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Paired TCR $\alpha\beta$ analysis of virus-specific CD8⁺ T cells exposes diversity in a previously defined ‘narrow’ repertoire

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Abstract

T cell receptor (TCR) usage plays an important role in determining the outcome of CD8⁺ cytotoxic T lymphocyte (CTL) responses to viruses and other pathogens. However, the characterization of TCR usage from which such conclusions are drawn, is based on exclusive analysis of either the TCR α or, more commonly, the TCR β chain. Here, we have used a multiplexed RT-PCR protocol to analyse the CDR3 regions of both TCR α and β chains from single naïve or immune epitope-specific cells to provide a comprehensive picture of epitope-specific TCR usage and selection into the immune response. Analysis of TCR repertoires specific for three influenza-derived epitopes (D^bNP₃₆₆, D^bPA₂₂₄, and D^bPB1-F2₆₂), showed preferential usage of particular TCR $\alpha\beta$ proteins in the immune relative to the naïve repertoire, in some cases resulting in a complete shift in TRBV preference or CDR3 length, and restricted repertoire diversity. The NP₃₆₆-specific TCR $\alpha\beta$ repertoire, previously defined as clonally restricted based on TCR β analysis, was similarly diverse as the PA₂₂₄- and PB1-F2₆₂-specific repertoires. Intriguingly, preferred TCR characteristics (Variable (V) gene usage, CDR3 length and Junctional (J) gene usage) appeared able to confer specificity either independently or in concert with one another, depending on the epitope specificity. These data have implications for established correlations between the nature of the TCR repertoire and response outcomes after infection, and suggest that analysis of a subset of cells or a single TCR chain does not accurately depict the nature of the antigen-specific TCR $\alpha\beta$ repertoire.

Introduction

Biased usage of TCRs is a fundamental characteristic of antigen-specific T cell responses, and has been observed against a wide spectrum of antigen types, including pathogen, tumor-derived, as well as innocuous environmental and self antigens (reviewed in ^{1, 2}). Such biases in TCR usage may be classified (according to the extent of bias) as Type I, II, III, or IV^{1, 2} ranging from preferential use of a particular V α or V β gene segment only (Type I), through to usage of identical TCR α or β clonotypes (V region, CDR3 region, and J region) (Type III)². Biases may be present in the preimmune repertoire^{3, 4}, due to a combination of structural constraints imposed by the need for peptide + Major Histocompatibility Complex (pMHC) recognition^{5, 6}, and convergent recombination, a process that results in the prevalence of more easily generated TCRs^{7, 8}. Preferential expansion of particular T cell clones from the naïve into the immune repertoire can then further skew the usage of particular TCRs or TCR characteristics^{3, 9–13}, thereby exacerbating antigen-driven TCR bias. Whether continued antigenic stimulation, such as in chronic infection, continues to narrow the TCR repertoire, as has been suggested for the HLA-B8 restricted response to Epstein Barr Virus (EBV) EBNA-3A FLRGRAYGL peptide where the vast majority of the antigen-specific response is clonal¹⁴, or whether such extreme bias occurs upon initial antigen encounter^{15–17}, is unclear.

Interestingly, while the nature of the TCR bias varies with antigen, antigen driven biases are highly conserved across individuals (in both animal models and humans), indicating the capacity of these biased subsets to mediate superior recognition of peptide + Major Histocompatibility Complex I complexes (pMHCI). Why is it important to understand bias in antigen-specific T cell responses? The extent to which an antigen-specific T cell response can utilize a broad range of TCRs, or relies only on a narrow subset of TCRs, has been shown to correlate with the outcome of infection. For example, in a number of viral infections, diversity in TCR usage has been positively linked to effective viral control, prevention of immune escape, and/or improved recognition of heterologous viruses^{18–24}. This has been suggested to be due to an increased structural capacity to recognize variant epitopes²⁵ or an increased likelihood that high affinity TCRs will be present²². In any case, it is clear that the composition of TCRs that make up an antigen-specific T cell repertoire impacts substantially on disease outcomes.

Interrogation of antigen-specific repertoires to date have predominantly relied on analysis of the TCR β chain, partly due to the early perception that this chain made a greater contribution to peptide binding, and therefore to pMHCI specificity². This was partly based on the greater diversity inherent in the CDR3 β due to the additional D region element, but also on the fact that CDR3 β loops contributed more than CDR3 α loops to peptide binding in several early TCR-pMHCI structures (mouse and human)^{26–29}. However, subsequent analyses of a growing number of TCR-pMHCI crystal structures (34 available to date) demonstrate that both CDR3 α and β are able to mediate significant contact with the peptide fragment and MHCI molecule, with 15 of those structures demonstrating a contribution of CDR3 α that is equivalent to or greater than that of CDR3 β ^{5, 6, 30}. In addition, multiple studies have shown an important functional role for the TCR α chain in conferring specificity^{31–35}. Thus, TCR specificity can be viewed as a product of complex interactions

between the TCR α and β chains. Analysis of TCR β chain repertoires has largely been limited to bulk T cell populations, or a previously identified dominant TRBV+ subset in single cell analyses. Even deep sequencing of antigen-specific TCRs, if performed using a multiplexed approach^{36, 37}, is prone to unequal amplification and does not typically provide information on TCR $\alpha\beta$ pairing. Only recently have strategies begun to emerge for analysis of both TCR α and β chains from individual cells^{24, 38–42}, and therefore accurate information on clonal frequency and TCR $\alpha\beta$ pairing is still lacking for the vast majority of antigen-specific T cell repertoires.

Following influenza virus infection, influenza virus derived NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific responses show clear preference for TRBV13-1+, 29+, and 19+ TCRs, respectively^{43–45}. Previous characterization of TCR β usage within these epitope specificities has largely been limited to these dominant TRBV+ subsets, and has also identified preferential usage of J β regions (NP₃₆₆ - TRBJ1-1/2-2, PA₂₂₄ - TRBJ1-1/2-6), and CDR3 β lengths (NP₃₆₆ 9 amino acids (aa), PA₂₂₄ 6 aa, PB1-F2₆₂ 8 aa), that have come to characterize these responses^{44, 46–48}. Importantly, based on these analyses the NP₃₆₆-specific response is characterized as narrow (utilizing relatively few TCR β clonotypes) and highly public (using clonotypes observed in multiple individuals)^{47, 48}. In contrast, the PA₂₂₄- and PB1-F2₆₂-specific TCR β repertoires are more diverse and typically unique to individuals (private)^{44, 46}. Given the biological significance of such characteristics of virus-specific T cell repertoires, we have used a multiplexed, nested RT-PCR protocol to amplify the CDR3 regions of the full range of both TCR α and β chains expressed by individual, epitope-specific CTLs both before and after infection, to get a complete and unbiased picture of three virus-specific CTL TCR $\alpha\beta$ repertoires, and to gain a better understanding of how key characteristics of antigen-specific TCR $\alpha\beta$ repertoires are selected in response to virus challenge.

Results

TRAV and TRBV usage is restricted in antiviral immune CTL TCR repertoires, compared to their naïve counterparts

Biased TRBV usage has been documented for all of the NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific populations. Analysis of TRBV usage from multiplex RT-PCR analysis of single antigen-specific cells³⁸, confirmed both the previously described bias toward TRBV13-1⁴³, 29⁴⁵, and 19⁴⁴ usage in NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific responses, respectively, and the exacerbation of these biases from naïve to immune populations^{3, 49} (Figure 1A-C and Supp. Figure 1A-C). In contrast, there is relatively little known about the TCR α chain usage in these populations, although a previous bulk analysis of NP₃₆₆-specific TCR α chains suggests the usage is relatively diverse⁵⁰.

Multiplexed analysis of TCR α chain usage in NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific immune populations also showed biased usage of particular TCR α chains (Figure 1D-F). NP₃₆₆- and PA₂₂₄-specific cells showed biased usage of TRAV16 (65 $\pm$ 2.4%) and TRAV6 (38 $\pm$ 5%), respectively, while PB1-F2₆₂-specific CTLs exhibited less dramatic preferences for TRAV5 (17 $\pm$ 8%) or TRAV8 (21 $\pm$ 16%). Each of these preferences was substantially increased in the immune response relative to the corresponding naïve populations (Supp.

Figure 1D-F), as was observed for TRBV bias. With the exception of NP₃₆₆-specific CD8⁺ T cells, the TRAV biases were not as pronounced as was typically observed for TRBV usage, nonetheless such TCR α chain enrichment implies its importance in conferring pMHC specificity.

The elevated usage of particular TRAV or TRBV chains in the immune, relative to the naïve, repertoire resulted in some cases in the specific diminution in usage of other chains (Supp. Figure 2A-F). For example, the enrichment of TRBV13-1 usage from naïve to immune NP₃₆₆-specific cells occurred at the specific expense of TRBV17 usage, which was the most prevalent TRBV in the naïve set ($26 \pm 13\%$), but was observed only once in the immune repertoire ($0.8 \pm 1.5\%$) ($p < 0.01$) (Supp. Figure 2A). These data suggest that some TCRs available for recognition in the naïve repertoire are avoided in the immune response.

We used Simpson's Diversity Index (SDI) to determine how such antigen-driven biases influence the diversity of TRAV and TRBV usage. SDI assesses both 'richness' (number of V gene segments used) and 'evenness' (the distribution of those V gene segments) to provide a measure of overall diversity^{51, 52}. All three epitope-specific populations showed significantly less diversity of TRBV usage in the immune compared to the naïve repertoires (Figure 1G). The effect on TRAV diversity was subtler, with only the NP₃₆₆-specific population exhibiting significantly reduced diversity in TRAV usage from naïve to immune sets ($p < 0.01$), despite a similar trend for PA₂₂₄ ($p = 0.11$) (Figure 1G). Thus, the selective expansion of CTL clones using particular TRBV and, to a lesser extent, TRAV chains, leads to more restricted TCR usage in response to antigen challenge, relative to what is available in the preimmune repertoire.

CDR3 length and J region analysis

Prior analysis of TCR CDR3 β loop length in influenza-specific CD8⁺ T cells showed preferential usage of CDR3 β lengths of 9, 6, and 8 aa in TRBV13-1+ NP₃₆₆-, TRBV29+ PA₂₂₄-, and TRBV19+ PB1-F2₆₂-specific cells, respectively⁴³⁻⁴⁵. Multiplex analysis of total NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific TCR β repertoires also revealed these CDR3 β length preferences (Figure 2A-C). TCR α chains specific for NP₃₆₆, PA₂₂₄, and PB1-F2₆₂ showed substantial biases toward CDR3 α lengths of 10, 8, and 7-8 aa, respectively (Figure 2D-F), to a similar extent as those observed for CDR3 β lengths. The prevalence of the dominant CDR3 β and α lengths observed in the immune populations was significantly increased relative to naïve repertoires (Figure 2). Interestingly, the corresponding naïve repertoires often showed distinct patterns of CDR3 length usage to those observed in the immune repertoires (Figure 2B, C, D, F). These data suggest that the highly reproducible CDR3 α and β length biases observed in epitope-specific CTL responses, while partly reflected in the naïve CTLp populations, are predominantly driven by preferential clonal expansion during the immune response.

Analysis of J region usage in the immune repertoires revealed a strong NP₃₆₆-specific preference for TRBJ2-2, a PA₂₂₄-specific preference for TRBJ2-7 and a slight bias toward TRBJ2-1 usage in the PB1-F2₆₂-specific population (Supp. Figure 2G-I). The NP₃₆₆-specific population exhibited a strong bias toward TRAJ42 (Supp. Figure 2J). While there appeared to be some biases noted in TRAJ usage for PA₂₂₄- and PB1-F2₆₂-specific cells,

they were less convincing and far more variable between mice (Supp. Figure 2K, L). Thus, unlike the NP₃₆₆-specific population, which exhibited TRBJ and TRAJ biases to a similar extent, the PA₂₂₄- and PB1-F2₆₂-specific populations showed no reproducible TRAJ preferences.

Pairing of dominant TRAVs and TRBVs

An important aspect of studying TCR at the single cell level is that it enables analysis of TCR $\alpha\beta$ pairing, thereby providing the full extent of epitope-specific TCR repertoire diversity. Given the strong antigenic selection for both TCR β and α chains in the antiviral response, we were interested in whether these chains were selected together or independently. For all 3 epitope-specific populations and in all mice analysed, usage of the dominant TCR β family was increased within the dominant TRAV populations compared to the total population (NP₃₆₆ - total 60 $\pm$ 29 vs TRAV16+ 78 $\pm$ 36 [p=0.06]; PA₂₂₄ - total 68 $\pm$ 5 vs TRAV6+ 95 $\pm$ 9.1 [p=0.01]; PB1-F2₆₂ - total 63 $\pm$ 7.7 vs TRAV8+ 96 $\pm$ 6.4 [p=0.07], using Students paired t-test) (Figure 1, compare left pie charts to right pie charts in each of A vs D, B vs E, and C vs F).

In contrast, enrichment of the dominant TRAV within the dominant TRBV population was less readily observed (Figure 1, compare right pie charts to left pie charts in each of A vs D, B vs E, and C vs F). Certainly, usage of TRAV16+ within the dominant TRBV13-1+ NP₃₆₆-specific set was greatly increased relative to total (total 67 $\pm$ 3.4 vs TRBV13-1+ 91 $\pm$ 14 [p=0.001]). However there was only slight enrichment of TRAV6 and TRAV8 usage in the dominant TRBV29+ PA₂₂₄-specific and TRBV19+ PB1-F2₆₂-specific populations, respectively, compared to the total set (PA₂₂₄ - total 38 $\pm$ 5.1 vs TRBV29+ 54 $\pm$ 10 [p=0.02]; PB1-F2₆₂ - total 21 $\pm$ 16 vs TRBV19+ 32 $\pm$ 19 [p=0.08]). These data collectively indicate that dominance of both TRAV16 and TRBV13-1 was due to their co-dependence in the NP₃₆₆-specific population, however, while the dominant TCR α chains were largely dependent on the dominant TCR β chains to confer PA₂₂₄ or PB1-F2₆₂ specificity, their TCR β chains were able to confer specificity when paired with a broad range of TCR α chains.

While these data indicate that selection of dominant TCR α and β chains is, to varying extents, co-dependent, it is possible that the dominance of the TCR α chain is simply due to its superior ability to pair with the dominant TRBV, and contributes minimally to pMHCI specificity. Analysis of TRAV usage in non-antigen-specific TRBV13-1+, TRBV29+, and TRBV19+ populations shows significantly reduced TRAV16, TRAV6, and TRAV8 usage, respectively, than in antigen-specific sets (Figure 1H), clearly demonstrating that the extent of pairing observed between the dominant TRAV and TRBV bearing TCR chains is antigen driven.

Collectively, these data suggest that there is an active selection of both the TRBV and TRAV families in antigen-specific CTL populations, and that the combination of preferred TCR α and β chains plays a role in conferring epitope specificity. However, the stringency of selection of particular TCR $\alpha\beta$ pairs occurs to different extents, with far greater flexibility in TRAV usage generally (and in the dominant TRBV+ set) in the PA₂₂₄- and in particular in the PB1-F2₆₂-specific populations, compared to NP₃₆₆. Such differences may indicate a more important role for the TCR α chain in NP₃₆₆, relative to PA₂₂₄ or PB1-F2₆₂.

recognition, or may simply indicate a greater range of TCR α chains with the capacity to impart PA₂₂₄ and PB1-F2₆₂ specificity.

Relationship between characteristic features of epitope-specific TCRs

To further determine whether the canonical features that characterize each of the epitope-specific immune CTL populations (V region usage, CDR3 length, J region usage) are co-selected, we analysed CDR3 length and J region usage as a function of V region usage (Figure 3). Importantly, this analysis was done irrespective of clonal abundance in order to eliminate the effect of individual clonal expansions. For the NP₃₆₆-specific population, both the preferred TRAV16+ and TRBV13-1+ TCRs showed an enrichment of TCRs with the preferred CDR3 lengths of 10 and 9 aa, respectively (Figure 3A, D). Similarly, analysis of preferred J region usage (TRAJ42 and TRBJ2-2/1-1) showed a substantial enrichment, in particular in the TRBV13-1+ (relative to TRBV13-1-) population, but also in the TRAV16+ population (Figure 3G, J). This indicates that the canonical features associated with the NP₃₆₆-specific TCR β chain (TRBV13-1 usage, 9 aa CDR3 β length and TRBJ1-1/TRBJ2-2 usage) and TCR α chain (TRAV16 usage, 10 aa CDR3 α length, and TRAJ42 usage) are selected together, suggesting that these features contribute to optimal pMHC binding in a coordinated inter-dependent fashion.

In contrast, for the PA₂₂₄-specific population, the dominant TRAV6+ and TRBV29+ subsets showed no selective enrichment of preferred CDR3 lengths (Figure 3B, E) or J region elements (Figure 3H, K) relative to the rest of the epitope-specific population. Similarly, for PB1-F2₆₂-specific cells, the only co-segregation of bias observed was an increased prevalence of 8aa CDR3 β lengths in the TRBV19+ (compared to TRBV19-) subset (Figure 3F), with all other preferences being observed equivalently in TRAV5/8+ compared to TRAV5/8-, or TRBV19+ compared to TRBV19-, subsets (Figure 3C, I, L). Thus, it appears that, for the PA₂₂₄-and PB1-F2₆₂-specific populations, the preferred elements observed in the TCR repertoire are more likely to either contribute independently to the recognition of pMHC, or mediate coordinated pMHC recognition in combination with a variety of different TCR elements.

Flexibility of CDR3 α and β pairing

One of the caveats of TCR β or α chain analyses performed independently of one another is that it provides no information on the promiscuity of pairing and therefore the true extent of clonal diversity. There is a wealth of data indicating that it is the combination of TCR α and β chains (rather than either chain alone) that imparts epitope specificity (reviewed in ^{6, 30}). Moreover, a recent study has demonstrated key structural interactions specifically between CDR3 α and CDR3 β regions that influence pMHC recognition⁵³. Our analysis of the extent of flexibility in CDR3 α and β pairing revealed that of the 11 CDR3 β that were observed more than once either within or between mice for NP₃₆₆, 6 of these were found to pair with multiple CDR3 α sequences, including CDR3 β sequences previously classified as 'public' (e.g. SGGANTGQL, SGGGNTGQL, SGGSTNTGQL) (Table 1, Supp. Table 1). Indeed, even dominance of a particular CDR3 β across multiple mice was not necessarily representative of a single TCR $\alpha\beta$ clonotype (e.g. SGGSTNTGQL) (Supp. Table 1). Similarly, 9/23 PA₂₂₄-specific and 3/15 PB1-F2₆₂-specific CDR3 β clonotypes that were observed more than once

within or between mice, were found to pair with multiple CDR3 α sequences (Table 1, Supp. Tables 2 & 3).

Analysis of the CDR3 α clonotypes revealed a similar situation with 6/17, 10/20, and 2/11 clonotypes observed more than once in the NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific repertoires, able to pair with more than one CDR3 β clonotype (Table 1). Collectively, these data demonstrate a large degree of promiscuity in CDR3 $\alpha\beta$ pairing, even for NP₃₆₆-specific TCRs where the TRBV13-1/TRAV16 pairing requirement is relatively stringent (Figure 1A, D). As a consequence, there appears to be substantial diversity in TCR $\alpha\beta$ clonotypes that were previously defined as ‘restricted’ by CDR3 β alone^{47, 48}.

Diversity and sharing in influenza-specific TCR $\alpha\beta$ repertoires

TCR repertoires are often characterised by the extent of clonotype diversity within individuals (the number and distribution of different clonotypes) and between individuals (the extent to which the repertoire is observed in multiple individuals (shared) or is unique to an individual (private))⁵⁴. Previous analyses of TCR β diversity in immune NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific repertoires (limited to the dominant TRBV families), clearly show that the TRBV13-1+ NP₃₆₆-specific repertoire is significantly more restricted in clonal diversity than either the TRBV29+ PA₂₂₄- or TRBV19+ PB1-F2₆₂-specific populations, which are both characterised as diverse and private^{44, 46, 47}. To determine whether these characteristics hold up upon analysis of the entire immune epitope-specific population and upon inclusion of both TCR α and β chains, we analysed diversity (using SDI) in the global TCR α and β chain repertoires for each of these specificities.

Looking within the dominant TRBV13-1+ NP₃₆₆-specific, TRBV29+ PA₂₂₄-specific, and TRBV19+ PB1-F2₆₂-specific sets, it is clear that TCR β chain diversity is significantly lower in the NP₃₆₆-specific population, compared to the relatively diverse PA₂₂₄- ($p < 0.027$) and PB1-F2₆₂- ($p < 0.003$) specific sets (Figure 4A-C, compare white bars, right plots). Diversity in the combined TCR $\alpha\beta$ clonotypes within the dominant TRBV+ subsets, remained relatively low for NP₃₆₆-, compared to PA₂₂₄- or PB1-F2₆₂-specific populations (Figure 4A-C, right plots), due to the relatively stringent TRAV16 usage in this population and the associated restriction in CDR3 α diversity therein (Figure 1) (Supp. Table 1).

Analysis of the total epitope-specific TCR $\alpha\beta$ repertoire (Figure 4A-C, left plots) revealed that while TCR $\alpha\beta$ diversity was generally increased (compared to the dominant TRBV subset) for all three epitope specificities (Figure 4A-C, total v TRBV sets), this increase was most pronounced for the NP₃₆₆-specific set. This was a consequence of the fact that TCR $\alpha\beta$ s outside of the TRBV13+ subset were characteristically distinct from those within it, and showed far greater CDR3 α and β diversity (Supp. Table 1).

Consequently, analysis of the absolute diversity for each of the epitope-specific populations (that is, unrestricted to the dominant TRBV set and inclusive of TCR α chain), demonstrates that the NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific populations show similar levels of overall TCR diversity (0.89, 0.95, 0.96, respectively) ($p > 0.2$ comparing NP₃₆₆- to PA₂₂₄- or PB1-F2₆₂-specific sets) (Figure 4A-C, left plots, compare black bars). This contrasts with previous characterisations of the NP₃₆₆-specific repertoire, based exclusively on CDR3 β

clonotype analysis within the TRBV13-1+ set, as being highly restricted in diversity. Indeed, comparison of SDI between NP₃₆₆-specific TRBV13-1+ TCR β and total TCR $\alpha\beta$ reveals a 1.8-fold difference ($p=0.03$), while comparison of SDI between PA₂₂₄-specific TRBV29+ TCR β and total TCR $\alpha\beta$, or between PB1-F2₆₂-specific TRBV19+ TCR β and total TCR $\alpha\beta$ showed little difference (1.07-fold [$p=0.12$] and 1.02-fold [$p=0.27$], respectively).

As previously documented^{7, 47, 48}, a significantly larger proportion of the TRBV13-1+ NP₃₆₆-specific TCR β chain sequences (88%) were observed in multiple mice (i.e. 'shared') compared to TRBV29+ PA₂₂₄-specific (32%) ($p=0.004$) or TRBV19+ PB1-F2₆₂-specific (26%) ($p=0.0002$) sequences (Figure 4D-F, white bars, right plots). However, the extent to which TRBV13-1+ CDR3 β clonotypes are shared between animals is atypical, since sharing of TCR α sequences (as defined by the Proportion of TCRs in Common (PTIC)) within dominant TRBV was more similar for the three epitope-specificities (Figure 4D-F, grey bars, right plots). Because of the influence of the TCR β chain, however, overall the extent of TCR $\alpha\beta$ sharing in the dominant TRBV population remained relatively (although not significantly) high for NP₃₆₆-specific cells (Figure 4D-F, right plots, black bars).

Analysis of TCR $\alpha\beta$ clone sharing in total epitope-specific responses revealed that while the NP₃₆₆-specific response was still the most shared response (27%, compared to 10% and 9% for PA₂₂₄ and PB1-F2₆₂, respectively), the extent to which it was shared was drastically reduced compared to what one might have predicted based on TCR β analyses within the TRBV13-1+ population (Figure 4D, right plot, white bar) or globally (Figure 4D, left plot, white bar). The general trend of a drop in $\alpha\beta$ clonotype sharing, relative to α or β clonotype alone (Figure 4D-F), reflects the promiscuity of TCR α /TCR β pairing, which is most obvious for the large public NP₃₆₆-specific TCR β clonotypes (Table 1).

In summary, analysis of TCR diversity and sharing in the global TCR $\alpha\beta$ repertoire reveals that the three epitope-specific CTL populations, formerly described as having distinct profiles of TCR diversity and sharing, are similarly diverse and are shared between individuals to a greater extent than would be predicted based on TCR β analysis alone.

Discussion

We describe the analysis of paired TCR $\alpha\beta$ clonotype usage in CD8+ T cell populations specific for 3 different D^b restricted influenza A virus-derived epitopes. These 3 repertoires were previously distinguished on the basis of CDR3 β clonotype diversity and sharing within the dominant TRBV subset, with the NP₃₆₆-specific repertoire characterized as restricted and public (highly shared), while the PA₂₂₄- and PB1-F2₆₂-specific repertoires are considered to exhibit diverse and private (unique to individuals) CDR3 β usage. The present analysis of the total TCR $\alpha\beta$ epitope-specific repertoire revealed that, unlike for PA₂₂₄- and PB1-F2₆₂-specific cells, the restricted pairing and TCR usage in the prevalent TRBV13-1+ subset of NP₃₆₆-specific cells bore little resemblance to the remainder of the NP₃₆₆-specific population. As such, analysis across the total epitope-specific TCR $\alpha\beta$ repertoires revealed that TCR $\alpha\beta$ clonotype diversity was remarkably similar for all 3 epitope specific CTL populations.

Prior analyses of the NP₃₆₆, PA₂₂₄- and PB1-F₂₆₂-specific predominant V β subpopulations (TRBV13-1, 29, and 19, respectively) reveal that the TRBV13-1+ NP₃₆₆-specific repertoire is notable for its lack of clonal diversity and high level of repertoire sharing between individuals, compared to the PA₂₂₄- and PB1-F₂₆₂-specific repertoires. Moreover, for each of these epitope-specific V β subsets, preferred J β region usage, CDR3 β lengths and conserved aa motifs have all been described^{44, 46–48}. However, much less is known about TCR α chain usage in these populations. An analysis of CDR3 α usage in bulk TRBV13-1+ NP₃₆₆-specific T cells demonstrated that TCR α clonal diversity was substantially greater than TCR β diversity in this subset of cells⁵⁰. Moreover, our own recent studies utilizing single chain retrogenic mice expressing a TRBV13-1+ or TRBV29+ TCR β specific for either NP₃₆₆ or PA₂₂₄, respectively, demonstrated that the TCR α chain was important for imparting specificity (only a small minority of the retrogenic TCR β -expressing cells were epitope specific), and that a range of selected TRAVs were able to do so⁵⁵. However, the current study indicates that TCR α usage is even more diverse than observed in NP₃₆₆- or PA₂₂₄-specific single TCR β chain retrogenic mice and, for all specificities, TCR α usage is at least as diverse as the corresponding TCR β chains.

Importantly, in the original description of the single cell multiplexed RT-PCR technique it was noted that 10% of all immune CD8+ cells expressed dual in-frame TCR α chains³⁸. While both TCR α chains have the potential to pair with the expressed TCR β chain, only one is likely to confer specificity. Thus, it remains a possibility that, from some cells within that subset, we are detecting the sequence of an irrelevant TCR α (from a viral antigen perspective). Despite this, we have previously randomly selected and expressed TCR $\alpha\beta$ heterodimers identified using this strategy⁵⁶, and all show the expected epitope specificity. Therefore, we feel confident that this strategy is providing an accurate global representation of epitope-specific TCR $\alpha\beta$ prevalence and pairing.

Our previous analysis of pMHC I crystal structures show that both the PA₂₂₄ and PB1-F₂₆₂ peptides bound to D^b present a relatively ‘featured’ surface for recognition by specific TCRs, by virtue of a prominent arginine residue at positions 7 and 4, respectively. In contrast, the lack of any such prominent features in the NP₃₆₆ peptide was associated with the highly restricted clonotype usage observed in the TRBV13-1+ NP₃₆₆-specific TCR repertoire and was thought to reflect the difficulty of navigating recognition of this relatively flat pMHC I complex⁵⁷. The current study demonstrates that there is in fact a wide range of TCRs capable of D^bNP₃₆₆ recognition, and the overall diversity of clonotype usage in this repertoire is not significantly different to that used in the highly diverse PA₂₂₄ and PB1-F₂₆₂ repertoires. However, despite the breadth of TCR clonotype usage, the clones that are apparently able to confer optimal NP₃₆₆ recognition (based on their preferential expansion in the immune repertoire) are a restricted and relatively uniform subset of NP₃₆₆-specific clonotypes (TRBV13-1+ set). Indeed, a key finding from this study is that the key characteristics of the prominent TRBV13-1+ subset in the NP₃₆₆-specific population (CDR3 length, TRAV pairing, J region usage) are distinct from the features associated with the remainder of the NP₃₆₆-specific population. This is not true of the PA₂₂₄- and PB1-F₂₆₂-specific populations, whose preferred TCR characteristics are prominent throughout the epitope-specific population. Thus, TCR recognition of NP₃₆₆ may not necessarily restrict the number of clones capable of recognition, but may limit the potential docking modes of

NP₃₆₆-specific TCRs, with a consequence being that the TCR populations exhibit restricted and synchronized characteristics.

A number of studies have demonstrated a link between TCR diversity and improved viral control^{18–24}, thought to be due to the larger number of TCR structures increasing the flexibility with which variant epitopes may be recognized²⁵. The observation that escape mutations occur more frequently in the NP₃₆₆, compared to the PA₂₂₄ epitope⁵⁸, was thus originally attributed to the restricted clonal diversity of the NP₃₆₆-specific population, which was thought to be less capable of structural flexibility as a consequence. Our observation that the global NP₃₆₆-specific TCR repertoire is not less clonally diverse than the PA₂₂₄- and PB1-F2₆₂-specific repertoires suggests that the presence or absence of clonal diversity *per se* may be an inadequate correlate of protection from virus. Rather, the number of ways that TCRs can recognize a specific pMHC I may be the key determinant of preventing immune evasion. As such, we would argue that despite its clonal diversity, TCR recognition of NP₃₆₆ is relatively inflexible, as evidenced by the stringent co-segregation of preferred TCR elements, and thus TCR ‘structural diversity’ (rather than clonal diversity) may represent a better correlate of immune protection.

Alternatively, other studies showing that particular TCRs are associated with improved control of virus infection^{59–63}, suggest that any benefit conferred by TCR diversity is likely to be due to the inclusion of TCRs with high affinity for pMHC I^{11, 22, 64}. Thus, it is possible that despite the clonal diversity of NP₃₆₆-specific TCRs, the ready emergence of viral variants for this epitope may be due to a poorer quality T cell response, rather than a lack of TCR diversity, compared to PA₂₂₄. This is partly supported by the observation that polyclonal NP₃₆₆-specific CTLs exhibit a more rapid off rate and greater dependence on the CD8 co-receptor relative to their PA₂₂₄-specific counterparts^{65–67}.

The strong bias toward TRAV16 in the NP₃₆₆-specific repertoire, contrasted somewhat with the less extensive TRAV biases in the PA₂₂₄- and PB1-F2₆₂-specific repertoires and inferred a greater importance of the TCR α chain in conferring pMHC I specificity in the NP₃₆₆-specific set. In fact, previous structural analysis of a single D^b PA₂₂₄-TCR $\alpha\beta$ ternary complex (TRBV29/BJ27, TRAV21/AJ53) showed that the CDR3 α loop mediated the majority (68%) of the PA₂₂₄ peptide contacts, while the CDR3 β loop provided only 17% of the peptide contacts⁶⁸. Moreover, a CDR3 α -encoded motif (SNY) mediated critical interactions with the PA₂₂₄ peptide, while the CDR3 β loop engaged the peptide only via main chain interactions. Thus it was suggested that the PA₂₂₄-specific CDR3 β length of 6 aa was important for recognition, while the CDR3 α aa sequence (in particular, Tyr112) was critical for PA₂₂₄ recognition. It remains to be seen whether similar pMHC I docking modes are utilized by all PA₂₂₄-specific TCRs. While our data certainly shows a broad preference for a CDR3 β length of 6 aa, the CDR3 α -encoded SNY motif was only found in 20% of all PA₂₂₄-specific CDR3 α clonotypes, with the critical Y aa in this position found in only 32% of those clonotypes (representing only a minor enrichment from 20% in non-antigen specific TCR α chains). Thus, either the mode of TCR $\alpha\beta$ docking is substantially altered between PA₂₂₄-specific TCRs, or other CDR3 α encoded aa residues (e.g. Asp, Arg, Lys) provide these seminal contacts to preserve the docking mode.

Collectively, this study is the first analysis of both naïve and immune epitope-specific TCR $\alpha\beta$ repertoires. Comparison of these repertoires clearly demonstrates a more restricted TCR usage in the immune, relative to naïve, epitope-specific populations, indicative of selective recruitment and/or expansion of CTLs with preferred TCR characteristics. The data also suggests that, in some cases, analysis of a subset of epitope-specific TCRs does not provide an accurate representation of TCR characteristics or diversity within the total epitope-specific repertoire. Moreover, while all epitope-specific populations analysed exhibited preferences for particular TCR characteristics, the stringency of the context in which those characteristics can be utilized to impart recognition may better define the flexibility of a TCR repertoire, despite similar levels of clonal diversity.

Methods

Mice and virus infections

Female C57BL/6J (H-2^b) mice were bred and housed in the animal facility of the Department of Microbiology and Immunology at the University of Melbourne (Parkville, Australia). Naïve 6–10 week-old mice were anesthetized by isoflurane inhalation and infected intranasally (i.n.) with 1×10^4 PFU of the HKx31 (H3N2) influenza A virus in 30 μ l of PBS. All animal experimentation was conducted following the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines for housing and care of laboratory animals and performed in accordance with Institutional regulations after pertinent review and approval by the University of Melbourne Animal Ethics Committee.

Tetramer and antibody staining

Spleens were collected from mice 10 d after primary influenza virus infection, processed into single cell suspension through a 70 μ m sieve, and enriched for T cells by panning on plates coated with Affinipure anti-mouse IgG/IgM Abs (200 μ g/ml) for 1 h at 37°C to remove B cells. Epitope-specific CD8⁺ T cells were identified by staining with PE conjugated tetrameric complexes of H-2D^b MHC class I glycoprotein + nucleoprotein derived NP_{366–374} (ASNENMETM), acid polymerase derived PA_{224–233} (SSLENFRAYV), or PB1-F2_{62–70} (LSLRNPILV), from the +1 reading frame of the influenza viral polymerase B subunit peptides (ImmunoID, University of Melbourne) for 1 h at RT. Cells were then co-stained with anti-CD8 α -FITC before entire samples were acquired on a FACS Aria III cell sorter using FACSDiva software (BD Immunocytometry Systems, San Jose, CA, USA). Data were analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Magnetic enrichment and isolation of epitope-specific CD8⁺ T cells

Tetramer-based magnetic enrichment was used for identification of naïve epitope-specific CD8⁺ T cell precursors, as described in detail previously^{3, 4, 69}. Briefly, single cell suspensions of pooled spleen and major LNs (auxiliary, brachial, cervical, inguinal and mesenteric) from naïve mice were stained with PE-labelled influenza-specific NP₃₆₆, PA₂₂₄, or PB1-F2₆₂ tetramers, washed and labelled with anti-PE conjugated magnetic microbeads, and tetramer-bound cells enriched over a magnetic LS column (Miltenyi Biotec). Enriched cells were then stained with a cocktail of conjugated antibodies to identify epitope-specific

cells (CD8 α + CD3 ϵ + CD62Lhi CD11b- CD11c- B220- F4/80- CD4-). Entire samples were acquired on a FACS Aria III cell sorter with FACSDiva software (BD Immunocytometry Systems, San Jose, CA, USA).

Single cell multiplexed RT-PCR

Individual NP₃₆₆-, PA₂₂₄- or PB1-F2₆₂-specific CD8+ T cells were sorted into wells of a 96-well plate using a BD FACS Aria (BD Biosciences). Multiplex single-cell reverse transcription and PCR amplification of TCR CDR3 α and CDR3 β regions was performed using a panel of TRBV and TRAV specific oligonucleotides, as described³⁸. Briefly, mRNA was reverse transcribed in 2.5 μ l using the Superscript III VILO cDNA Synthesis Kit (containing 1 $\times$ Vilo reaction mix, 1 $\times$ superscript RT, 0.1% Triton X-100), and incubated at 25°C for 10 min, 42°C for 120 min, and 85°C for 5 min. The entire volume was then used in a 25 μ l first round PCR reaction with 1.5U Taq DNA polymerase, 1 $\times$ PCR buffer, 1.5 mM MgCL2, 0.25mM dNTPs and a mix of 23 TRAV external sense primers and a TRAC external antisense primer³⁸, along with 19 TRBV external sense primers and a TRBC external antisense primer (each at 5 pmol/ μ l)³⁸, using standard PCR conditions. For the second round nested PCR, a 2.5 μ l aliquot of the first round PCR product was used in separate TRBV and TRAV specific PCRs, using the same reaction mix described above, however a set of 23 TRAV internal sense primers and a TRAC internal antisense primer, or a set of 19 TRBV internal sense primers and a TRBV internal antisense primer, were used³⁸. Second round PCR products were visualized on a gel and positive reactions were purified with ExoSAP-IT reagent. Purified products were used as template in sequencing reactions with internal TRAC or TRBC antisense primers, as described^{38, 46}. TCR gene segments were assigned using the International ImMunoGeneTics (IMGT) database⁷⁰.

Statistical analyses

Simpson's Diversity Index (SDI) was used to measure the extent of diversity (i.e. a combination of the number and distribution of species) in TRBV and TRAV gene segment usage, and clonotype usage^{49, 51, 52}. SDI is calculated as $D = \sum_i [(n_i(n_i - 1)) / (N(N - 1))]$ where n_i is the number of sequences in the i th clonotype and N is the total number of sequences in the whole population. To measure clonotype sharing, we determined the proportion of sequences that are found in a specific percentage (q) of the mice analysed, referred to as PTIC _{q} (Proportion of TCRs in Common)^{49, 71}. The Mann-Whitney test, or Students unpaired t-test, was used to determine significance for all individual comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TRAV and TRBV bias, pairing, and diversity in immune antiviral CTL responses
 TRAV and TRBV usage was determined from CDR3 sequences generated using multiplexed nested RT-PCR from individual NP₃₆₆-, PA₂₂₄-, or PB1-F2₆₂-specific CD8⁺ T cells from d10 immune splenocytes, as described. Shown is data from all cells that yielded both CDR3α and CDR3β sequences. (A-C) TRBV usage in the total epitope-specific populations and TRAV usage within the dominant TRBV subset. (D-F) TRAV usage in the total epitope-specific populations and TRBV usage within the dominant TRAV subset. (G) SDI analysis of TRAV and TRBV usage in naïve and immune epitope-specific CD8⁺ T cell

populations. (H) Analysis of TRAV16, TRAV6, and TRAV8 usage within the TRBV13-1+, TRBV29+, and TRBV19+ subsets, respectively, of non-epitope specific, naive epitope-specific and immune epitope-specific populations. Shown are the data obtained from 4, 4, and 4 immune mice and 6, 5, and 8 naive mice, for NP₃₆₆, PA₂₂₄, and PB1-F₂₆₂, respectively. N=69 NP₃₆₆-specific, 98 PA₂₂₄-specific, and 139 PB1-F₂₆₂-specific naïve sequences analysed. Non-antigen-specific TCRαβ sequences were obtained by single cell sorting on TRBV13-1+ (n=83), TRBV29+ (n=107) or TRBV19+ (n=121) populations from 3 uninfected B6 mice and performing nested RT-PCR using TRBVspecific oligos and a panel of multiplexed TRAV oligos. ND = not detected.

naïve n=155, immune n=161; PB1-F2₆₂ CDR3 β : naïve n=269, immune n=127; PB1-F2₆₂ CDR3 α : naïve n=271, immune n=136.

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Figure 3. Co-selection of TCR biases in epitope-specific populations

TCR clonotypes from d10 immune epitope-specific populations were used to plot CDR3 length or J region usage for cells using the dominant TRAV or TRBV, and compared to CDR3 length and J region usage in cells outside of the dominant TRAV or TRBV subsets. The analysis was done irrespective of clonotype abundance. CDR3α length distribution (A-C) and TRAJ usage (G-I) in TRAV16+/-, TRAV6+/-, and TRAV5 or 8 +/- subsets of NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific populations, respectively. CDR3β length distribution (D-F) and TRBJ usage (J-L) in TRBV13+/-, TRBV29+/-, and TRBV19 +/- subsets of NP₃₆₆-, PA₂₂₄-, and PB1-F2₆₂-specific populations, respectively. CDR3α clonotypes: NP₃₆₆ - n=48, PA₂₂₄ - n=71, PB1-F2₆₂ - n=78; CDR3β clonotypes: NP₃₆₆ - n=28, PA₂₂₄ - n=83, PB1-F2₆₂ - n=76.

Figure 4. TCRαβ diversity and sharing in global epitope-specific CTL repertoires
Epitope-specific CDR3α, β, and αβ clonotypes determined by single cell multiplexed RT-PCR, were analysed for diversity using SDI (A-C), and for sharing between individuals using PTIC_{0.33} (D-F), as described in the Methods. Number of sequences are as follows: TOTAL: NP₃₆₆ β (n=149) α (n=174) αβ (n=104); PA₂₂₄ β (n=166) α (n=161) αβ (n=116); PB1-F₂₆₂ β (n=127) α (n=136) αβ (n=80). TRBV13-1+ NP₃₆₆ β (n=93) α (n=68) αβ (n=68);

TRBV29+ PA₂₂₄ β (n=112) α (n=79) $\alpha\beta$ (n=79); TRBV19+ PB1-F2₆₂ β (n=84) α (n=51) $\alpha\beta$ (n=51). Statistical analyses were performed using Student's t-test.

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Table 1

Degeneracy of TCR $\alpha\beta$ pairing in epitope-specific immune populations

NP ₃₆₆ -specific											
TCRβ chain		Paired TCRα chains				TCRα chain			Paired TCRβ chains		
Vβ	CDR3β	Jβ	Vα	CDR3α	Jα	Vα	NP CDR3α	Jα	Vβ	CDR3β	Jβ
13-1	SGGSNTGQL	2-2	16	RAAGGSNAKL	42	7	MSNYNVL	21	12-2	FPDRRNSYNSPL	1-6
			16	REALGEGGRAL	15			16	SFGAANTEV	1-1	
			16	REPSGTQ	13						
			16	RETRSGTYQ	13						
			16	RGSGGSNAKL	42						
			16	RNSGGSNAKL	42						
			16	RVSGGSNAKL	42						
			15-1	WELDTNAYKV	30						
13-1	SGGANTGQL	2-2	16	RDQGRAL	15	16	REALGEGGRAL	15	13-1	SGGNTGQL	2-2
			16	RNSGTQ	13			13-1	SGGSNTGQL	2-2	
			16	RGSGGSNAKL	42						
			16	RVSGGTQ	13						
			16	RVSGGSNAKL	42						
2	SQDRRNSYNSPL	1-6	16	RVSGGSNAKL	42	16	REGKGGGSNYKL	53	19	RMGVYNSPL	1-6
			7-4	SAIASSFSKL	50			19	SPLGGGAETL	2-3	
			7-3	SGQGRAL	15			5	SQDLGGTYEQ	2-7	
			7-4	SPSSGSWQL	22			12-1	SPWGEYEQ	2-7	
			7-4	STMNNAGAKL	39						
16	SFGAANTEV	1-1	7	MSNYNVL	21	16	RGSGGSNAKL	42	16	SFGAANTEV	1-1
			16	REPSGTQ	13			13-1	SGGANTGQL	2-2	
			16	RGSGGSNAKL	42			13-1	SGGSNTGQL	2-2	
			16	RVSGGSNAKL	42			26	SLWRDTL	2-4	
26	SLWRDTL	2-4	7-3	RAOTGFASAL	35	16	RVSGGSNAKL	42	16	SFGAANTEV	1-1

NP ₃₆₆ -specific										
TCRβ chain			Paired TCRα chains				TCRα chain			
Vβ	CDR3β	Jβ	Vα	CDR3α	Jα	Vα	NP CDR3α	Jα	Vβ	CDR3β
			16	RGSGGSNAKL	42				13-1	SGGANTGQL
			6	RRNTGGLSGKL	2				13-1	SGGGNTGQL
			16	VSGGSNAKL	42				13-1	SGGSNTGQL
13-1	SGGGNTGQL	2-2	16	RVSGGSNAKL	42					
			16	REALGEGGRAL	15					

PA ₂₂₄ -specific										
TCRβ chain			Paired TCRα chains				TCRα chain			
Vβ	CDR3β	Jβ	Vα	CDR3α	Jα	Vα	NP CDR3α	Jα	Vβ	CDR3β
29	SHDRGQL	2-2	6	GAQGGRAL	15	6	GAQGGRAL	15	13-1	SGNAEQ
			21	RVGATGGNNKL	56				29	SHDRGQL
19	SIGDEQ	2-7	13-1	SNNRI	31	6	GGGSNYKL	53	29	SLGERL
			7-2	SRGSNNRI	31				29	TAGERL
19	SIGGEV	1-1	9D-4	SAWGRAL	15	6	HSGGSNAKL	42	29	SSYEQ
			7-2	SRGSNNRI	31				29	TMGERL
			15D-1	WEPDTNAYKV	30					
29	SLGERL	1-4	6	GGGSNYKL	53	6	IASSGSWQL	22	29	SGGKAP
			9D-4	SPSNTNKV	34				29	SGGQAP
29	SLSGFEQ	2-7	6D-4	APSNTNKV	34	21	NGGSNYKL	53	29	SFGQAP
			6D-4	VPSNTNKV	34				29	SPDRGAL
29	SPDRGAL	2-4	21	NGGSNYKL	53	21	RVGATGGNNKL	56	29	SHDRGQL
			21	RVGATGGSSKL	56				29	SLDRGQV
			3-4	SGGATGGNNKL	56				29	SPDRGRL
29	SSGETL	2-3	6	GAGSNYKL	33	21	SGGSNYKL	53	29	SLGAEQ

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PA ₂₂₄ -specific												
TCRβ chain			Paired TCRα chains				TCRα chain			Paired TCRβ chains		
Vβ	CDR3β	Jβ	Vα	CDR3α	Jα	Vα	NP	Vα	Jα	Vβ	CDR3β	Jβ
19	SIGDSYEQ	2-7	7	TDHQPGTGSNR L	28			19	SWGGEQ	2-7	29	SWGGEQ
								29	SWGGEQ	2-7	29	SWGGEQ
								29	SWGVEQ	2-5		
29	SSYEQ	2-7	6	GEGSNYQL	33	7	SRGSNNRI	31	SIGDEQ	2-7		
			6	HSGGSNAKL	42			19	SIGGEV	1-1		
								19	SIGNEQ	2-7		
								19	SIGSEQ	2-7		
								29	SLGGYEQ	2-7		
								19	SMGNEQ	2-7		
29	SWGGEQ	2-7	53	SGGSNYKL	19	12	SRTNAYKV	30	SAGSDY	1-2		
			53	SKTNTNKV	29			29	SLHTEV	1-1		
								29	TCTEEQ	2-7		
						6	VPSNTNKV	34	LGGDNQAP	1-5		
								29	SLSGFEQ	2-7		
								29	SLSGYEQ	2-7		

PB1-F2 ₆₂ -specific												
TCRβ chain			Paired TCRα chains				TCRα chain			Paired TCRβ chains		
Vβ	CDR3β	Jβ	Vα	CDR3α	Jα	Vα	NP	Vα	Jα	Vβ	CDR3β	Jβ
19	SIGDSYEQ	2-7	8-2	DTGNYKY	40	8	DTGNYKY	40	13-2	GELGASAEVL	2-3	
			6-6	GENNYAQGL	26			19	SIGDNYEQ	2-7		
								19	SIGDSYEQ	2-7		
								19	SIGDTTEV	1-1		
								19	SMGTDITQ	2-5		
								19	SPGDYAEQ	2-1		
								19	SRGDYAEQ	2-1		
								19	SWGDYAEQ	2-1		

PB1-F2 ₆₂ -specific												
TCRβ chain			Paired TCRα chains			TCRα chain			Paired TCRβ chains			
Vβ	CDR3β	Jβ	2-5	Vα	CDR3α	Jα	Vα	NP CDR3α	Jα	Vβ	CDR3β	Jβ
29	SLQLQDTQ			5	SAANKM	47	12	SDYGSSGNKL	32	19	SMGAEV	1-1
				10	SSGGNYKP	6				19	SMGMEAP	1-5
19	SWG DYAEQ		2-1	4-3	DQDSNYQL	33						
				8	DTCNYKY	40						
				9	RNSNYQL	33						