2^{+}$, TCRV$\alpha3^{+}$, and TCRV$\alpha2^{+}$V$\alpha3^{+}$ dual TCR cells from B6 T splenocytes. (E) Representative flow cytometry histogram comparing expression of CD3 as a measure of total TCR complex in TCRV$\alpha2^{+}$, TCRV$\alpha3^{+}$, and TCRV$\alpha2^{+}$V$\alpha3^{+}$ dual TCR cells (F) CD3 expression across three independent experiments (n = 9 mice) was compared by analysis of ratio CD3 MFI for TCRV$\alpha2^{+}$V$\alpha3^{+}$/TCRV$\alpha2^{+}$ T cells for individual mice. Data shown as mean $\pm$ s.e.m. Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

Next, we examined ERK phosphorylation in B6 and TCR$\alpha^{+/-}$ T cells as a measure of TCR signaling capability. ERK phosphorylation (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. 4.5A). 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 $\pm$ 1.74% pERK^{+} in B6 T cells compared to 39.88 $\pm$ 3.31% in TCR$\alpha^{+/-}$ T cells responding to PMA/ionoycin; 16.20 $\pm$ 1.01% pERK+ B6 T cells compared to 14.57 $\pm$ 3.8% TCR$\alpha^{+/-}$ T cells responding to anti-CD3/anti-CD28, n.s.) (Fig. 4.5B). The amount of pERK in each cell was also examined by analysis of pERK MFI, to discern whether differences in signal strength existed between B6 and TCR$\alpha^{+/-}$ T cells (Fig. 4.5C). 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 $\pm$ 101.5 MFI vs. TCR$\alpha^{+/-}$ 1348 $\pm$ 95.47 MFI, n.s.) or anti-CD3/anti-CD28 (B6 1532 $\pm$ 36.51 MFI vs. TCR$\alpha^{+/-}$ 1470 $\pm$ 25.74 MFI, n.s.). Together, we interpret these results, in conjunction with our previous examinations of TCR expression and responsiveness to non-specific 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.

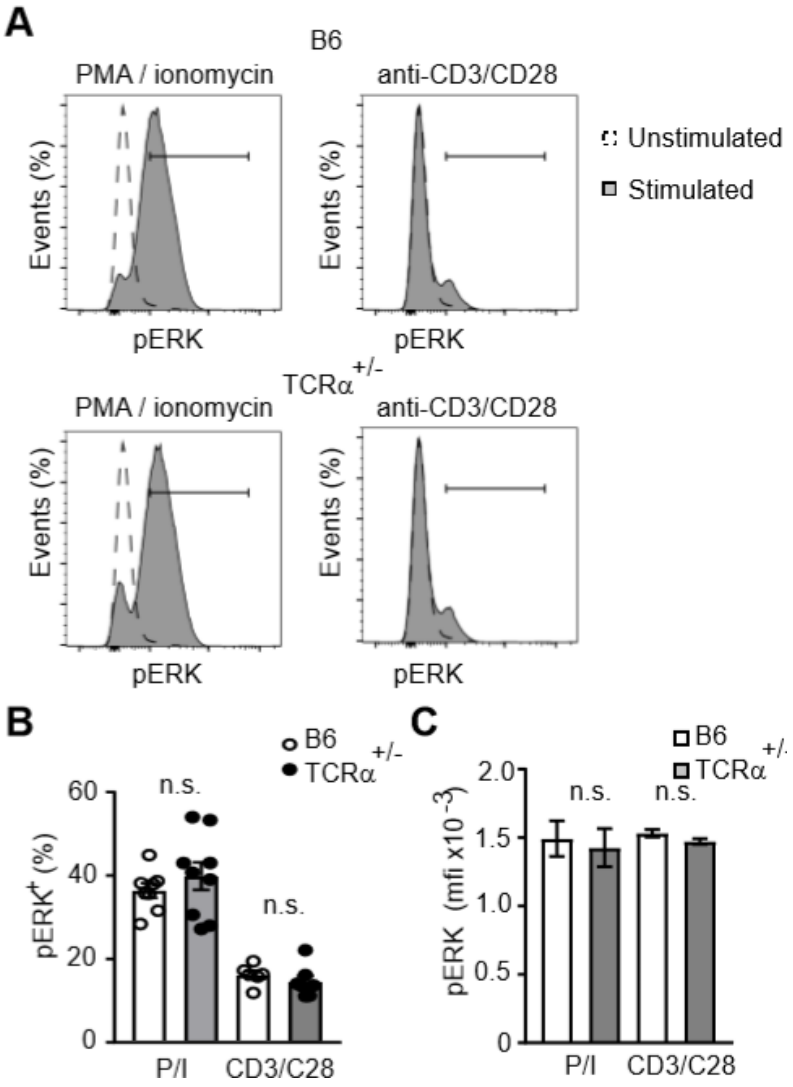

Figure 4.5 Elimination of secondary TCRs does not alter TCR signaling. (A) TCR signaling was assessed by measuring ERK1/2 phosphorylation 15 min after stimulation with PMA and ionomycin or 10 min after stimulation with anti-CD3 and anti-CD28 bound to microsspheres. Representative flow cytometry histogram comparing phosphorylation of ERK1/2 in B6 or TCRα$^{+/-}$ T cells. (B) Percentage of B6 and TCRα$^{+/-}$ 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. (C) Quantification of ERK phosphorylation by mean fluorescence intensity (MFI) on B6 or TCRα$^{+/-}$ T cells. Data are mean $\pm$ s.e.m. from all experiments (n = 9-10 mice/group). Statistical analysis performed using Mann Whitney rank-sum test.

4.4.4 Decreased LIP by TCR$\alpha^{+/-}$ T cells results from absence of dual TCR cells with increased affinity for self-pMHC

The decreased ability of TCR$\alpha^{+/-}$ T cells to undergo LIP, particularly in the aLIP model (Fig. 4.1), suggests a defect in the ability of these cells to compete for factors necessary to promote homeostasis. The specificity of this finding for aLIP, combined with our previous findings demonstrating that TCR$\alpha^{+/-}$ thymocytes have a decreased ability to mediate low-affinity interactions with self-pMHC ligands in the thymus necessary for positive selection (7), suggested that the deficit in aLIP may be attributable to a decreased affinity of TCR$\alpha^{+/-}$ T cells for self-pMHC ligands. To formally test 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. 4.6, ratio TCR$\alpha^{+/-}$/B6 CD4^{+} T cells 0.88 $\pm$ 0.03) compared to wild-type recipients (0.64 $\pm$ 0.05, $P < 0.001$), indicating that recognition of self-pMHC is the mechanistic cause for differences in LIP attributable to dual TCR expression.

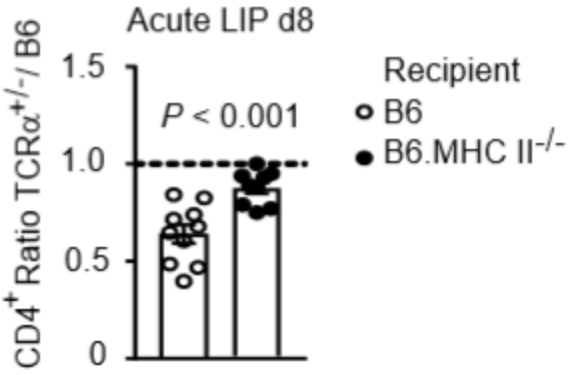

Figure 4.6 Homeostatic advantage of dual TCR expression depends on interaction with self-pMHC. Effects of homeostatic proliferation were assessed by enumerating congenically marked B6.Ly5.1 and B6.TCR$\alpha^{+/-}$.Thy1.1 cells transferred at a 1:1 ratio into sublethally irradiated B6.MHC $II^{-/-}$ recipients d 8 post-transfer. 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 are presented for comparison. Statistical analyses performed using Mann Whitney rank-sum test.

To evaluate possible differences in affinity for self-pMHC ligands, we measured expression of CD5 on $CD4^+$ and $CD8^+$ T cells from B6 and $TCR\alpha^{+/-}$ mice (Fig. 4.7A). The expression level of CD5 is determined during positive selection based on the TCR affinity to the selecting self-peptide MHC (20, 21), and high CD5 expression is correlated with increased potential for homeostatic proliferation (19, 22-24). Within individual experiments, $CD4^+$ and $CD8^+$ T cells from $TCR\alpha^{+/-}$ mice (n = 11) demonstrated a trend toward decreased CD5 expression as compared to wild-type B6 mice (n = 10) mice (Fig. 4.7B). However, when the ratios of $TCR\alpha^{+/-}$ / B6 T cell CD5 MFI were compared across independent experiments, we did not observe a statistically significant difference in CD5 expression (0.97 $\pm$ 0.05 $TCR\alpha^{+/-}$ / B6 $CD4^+$ CD5 MFI, 1.00 $\pm$ 0.07 $TCR\alpha^{+/-}$ / B6 $CD4^+$ CD5 MFI) (Fig. 4.7C).

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 by $TCRV\alpha2^+V\alpha3^+$ dual TCR $CD4^+$ cells (Fig. 4.4D) as compared to $TCRV\alpha2^+$ or $TCRV\alpha3^+$ $CD4^+$ T cells from B6 mice (n = 11) (Fig. 4.7D). 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 a trend for increased expression of CD5 in individual experiments (Fig. 4.7E) that was statistically significant when the data were examined across experiments (ratio CD5 MFI dual TCR/$V\alpha2^+$ cells 1.25 $\pm$ 0.11, P = 0.039) (Fig. 4.7F). These findings support the hypothesis that dual TCR expression causes increased affinity for self-pMHC ligands, such as those necessary for positive selection (7) or aLIP. The absence of dual TCR cells in $TCR\alpha^{+/-}$

mice removes these cells with increased affinity for self-pMHC, providing a mechanistic explanation for the deficit in LIP.

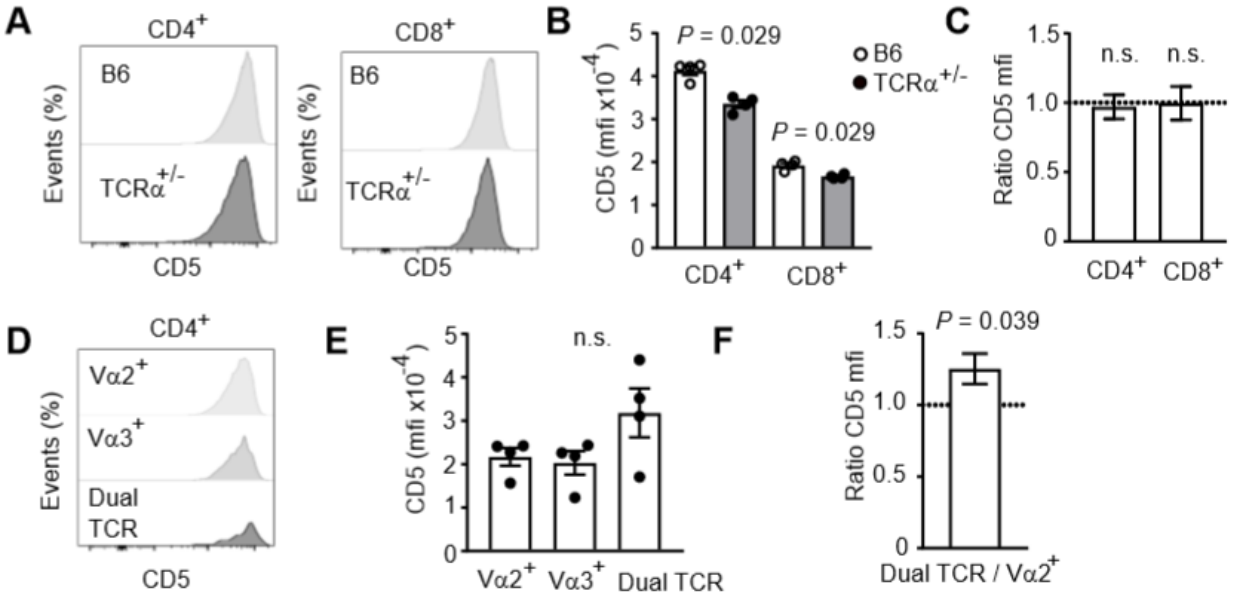

Figure 4.7 Dual TCR cells have higher expression of CD5, indicating increased affinity for self-antigens. (A) Representative flow cytometry histogram comparing CD5 expression by $CD4^+$ and $CD8^+$ T cells from B6 or $TCR\alpha^{+/-}$ mice. (B) Quantification of CD5 expression by mean fluorescence intensity (MFI) on B6 and $TCR\alpha^{+/-}$ T cells. Data are individual mice from representative experiment of 3 independent experiments. Statistical analysis performed using Mann Whitney rank-sum 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 $\pm$ s.e.m. Statistical analysis performed using Wilcoxon matched-pairs signed rank test. (D) Representative flow cytometry histogram comparing expression of CD5 as a measure of total TCR complex in $TCRV\alpha2^+$, $TCRV\alpha3^+$, and $TCRV\alpha2^+V\alpha3^+$ dual TCR cells (E) CD5 expression by $TCRV\alpha2^+$, $TCRV\alpha3^+$, and $TCRV\alpha2^+V\alpha3^+$ T cells was quantified by MFI. Data shown are individual mice from representative experiment. (F) CD5 expression across three independent experiments (n = 11 mice) was compared by analysis of ratio CD5 MFI for $TCRV\alpha2^+V\alpha3^+/TCRV\alpha2^+$ T cells for individual mice. Data shown as mean $\pm$ s.e.m. Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

4.5 Discussion

Previous examinations of mice genetically deficient for dual TCR expression have not identified overt T cell compartment defects (7, 9, 35), 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. Previously, we observed in a competitive model that the absence of dual TCR expression results in a significant deficit in the ability of developing thymocytes to recognize positively selection self-pMHC ligands in the thymus and mature to single-positive thymocytes (7). The same types of low-affinity TCR:self-pMHC interactions required for positive selection, potentially even the same ligands, are important in peripheral homeostasis (16 , 17). This prompted us to use a similar competitive co-transfer approach to examine the effects of dual TCR expression in homeostasis. The significant deficit in homeostatic proliferation by TCR$\alpha^{+/-}$ T cells in aLIP after transfer to sublethally-irradiated mice (Figs. 4.1, 4.2) indicates that the absence of dual TCR expression reduces the ability of T cells to compete for the self-pMHC ligands that are essential for aLIP. 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.

A similar, though less pronounced, effect was observed after transfer to a chronically-lymphopenic environment of T cell-deficient TCR$\alpha^{-/-}$ mice (Figs. 4.1C, 4.2C). The defect in

cLIP was less expected, as cLIP has been attributed to T cell recognition of endogenous commensal bacterial antigens (19, 36), and previous work from our lab has not observed a difference in the ability to recognize foreign antigen by dual TCR cells and T cells lacking dual TCR expression, though we have observed increased cross-reactivity of dual TCR cells for presented peptide antigens (7). However, our interrogation of the antigenic reactivity of dual TCR cells and T cell repertories lacking dual TCR expression is not exhaustive, and it is unknown whether dual TCR expression affects recognition of antigens derived from endogenous commensal organisms. Studies examining this question, particularly given the importance of endogenous commensal organism antigens in establishing and maintaining immunologic tolerance (37) in the gut may be important for understanding the loss of tolerance in disease states.

The deficit in homeostatic proliferation by TCR$\alpha^{+/-}$ T cells was not attributable to differences in expression of total TCR complex at the cell surface (evidenced by CD3 expression) (Fig. 4.4) or TCR signaling as measured by ERK phosphorylation (Fig. 4.5). These data are consistent with previous demonstrations that dual TCR expression does not affect total TCR expression or general responsiveness through the TCR (7, 9, 33, 34). In the setting of dual TCR cells, the 2 TCRα (or TCRβ) chains are in competition to pair with the single partner chain to form a heterodimer and the TCR:CD3 signaling complex (32). While the overall stoichiometry must be regulated by expression of the limiting shared components (38), it is unclear what factors regulate TCR$\alpha\beta$ heterodimer formation and whether the ratios of heterodimers present are stable or are dynamic during the life of a T cell. While fluctuations in the TCR$\alpha\beta$ heterodimer composition would not affect the overall abundance or activity of T cell signaling, they could possibly influence the antigenic reactivity of dual receptor cells.

Attenuation of the effect of dual TCR expression on aLIP in a MHC II-deficient model (Fig. 4.6) supports the hypothesis that the promotion of LIP by dual receptor expression is a result of increased recognition of self-pMHC ligands. The iincreased expression of CD5 by dual TCR cells (Fig. 4.7) further indicates that these cells have increased affinity for self-pMHC ligands, supporting our hypothesis for the mechanism responsible for deficit in LIP by TCR$\alpha^{+/-}$ T cells (Figs. 4.1, 4.2). Importantly, this provides a novel demonstration of the unique contribution of dual TCR cells to shaping the T cell repertoire. The increased affinity for self-pMHC ligands by dual TCR cells provides a beneficial advantage for dual receptor expression in homeostasis as described here, as well as in positive selection (7). Conversely, this reactivity against self-antigens The potential for increased reactivity to self-pMHC underlies the potential for autoreactivity. 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.

The reactivity against self-antigens promoted by dual TCR expression could also have a beneficial effect promoting regulatory T cell (Treg) development and differentiation, as this process depends on high-affinity interaction with self-pMHC ligands (39). The role of dual TCR expression in Treg development and function is as yet unclear. The description that more than 50% of Tregs in human peripheral blood express dual TCRs (40) suggests that the increased reactivity of dual TCR cells against self-antigens may promote differentiation to the Treg lineage, though it is unknown whether this is a result of agonist selection in the thymus or peripheral differentiation to Tregs. It has been shown that elimination of dual TCR expression can increase Treg lineage commitment in the thymus, presumably through forced diversion of thymocytes with autoreactive TCRs to agonist selection or negative selection (35). This suggests

that the increased frequency of dual TCR expression observed in human Tregs may result from peripheral conversion, highlighting an important potential target for immune therapy to enhance immunologic tolerance, particularly in transplantation where T cells respond to self- and allogeneic pMHC ligands in the setting of post-transplant lymphopenia.

4.6 Acknowledgements

Chapter 4, in full is currently being prepared for submission for publication by Balakrishnan, A. Amritha Balakrishnan was the primary investigator and author of this material. I would like to thank Mr Burhan Jama for his assistance and contributions to this paper.

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