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The highly alloreactive nature of dual TCR T cells

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Abstract

Purpose of review—T cells can mediate allograft rejection and graft versus host disease (GVHD), but are necessary for tolerance and protective immunity. Identifying T cell populations differentially responsible for these effects has been a goal in transplant research. This review describes investigation of a small subset of T cells naturally predisposed toward alloreactivity, cells expressing two T cell receptors (TCRs).

Recent findings—Rare peripheral T cells express two $\alpha\beta$ TCRs. Their impact on T cell development and function has been uncertain. Recent work demonstrates an important role for these cells in mouse models and human hematopoietic stem cell transplant (HSCT) patients with acute GVHD. Dual receptor T cells are preferentially activated and expanded *in vitro* and *in vivo* by allogeneic stimulation. Genetic elimination of dual TCR expression results in loss of approximately half of the alloreactive repertoire and impedes the earliest steps of GVHD.

Summary—Identification of dual TCR T cells as predisposed to alloreactivity provides opportunity to examine responses limiting transplantation. Continued investigation will reveal significant fundamental features of T cell alloreactivity and important information about the earliest events determining allograft rejection and self-tolerance.

Keywords

T cell; TCR; alloreactivity; transplantation; GVHD

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

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There are no relevant conflicts of interest to disclose.

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.

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 α , along with a single in-frame TCR β rearrangement [11]. Both TCR α proteins were detected in the cell, providing potential for expression of two $\alpha\beta$ TCR heterodimers. However, only a single $\alpha\beta$ TCR 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 TCR α 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 TCR α is a physiologically normal process.

The first evidence for the functionality of naturally co-expressed TCRs came from examination of TCRV α expression on human peripheral blood lymphocytes [8]. Approximately 10^{-3} – 10^{-4} peripheral blood T cells exhibited coincident labeling with two anti-TCRV α (TCRV α 2, TCRV α 12, or TCRV α 24) monoclonal antibodies (mAbs). Based upon the frequencies of labeling with each of the TCRV α 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 TCR α chains on the cell surface [17,18], and revealed the existence of T cells expressing two TCR β 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.

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) is begun in CD4⁻CD8⁻ double-negative (DN) thymocytes, where the RAG genes act to recombine germline-encoded TCR β variable (V), diversity (D), and joining (J) gene segments to form a functional TCR β protein. Typically, only one TCR β locus is in an open chromatin state to enable recombination, ensuring allelic exclusion [23–25]. After functionality of the TCR β chain is ensured via β -selection, the thymocyte proliferates and progresses to the CD4⁺CD8⁺ double-positive (DP) stage. DP thymocytes recombine the TCR β V and J segments to produce a TCR α protein.

Functionality of the $\alpha\beta$ TCR 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, TCR α gene rearrangement is not subject to the allelic exclusion observed for TCR β [27]. Both TCR α 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 TCR α capable of paring with the TCR β and mediating positive selection.

Consequences of TCR allelic inclusion were first evidenced in transgenic TCR systems. While the presence of a transgenic TCR β results in few endogenous TCR β gene rearrangements, endogenous TCR α rearrangements are relatively common [23,27,32], highlighting differences between TCR β and TCR α 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 TCR α (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 TCR α 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 TCR α 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 TCR β 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, insulinitis 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 MOG38-49:I-A^b by 70% while not affecting response to a foreign antigen, LCMV66-77: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 2).

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 MBP79-87: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 TCR α sequencing revealed the presence of high-frequency TCR α 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 (LCMV66-77:I-A^b), but TCR $\alpha^{+/-}$ T cells had a 70% reduction in recognition of autoantigen (MOG38-49:I-A^b) and 49% and 57% reductions in response to MHC-mismatched alloantigens (CD22654-666:I-E^k and TFR231-244:I-E^k respectively). These differences in antigen-reactive T cell frequency could affect pathogenesis of autoimmune and alloreactive responses.

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 NP366-374:H2-D^b) [45]. T cells isolated from rejected grafts expressed endogenous TCR α and TCR β , 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 TCR α chains (V α 2 and V α 4) and a single TCR β (V β 1). The V α 4V β 1 TCR mediates thymic selection, is reactive to a foreign protein hemoglobin64-76:I-E^k and alloreactive to I-E^p, while the secondary V α 2V β 1 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 E α 52-68:I-A^b [35], while human dual TCR T cells were found to respond to the miHA HA-1^H137-145: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-F1 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 α ^{+/-} 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±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.

Conclusions

The potential for dual TCR T cells to contain unwanted reactivity has been theorized since their discovery. Recent demonstration of the importance of TCR α allelic inclusion for efficient thymic selection indicates secondary TCR α 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 TCR α rearrangements in TCR $\alpha^{+/-}$ 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 TCR $\alpha^{+/-}$ 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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Key Points

- A small percentage of peripheral T cells express two $\alpha\beta$ TCRs. Both of these receptors are functional and can mediate immune responses.
- Dual TCR expression arises naturally as a consequence of simultaneous rearrangement of both TCR α loci which promotes efficient positive selection.
- The TCR repertoire of dual TCR T cells has significantly increased frequencies of receptors recognizing allogeneic ligands. This translates to disproportionate contribution of dual TCR T cells to *in vitro* and *in vivo* alloreactive responses including GVHD.

Figure 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.

Positive Selection

The single TCR must respond to positively-selecting self-pMHC ligand, ensuring self-MHC restriction

Only one TCR must respond to positively-selecting self-pMHC ligand, ensuring self-MHC restriction. Secondary TCR not selected by self-MHC

Negative Selection

Thymocytes strongly reacting with self-pMHC ligands are deleted, removing autoreactive and highly cross-reactive TCRs from repertoire

Secondary TCRs expressed at lower levels on cell surface, potentially masked from negative selection, permitting T cells with autoreactive and cross-reactive TCR specificities

Figure 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.