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Tracking phenotypically and functionally distinct T cell subsets via T cell repertoire diversity

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Abstract

Antigen-specific T cell receptors (TCRs) recognise complexes of immunogenic peptides (p) and major histocompatibility complex (MHC) glycoproteins. Responding T cell populations show profiles of preferred usage (or bias) toward one or few TCR β chains. Such skewing is also observed, though less commonly, in TCR α chain usage. The extent and character of clonal diversity within individual, antigen-specific T cell sets can be established by sequence analysis of the TCR V β and/or V α CDR3 loops. The present review provides examples of such TCR repertoires in prominent responses to acute and persistent viruses. The determining role of structural constraints and antigen dose is discussed, as is the way that functionally and phenotypically distinct populations can be defined at the clonal level. In addition, clonal dissection of "high" versus "low" avidity, or "central" versus "effector" memory sets provides insights into how these antigen specific T cell responses are generated and maintained. As TCR diversity potentially influences both the protective capacity of CD8⁺ T cells and the subversion of immune control that leads to viral escape, analysing the spectrum of TCR selection and maintenance has implications for improving the functional efficacy of T cell responsiveness and effector function.

Keywords

CD8⁺ cells; T cell receptor repertoire; CD62L; memory; influenza A virus

INTRODUCTION

Immune T cells play a crucial role in limiting viral, bacterial and parasitic infections, and in the induction of immune responses to some tumours. Both the CD8⁺ and CD4⁺ sets use specific T cell receptors (TCRs) to recognise complexes of peptide (p) bound to major histocompatibility complex (MHC) class I (CD8) or class II (CD4) glycoproteins on the surface of infected cells. Following ligation to these pMHC complexes, T cells proliferate and initiate the production of cytokines (e.g. IFN- γ , TNF and IL-2) and cytotoxic effector molecules that control infectious processes and, in the longer term, establish immune memory. Such responses are often directed at peptides from relatively conserved, internal viral proteins, providing a

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measure of protection against pathogens that rapidly mutate their surface glycoproteins and thus circumvent antibody neutralisation.

Antigen-specific TCR $\alpha\beta$ heterodimers are comprised of variable ($V\alpha$ and $V\beta$) and constant ($C\alpha$ and $C\beta$) regions that are spliced together in the thymus. Both the $V\beta$ and the $V\alpha$ chains are encoded by variable (V) and junctional (J) gene segments, while additional, diversity (D) gene segments contribute to the nature of the $V\beta$ chain. These V-gene segments contain three regions of hypervariability, known as complementarity-determining regions (CDR1, CDR2 and CDR3), that are involved in the TCR-pMHC interaction (Chothia et al., 1988). The TCRs specific for a particular pMHC complex normally select one, or a couple, of $V\beta$ or (less commonly) $V\alpha$ regions, a phenomenon referred to as $V\beta$ or $V\alpha$ bias. Sequence analysis of the CDR3 β or CDR3 α regions can be used to determine the clonal characteristics of a particular T cell response.

The present review discusses the main determinants, consequences and constraints governing TCR repertoire diversity, and provides examples of distinct TCR repertoires generated and maintained in response to acute or persistent viral infections. The ways that this clonotypic approach can be used to both track and define functional subsets within phenotypically distinct T cell populations is also described.

DETERMINANTS OF TCR REPERTOIRE DIVERSITY

Epitope-specific TCR repertoires are selected from a pool of naïve precursors estimated to range in size from $\sim 10^7$ (in mice) and $\sim 10^8$ (in humans) distinct TCR $\alpha\beta$ heterodimers (Arstila et al., 1999; Casrouge et al., 2000). Despite this vast potential in the naïve repertoire, T cell responses are frequently characterised by reproducible antigen-specific biases in TCR $V\beta$ usage (Acha-Orbea et al., 1988; Deckhut et al., 1993; Imarai et al., 1995; Kjer-Nielsen et al., 2003; Maryanski et al., 1996; Stewart-Jones et al., 2003; Turner et al., 2003; Urban et al., 1988; Wallace et al., 2000; Yanagi et al., 1990) and, less often, by a limited spectrum of TCR $V\alpha$ selection (Davis et al., 1995; Kjer-Nielsen et al., 2003), reviewed in (Turner et al., 2006). Most of the diversity in TCR/pMHC interactions rests in the CDR3 regions, with the relatively greater importance of CDR3 β , reflecting the availability of additional D elements, the numbers of D and J elements, D-element reading frame junctional diversity and N-nucleotide addition. The CDR3 β loop is generally positioned over the centre of the antigenic peptide bound inside the groove of the MHC molecule, and hence makes the predominant contact with the pMHC complex (Garboczi and Biddison, 1999; Garboczi et al., 1996). However, the CDR1 and CDR2 loops also mediate important interactions with antigenic peptides and/or MHC molecule components (Garcia et al., 1996; Kjer-Nielsen et al., 2003; Stewart-Jones et al., 2003).

Epitope-specific TCR repertoire diversity can be measured at a number of levels. Particularly for the $V\beta$ chains, a range of monoclonal antibodies (MAbs) is available to identify different TCR families. The greatest acuity is, however, achieved by first sorting with a TCR-specific MAb, then sequencing the CDR3 loop (that is, CDR3 sequence, length and J β usage) within the $V\beta$ or $V\alpha$ segments. Since the fundamental determinant of T cell diversity is the clonotypic TCR, clonality is defined by the CDR3 profiles within a particular TCR $V\beta$ (or TCR $V\alpha$) staining profile. Given that the majority of clonotypes are encoded by only one nucleotide sequence it is considered that every amino acid (aa) clonotype in an endogenous T cell response is the progeny of a single T cell precursor. However, since some dominant, or “public” clonotypes (Kedzierska et al., 2004) can be represented by several different nucleotide sequences, clonality needs to be addressed at the nucleotide level for such T cell responses.

Patterns of TCR CDR3 β usage can be defined in different ways, including the determination of amino acid (aa)/nucleotide (nt) sequences or by the spectratyping approach (Fig 1). The spectratyping or “immunoscope” technique was used extensively in the early 1990s to measure

CDR3 β length and J β usage (Lin and Welsh, 1998; Pannetier et al., 1993; Pannetier et al., 1995; Sourdivé et al., 1998) (Fig. 1A). This method relies on the use of a fluorescently-labelled primers for individual V β genes, together with a primers specific for the constant regions of the TCR β chain to amplify particular CDR3 β regions. These PCR products are then run on an automated sequencer and analysed for CDR3 β length. Spectratypes determined as CDR3 β length from a naïve TCR V β repertoire follow a classical Gaussian distribution (Fig. 2, representative naïve V β 8.3⁺CD8⁺ and V β 7⁺CD8⁺ TCR repertoire). Conversely, epitope-specific TCRs are characterised by biased, selected CDR3 β profiles (Fig. 3; representative immune V β 8.3⁺CD8⁺ and V β 7⁺CD8⁺ TCR repertoire).

Because the early TCR repertoire analyses were generally performed on total lymphocyte populations recovered during the course of immune responses, they did not specifically target pMHC (epitope)-specific T cells. Once tetrameric pMHC complexes became available for staining, it was then possible to use the spectratyping approach with sorted, epitope-specific T cells (Lim et al., 2002). However spectratyping does not define clonality as it cannot assess the extent of heterogeneity within a particular CDR3 β length. Sequencing the CDR3 β region of tetramer⁺ antigen-specific T lymphocytes overcomes this problem. Using this approach, tetramer⁺V β ⁺CD8⁺ cells can be bulk-sorted into tubes, followed by RNA isolation, cDNA synthesis and amplification of V β segment in a single (not-nested) PCR (Fig. 1B). The positive V β products are then cloned into a vector such as TOPO and colonies are selected for analysis. This technique is particularly useful for dominant T cell responses utilising more than one V β segment.

An alternative approach is to sort single tetramer⁺V β ⁺CD8⁺ T cells into the wells of 96-well plates, followed by cDNA synthesis and nested PCR to amplify the V β regions of interest. The PCR products are run on a gel and positive PCR products sequenced and translated (Fig. 1C). Several groups (including ours) have used this protocol to analyse the clonal characteristics of epitope-specific T cells. Recently, we compared single-cell V β sequencing with bulk-analysis for the same CD8⁺ T cell responses, and found comparable results when substantial, first-round PCR products were obtained (Kedzierska et al., 2006a). Thus, it appears that the real advantage of single-cell RT-PCR over bulk PCR analysis is for studies analysing low-frequency T cell populations, such as distinct subsets of memory T cells. Using the single-cell TCR repertoire approach, a small number of antigen-specific cells (<100 cells) can be sufficient to provide a comprehensive analysis of a particular response.

TCR REPERTOIRES IN VIRUS-SPECIFIC T CELL RESPONSES

A number of studies have looked at TCR diversity and the composition of repertoires for virus- and tumour-specific responses. In general, TCR repertoires can be either broad, consisting of numerous clonotypes of different CDR3 aa sequences and J β regions, or restricted to a few clonotypes that generally show similar J β and CDR3 β characteristics. In addition to the overall extent, TCR repertoires can be predominantly “public” (same clonotypes found in all the individuals) or completely “private” (unique to the individual). Examples of both types of TCR repertoires are discussed here.

(a) TCR repertoire in acute viral infections

Influenza A virus infection of C57Bl/6J (B6) mice provides a well-defined “natural” (non-TCR-transgenic) model for dissecting CD8⁺ T cell responses at the clonotypic level. This respiratory challenge system is characterized by an acute, transient, localized pneumonia, and virus clearance by day (d) 10 after primary infection (Allan et al., 1990; Doherty and Christensen, 2000). Prominent CD8⁺ sets are specific for epitopes derived from the viral nucleoprotein (D^bNP₃₆₆₋₃₇₄) (Falk et al., 1991) (Townsend et al., 1986) and acid polymerase (D^bPA₂₂₄₋₂₃₃) (Belz et al., 2000b) proteins. These D^bNP₃₆₆⁺CD8⁺ and D^bPA₂₂₄⁺CD8⁺ T cells

display strong biases in TCR β usage towards V β 8.3 (Belz et al., 2000a; Deckhut et al., 1993) and V β 7, respectively (Belz et al., 2000a; Turner et al., 2003), that are stable over time following both primary and secondary virus challenge (Kedzierska et al., 2006b; Turner et al., 2004).

The clonal characteristics of these D^bNP₃₆₆⁺CD8⁺V β 8.3⁺ and D^bPA₂₂₄⁺CD8⁺V β 7⁺ T cell responses were analysed, subsequent to FACS sorting, by single cell RT-PCR to determine CDR3 β profiles. The CD8⁺D^bPA₂₂₄⁺V β 7⁺ response (Turner et al., 2003; Turner et al., 2005) is essentially diverse and private, while a limited and predominantly public (found in all the mice) repertoire is consistently detected for the D^bNP₃₆₆⁺V β 8.3⁺CD8⁺ set (Kedzierska et al., 2004; Zhong and Reinherz, 2004). Compared to the Gaussian distribution in CDR3 β length (Fig. 2A) and J β region (Fig. 2C) found in naïve V β 8.3⁺CD8⁺ T cells, the high frequency public and repeated clonotypes in the CD8⁺D^bNP₃₆₆⁺V β 8.3⁺ populations are heavily biased toward usage of a 9aa CDR3 β loop (Fig. 3A) and J β 2.2 (Fig. 3B).

Apart from the high frequency public/repeated clonotypes, a small component (<10%) of the D^bNP₃₆₆⁺V β 8.3⁺CD8⁺ repertoire detected during the acute phase (d10) of the primary response is comprised of “private” clonotypes. These unique clonotypes are more likely to express TCRs that are “non-consensus” for CDR3 β length, sequence and J β usage (Kedzierska et al., 2006b). In contrast to the restricted TCR β repertoire for CD8⁺D^bNP₃₆₆⁺V β 8.3⁺ cells, the private and diverse TCR β sequences for the D^bPA₂₂₄⁺CD8⁺V β 7⁺ cells are characterized by a variety of J β elements (with a minor J β 1.1 and J β 2.6 bias) and a predominant 6aa CDR3 β loop (Fig. 3A) (Turner et al., 2003; Turner et al., 2005). Again, the CDR3 β lengths (Fig. 2B) and J β regions (Fig. 2D) found in naïve V β 7⁺CD8⁺ T cells followed the Gaussian distribution. Comparison of T cells recovered from different anatomical compartments, including the site of infection (lungs and BAL), draining lymph nodes (MLN), lymphoid organs (spleen) and non-lymphoid tissue (liver) demonstrated, for both the D^bPA₂₂₄⁺CD8⁺ (Turner et al., 2003) and the D^bNP₃₆₆⁺CD8⁺ (Kedzierska et al., 2004) sets, that the clonotypes are widely dispersed, with no apparent selection for particular tissue localization profiles. Taken together, these two prominent influenza-specific CD8⁺ T cell responses are characterised by distinct TCR repertoires: public and restricted for the CD8⁺D^bNP₃₆₆⁺V β 8.3⁺ response and private and diverse for the D^bPA₂₂₄⁺CD8⁺V β 7⁺ T cells. These very different, yet highly reproducible patterns offer obvious possibilities for dissecting the mechanisms underlying both selection as well as the use and persistence of available TCR repertoires.

Following influenza A virus infection in humans, CD8⁺ T cell responses in HLA-A2⁺ individuals are directed towards a dominant epitope derived from influenza virus matrix protein, MP₅₈₋₆₆ (GL9) (Gotch et al., 1987; Morrison et al., 1992). These CD8⁺ T cells specific for MP₅₈ complexed with HLA-A2 are characterised by highly restricted V β usage and remarkably similar CDR3 β sequences (Lehner et al., 1995; Moss et al., 1991; Stewart-Jones et al., 2003). The majority of MP₅₈CD8⁺ T cells express a V β 17 bias, while the CDR3 β sequences are highly conserved and contain the aa motif IRSSY (Lehner et al., 1995; Moss et al., 1991). In this restricted TCR repertoire, the same dominant aa sequences are encoded by different nucleotide sequences, similar to the public clonotypes detected for D^bNP₃₆₆-specific CD8⁺ T cell responses in B6 mouse model.

(b) TCR repertoire in persistent viral infections

As with the TCR repertoires selected for acute, readily resolved infections, the spectrum of clonotype diversity and composition following infection with persistent viruses can be diverse or restricted, private or public. A well-defined example of a persistent viral infection that induces two prominent CD8⁺ T cell responses characterised by different TCR repertoires is available for simian immunodeficiency virus (SIV) infection of the rhesus macaques. These SIV-specific CD8⁺ T cell responses are directed against Tat₂₈₋₃₅ (TL8) and Gag₁₈₁₋₁₈₉ (CM9)

peptides presented by the Mamu-A*01 MHC class I molecule (Price et al., 2004). Both CD8⁺ T cell responses display a strong V β bias; V β 6.5 and V β 27 (IGMT nomenclature), respectively. The TL8-Mamu-A*01-specific CD8⁺ T cells are characterised by a conserved CDR3 β region, both within and between individual monkeys, while the CM9-Mamu-A*01-specific clonotypes display diverse and private CDR3 β sequences.

Public clonotypes are also prominent in HLA-B8 individuals persistently infected with Epstein-Barr virus (EBV). This CD8⁺ T cell responses directed at a peptide derived from EBNA3 (FL9) selects a particular T cell sequence represented by the clonotype LC13 (Argaet et al., 1994), which can be encoded by several nucleotide sequences. Furthermore, LC13 TCR recognises both the EBV epitope (FL9-HLA-B8) and the HLA-B*4402 alloantigen on uninfected cells (Burrows et al., 1994). Thus, the LC13 TCR is not found in individuals expressing HLA-B*4402, presumably as a consequence of negative selection in thymus. In individuals expressing both HLA-B8 and HLA-B*4402, an array of diverse TCR clonotypes emerges that can recognise the FL9-HLA-B8 complex as efficiently as LC13 clonotype (Burrows et al., 1995), suggesting that availability of this dominant TCR is not critical for EBV-specific CD8⁺ T cell responses.

However, contrasting effects have been observed with herpes simplex virus (HSV) infection of B6 mice. The predominant H2-K^b-restricted CD8⁺ T cell response to HSV is directed at a peptide derived from the HSV-1 glycoprotein B (gB₄₉₅₋₅₀₂; SL8) (Cose et al., 1995; Wallace et al., 1999). These gB-specific CD8⁺ T cells are characterised by a strong V β 10 bias, while particular clonotypes use a common germline TCR D β gene segment, with a conserved junctional sequence at position 3 and 4 (Cose et al., 1995). Interestingly, deletion of this germline D β region led to decreased V β bias as well as reduction overall in the magnitude of the CD8⁺ T cell responses to gB (Wallace et al., 2000), suggesting that the deletion of immunodominant clonotypes does not always lead to a compensatory increase in other responses.

Virus-specific CD8⁺ T cell responses to the cytomegalovirus (CMV) NLV epitope in HLA-A2⁺ individuals are characterised by a diverse TCR repertoire that is maintained in latent CMV infection. However, following CMV reactivation, this TCR repertoire narrows and, due to the high avidity of this clone for the NLV-HLA-A2 complex (Trautmann et al., 2005), is dominated by a single T cell clone. Narrowing of TCR repertoire in the chronic phase of persistent infection has also been described by others for SIV (Price et al., 2004), HIV (Pantaleo et al., 1994; Pantaleo et al., 1997), CMV (Trautmann et al., 2005; Wills et al., 1996) and EBV (Argaet et al., 1994; Callan et al., 1996). Emerging evidence suggests that this focusing effect reflects the preferential selection of high avidity TCR clonotypes (Price et al., 2005; Trautmann et al., 2005) as discussed later in more detail.

STRUCTURAL DETERMINANTS OF TCR DIVERSITY

Differences in the nature of responding TCR repertoires may, at least in part, be due to the structural characteristics of the peptide-MHC class I complex (Kjer-Nielsen et al., 2003; Stewart-Jones et al., 2003; Turner et al., 2005). Structural analyses of D^bNP₃₆₆ and D^bPA₂₂₄ have established that D^bNP₃₆₆ is relatively flat and featureless from the aspect of the TCR contact site (Young et al., 1994), while D^bPA₂₂₄ is much more “featured” with a prominent P7-arginine (Meijers et al., 2005; Turner et al., 2005). The consequence is that an appropriate TCR “fit” with the relatively “bland” D^bNP₃₆₆ is probably harder to achieve and thus D^bNP₃₆₆ selects a much less diverse, and more “public” spectrum of TCRs than D^bPA₂₂₄. The substantially greater diversity of the D^bPA₂₂₄ response fits with the idea that the TCR constraints governing recognition of the “prominent” D^bPA₂₂₄ are indeed less demanding than those required for the “featureless” D^bNP₃₆₆. Modifying the P7-arginine to a less prominent

alanine (R7A substitution) generates a topographically “flatter”, but still immunogenic P7Ala-D^bPA₂₂₄ which, following infection with a modified virus, leads to the selection of a significantly narrower TCR repertoire (Turner et al., 2005).

At the other end of the spectrum, peptides that orient in the MHC class I groove in a way that causes them to protrude too far can be obstructive and also (like D^bNP₃₆₆) cause the selection of a restricted TCR repertoire. This can be seen for some longer peptides, which overcome the “length” constraint for binding to MHC class I by “anchoring” the two ends in pockets and allow the intervening peptide residues to “bulge out” in a way that limits recognition by clonotypic TCRs. An excellent example is the 13aa EBV peptide that binds to HLA-B*3508 to give a “bulged”, rigid conformation that is recognized almost exclusively by the dominant LY13 TCR (Tynan et al., 2005a; Tynan et al., 2005b).

Recent crystallographic analysis of the influenza-specific GL9 peptide in HLA-A2⁺ individuals provided further insights into the structural basis of restricted TCR selection (Stewart-Jones et al., 2003). The crystal structure of the GL9-HLA-A2 complex ligated to specific Vβ17 TCRs is characterised by a highly conserved CDR3β sequence, **IRSSY** that utilizes a novel “peg-notch” binding mode (Stewart-Jones et al., 2003). In this conformation, the side-chain of the conserved arginine residue in the CDR3β loop inserts into a notch formed by GL9-HLA-A2 complex. Crystal structure analysis of the EBV-specific FL9-HLA-B8 complex also showed a similar peg-notch binding conformation (Kjer-Nielsen et al., 2003). In this case, however, it was a prominent tyrosine residue of the viral FL9 peptide (and not TCR as observed for GL9 peptide) forming a peg and being inserted into the CDR loops of the LC13 TCR.

Taken together, published studies provide solid evidence for a strong linkage between TCR diversity and peptide-MHC complex topology (reviewed in more details in (Turner et al., 2006)). Prominent features lead to selection of more diverse TCR repertoires for canonical peptides of 8 to 10 aa, while excessive bulging in long peptides (such as 13 aa) may inhibit binding and lead to the selection of a very restricted TCR repertoire. Furthermore, a prominent residue in either the antigenic peptide (eg FL9 peptide) or the CDR3β loop of the TCR (eg GL9 peptide) can form a peg that inserts into a CDR region or a peptide-MHC complex, respectively. A primary constraint in the generation of any TCR repertoire thus reflects the capacity of complementary “shapes” to fit together. The spectrum of potential interface in the naive TCR repertoire will be determined by selective events in the thymus, while the binding of adventitious peptides derived from pathogens gives a diverse spectrum of conformations that will, in turn, determine the spectrum of “selectable” TCR repertoire.

ANTIGEN DOSE AND TCR REPERTOIRE

Does antigen dose influence the diversity of responding T cell populations? Given the established relationship between antigen dose and TCR/epitope avidity, it seems possible that the extent of immunogenic epitope availability could play a determining role in TCR selection. This question was addressed for the influenza A viruses by altering the viral context of the prominent D^bNP₃₆₆ and D^bPA₂₂₄ epitopes by inserting the immunogenic peptides in the stalk of the surface neuraminidase (NA) protein (La Gruta et al., 2006b). Compared to control viruses expressing NP₃₆₆ (NP⁺PA⁻) and PA₂₂₄ (PA⁺NP⁻) in their native configuration, there was a 5-fold decrease in the D^bNP₃₆₆-specific CD8⁺ response with the NA virus and a 2-fold increase in the D^bPA₂₂₄-specific response (2-fold). Despite the alteration in response magnitude, there was no effect on the clonal composition or diversity of TCR repertoire for either the D^bPA₂₂₄- or D^bNP₃₆₆-specific CD8⁺ T cell populations (La Gruta et al., 2006b). As following infection with wild type influenza viruses, the D^bNP₃₆₆⁺Vβ8.3⁺CD8⁺ T cell set showed evidence of limited diversity and domination by public and repeated clonotypes characterised

by J β 2.2 and CDR3 β loop of 9aa, while the D^bPA₂₂₄⁺V β 7⁺CD8⁺ TCR repertoire was diverse and predominantly private in nature. Thus, this study suggests that altered antigen dose leading to increased/decreased magnitude of CD8⁺ T cell responses does not affect either the clonal composition or diversity of a responding CD8⁺ T cell repertoire.

DISSECTING MEMORY T CELL RESPONSES

Detailed dissection of TCR repertoires during the acute phase of primary infection provides a baseline for following T cell populations through the course of the immune response, into long term memory, then following secondary challenge (Kedzierska et al., 2005; Kedzierska et al., 2004; Kedzierska et al., 2006c; Malherbe et al., 2004; McHeyzer-Williams and Davis, 1995; Turner et al., 2003). If, for instance, the same individuals are sampled repeatedly (using blood) individual clonotypes can be followed using single cell FACS sorting, PCR and CDR3 β sequencing.

(a) Longitudinal analysis of TCR repertoire

Longitudinal analysis allows the fate of T cell clones to be tracked within the individual. Several studies have used this approach to follow clonotypic composition from an acute primary time-point through to early and long-term memory, and for secondary recall responses. This is an excellent approach to determine whether the same clonotypes persist from the acute response into the memory phase, then follow the recall response within the same individuals. Mice are bled at different time-points after primary infection, the T cells are stained with tetramer, antibodies to V β and CD8, and then single cell sorting and PCR-based technology is used to determine the clonal composition and diversity of the responding populations. Experiments of this type have established that the TCR repertoire within peripheral blood reflects that found in the spleen (Kedzierska et al., 2004; Turner et al., 2003). Once the memory phase is analysed, mice can be subsequently challenged and the clonotypes contributing to the recall responses identified in blood, spleen and other organs of interest.

Sequential, single-cell analysis of CDR3 β profiles for the D^bPA₂₂₄⁺V β 7⁺CD8⁺ and D^bNP₃₃₆⁺V β 8.3⁺CD8⁺ responses in individual mice have demonstrated that TCR β s prominent in the antigen-driven phase persist into memory, and are again expanded following secondary challenge (Kedzierska et al., 2004; Turner et al., 2003). The TCR β repertoires for both epitopes demonstrated equivalent levels of clonotypic diversity at every time-point during the primary or secondary response (Kedzierska et al., 2004; Turner et al., 2003). Prominent CD8⁺ TCR β clonotypes found at the peak of the primary response could always be detected in the blood of mice in the memory phase, even though the numbers of T cells recovered by this procedure were fairly small. The clonal stability from the acute response through to long-term memory found for both D^bNP₃₆₆ CD8⁺ and D^bPA₂₄₄CD8⁺ TCR repertoires is in agreement with previous published evidence from other infectious systems (Blattman et al., 2000; Lin and Welsh, 1998; Maryanski et al., 1996; Sourdive et al., 1998). Following secondary challenge, the relative frequencies of particular TCR β clonotypes were occasionally altered, although the extent of TCR β diversity was not substantially modified for either response. Prominent TCR β clonotypes found at high frequency in the primary and memory TCR β repertoires were not always dominant in the recall response. Conversely, some of the TCR β clonotypes detected at low frequency in the primary and memory CD8⁺ TCR β repertoires were greatly expanded following secondary challenge. This effect was observed for both the D^bNP₃₆₆CD8⁺ (Kedzierska et al., 2004) and D^bPA₂₄₄CD8⁺ T cell responses (Turner et al., 2003). Thus, while the majority of the secondary CD8⁺ TCR β repertoire is derived from antigen-specific CD8⁺ clonotypes found in the memory phase following influenza infection, the selection and subsequent expansion of particular CD8⁺ TCR β clonotypes following challenge appears to be a stochastic process.

In contrast, analysis of antigen-specific CD8⁺ T cell repertoires in other experimental systems has indicated narrowing during memory differentiation and subsequent secondary challenge (Busch et al., 1998; McHeyzer-Williams et al., 1999; McHeyzer-Williams and Davis, 1995). This, in turn indicates preferential selection of particular clonotypes upon secondary challenge. The underlying reasons are unknown but could include selection of high TCR affinity/avidity clonotypes.

(b) Central and effectot memory T cell subsets

Since memory T cells are known to be heterogenous populations with distinct lymph-node homing properties, anatomical locations and functions (Sallusto et al., 1999), it is of interest to understand how these distinct memory sets relate to one another. Based on the expression of lymph-node homing markers, human and murine memory T cells have been classified as “central memory” (T_{CM}, CD62L^{hi}, CCR7^{hi}), circulating between lymphoid organs, and “effector memory” (T_{EM}, CD62L^{lo}, CCR7^{lo}), residing in peripheral tissues (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 1999). Both the T_{CM} and T_{EM} subsets can be found in the peripheral blood and spleen (Sallusto et al., 1999; Wherry et al., 2003). Apart from their anatomical compartmentalization, T_{CM} and T_{EM} memory populations are also considered to display different functional properties (Masopust et al., 2001; Sallusto et al., 1999; Wherry et al., 2003).

There is no general agreement on the development of T_{CM} and T_{EM} subsets. While most accept that a proportion of the T_{CM} precursors can become effector and/or T_{EM} cells following secondary challenge (Bouneaud et al., 2005; Roberts et al., 2005; Wherry et al., 2003), some experiments suggest that a T_{EM}→T_{CM} transition occurs in the long-term (Bouneaud et al., 2005; Wherry et al., 2003). Conversely, others propose that diverse T_{CM} populations include a range of partially differentiated phenotypes that reflect a more limited and varied “signalling experience” and are a continuous source of distinct effector and T_{EM} sets (Lanzavecchia and Sallusto, 2000; Lanzavecchia and Sallusto, 2002). A further opinion is that the T_{EM} and T_{CM} populations divide into distinct lineages from the time of primary antigen exposure (Marzo et al., 2005). Most of the mouse experiments performed to investigate the relationship between T_{CM} and T_E subsets utilize adoptively transferred TCR-transgenic T cells (Dumortier et al., 2005; Marzo et al., 2005; Wherry et al., 2003). While, this is sufficient in some circumstances, defining the relationship between distinct subsets is rather difficult using a clonotypic TCR.

An alternative approach is to study the TCR diversity of an unmanipulated, endogenous, antigen-specific response to define the clonotypic character (including possible lineages) for the T_{CM} and T_{EM} subsets. It is important to note that only a small number of studies have been published using this approach. In humans, a limited long-term memory analysis of TCRs expressed on influenza-virus specific CD8⁺ memory T cells identified two signatures that were shared by stable CD62L^{lo} and CD62L^{hi} clones, but found no evidence that other T cells were converting from CD62L^{lo} to CD62L^{hi} over time in long-term memory (Baron et al., 2003). The memory CD62L^{hi} subset was, however, characterised by a greater clonal diversity than the CD62L^{lo} population. A further study from the same group, with adoptively transferred murine TCRβ transgenic lymphocytes with a variable TCRα chain, and analysed H-Y-specific CD62L^{hi} and CD62L^{lo} memory T cell clonotypes defined by sequence variation in the CDR3α region (Bouneaud et al., 2005). Two thirds of the T_{EM} and T_{CM} clones isolated at 6 to 10 weeks after priming shared a common naïve precursor. Unique CD8⁺ T cell clonotypes were present predominantly in the CD62L^{hi} subset, similar to the findings by Baron *et al* (Baron et al., 2003), indicating that the CD62L^{hi} population is significantly more diverse than the CD62L^{lo} set during the memory phase of the response.

Our recent, single cell analysis of TCR CDR3β profiles in influenza A virus-infected mice utilized partitioning into tetramer⁺ CD62L^{hi} and CD62L^{lo} subsets specific for the D^bNP₃₆₆

and D^bPA₂₂₄ epitopes to determine the clonotypic diversity and composition of these populations from the earliest phase of the acute response (d8) through to very long-term memory (d690). The predominant D^bNP₃₆₆+Vβ8.3+CD8⁺ and D^bPA₂₂₄+Vβ7+CD8⁺T cell populations were sorted as single CD62L^{hi} or CD62L^{lo} T cells, and the extent of TCR diversity was assessed by RT PCR and CDR3β sequencing. Our data showed that the same prominent clonal expansions bearing “public” or “shared” TCRs could be found in both CD62L^{lo} and CD62L^{hi} T cells specific for the influenza A virus D^bNP₃₆₆ and D^bPA₂₂₄ epitopes, although the CD62L^{hi} sets showed evidence of a more diverse repertoire than the comparable CD62L^{lo} populations, a profile that remained consistent through the acute and resolution phases of the primary response (Kedzierska et al., 2006c) (Table 1; a representative mouse for D^bPA₂₂₄+Vβ7+CD8⁺T cells; d28).

Detailed (>2,000 sequences from 13 mice) dissection indicated that a substantial component of the more diverse CD62L^{hi} T_{CM} pool is comprised of a very small number of clones that, perhaps, express sub-optimal TCRs. This high frequency of small CD62L^{hi} clones with “unique” TCRs reflected the TCR diversity range per mouse. The diversity was generally 2-fold greater within the CD62L^{hi}CD8⁺D^bNP₃₆₆⁺ set and 1.6-fold greater for CD62L^{hi}CD8⁺D^bPA₂₂₄⁺ population. Additionally, analysis of different time-points throughout the primary response revealed that this relationship was maintained from d8 into long-term memory (>d180 to d560). Our clonotypic analysis of acute and memory T_{CM} and T_{EM} populations suggested early establishment of both CD62L^{lo} and CD62L^{hi} memory CD8⁺ T cell populations (during the first week) and indicated that influenza-specific memory within the stable CD8⁺CD62L^{hi} subset preserves clonal diversity and prevents “overdominance” by a few public, or shared, clones.

Thus, all three studies (Baron et al., 2003; Bouneaud et al., 2005; Kedzierska et al., 2006c) suggest that the majority (at least 2/3) of the TCR repertoire in both CD62L^{lo} and CD62L^{hi} memory subsets are shared, and therefore cannot be from separate lineages, while the clonotypic composition of the CD62L^{hi} repertoire is more diverse and contains additional clonotypes, which are often characterised by sub-optimal TCRs. These clonotypes are not preferentially recalled following subsequent challenge (Bouneaud et al., 2005; Kedzierska et al., 2006b). Interestingly, these unique CD62L^{hi} clonotypes found throughout the primary response cannot be recalled (even within the CD62L^{hi} subset) after secondary challenge and, as a consequence, can not be detected at secondary long-term memory time-points (Kedzierska K *et al*, unpublished data).

DISSECTION OF FUNCTIONALLY DISTINCT SUBSETS OF EFFECTOR T CELLS

Characteristics of the TCR repertoire may also be correlated with functional differences within T cell populations. Are “best-fit” TCR clonotypes functionally superior?

High affinity/avidity T cells

Published evidence suggests that optimal virus-specific CD8⁺ T cell responses are of relatively high avidity (Alexander-Miller et al., 1996; Gallimore et al., 1998; Valmori et al., 2002; Zeh et al., 1999). As high avidity CD8⁺ T effectors can kill virus-infected cells 1000 more efficiently than low avidity populations (Alexander-Miller et al., 1996), these cells are of considerable interest for vaccine development. Clonotypic dissection of high affinity/avidity T cell populations can provide important insights into how protective CD8⁺ T cell responses might be generated.

Single-cell analysis was used to assess the contribution of particular clonotypes to high avidity T cell populations, with avidity being defined by binding characteristics under conditions of limiting tetramer availability. Based on published evidence (Busch and Pamer, 1999; Crawford et al., 1998; Daniels and Jameson, 2000; Yee et al., 1999), diminished access to low concentrations of tetramer selects a subset of T cells expressing high avidity TCRs. Thus, the tetramer dilution assay provides a potential mechanism for dissecting TCR clonal diversity for within the total, antigen-specific CD8⁺ T cell pool (stained with tetramers at saturating levels) and high avidity cells (stained under limiting tetramer conditions). A subset of D^bPA₂₂₄⁺Vβ7⁺ CD8⁺ T cell clonotypes was preferentially selected under conditions of limited tetramer availability, that is there was selection of particular D^bPA₂₂₄-specific clonotypes such that they were significantly over-represented in the putative “high avidity” population comparing to the total epitope-specific CD8⁺ set. Conversely, prominent, public D^bNP₃₆₆⁺Vβ8.3⁺ CD8⁺ T cell clones were found at essentially equivalent prevalence in effector T cells sorted from the same mice at the same time under conditions of saturating and minimal tetramer availability. These results suggest there is a pattern of differential TCRβ clonotype distribution based on the overall profile TCR-pMHC avidity. Furthermore, it appears the extent of such contribution may depend on the degree of clonal TCRβ diversity within the particular epitope. Thus, whilst TCR sequence appears to be the major contributor to avidity in diverse virus-specific CD8⁺ populations, other mechanisms may be predominant determinants of avidity in the absence of TCR diversity. Since our previous results showed a significantly higher dependence of D^bNP₃₆₆⁺CD8⁺ T cells on the CD8 co-receptor when compared to the D^bPA₂₂₄⁺CD8⁺ T cell population (La Gruta et al., 2006a; La Gruta et al., 2004), we can speculate that CD8 contributes significantly to overall avidity in the CD8⁺D^bNP₃₆₆⁺ set.

A similar approach using single-cell RT-PCR combined with affinity measurements was used by Malherbe and colleagues to directly correlate pMHCII binding affinities and clonal diversity of responding CD4⁺ T cell populations (Malherbe et al., 2004). Although naïve CD4⁺ T cells specific for pigeon cytochrome c (PCC) displayed a wide array of TCR affinities before and soon after antigenic exposure, clonotypes with low affinity TCRs were selectively lost during the immune response. Thus, it appears that the transition of PCC-specific CD4⁺ T cells from naïve to the immune repertoire requires that specific TCRs reach a certain affinity threshold for peptide-MHC class II complexes. This, in turn, suggests that the clonotypes with best-fit TCRs possess a selective advantage over less-fit clonotypes in the naïve T cell repertoire (Malherbe et al., 2004). Further, published evidence for prominent immune TCR clonotypes expressing high avidity for peptide-MHCI comes from a recent analysis of CD8⁺ T cell responses in individuals with chronic CMV or EBV infection (Price et al., 2005). In this study, the emergence of dominant TCR clonotypes during infection correlated with higher TCR avidity for peptide-MHC class I tetramers.

CONSEQUENCES OF TCR DIVERSITY

There is some evidence that the difference between immune protection and escape in CD8⁺ T cell responses can be related to the extent of TCR diversity (Messaoudi et al., 2002; Price et al., 2004). The SL8 peptide of HSV is presented equally well by both the wt H2K^b and the mutant H2-K^{bm8} MHC class I glycoproteins, which differ by four aa in the peptide-binding groove (Webb et al., 2004). Comparison of the wt B6 and congenic H2-K^{bm8} mutant strains showed that the wt mice were much more susceptible to HSV infection. Dissection of the SL8-specific TCR repertoires established that SL8-H2K^{bm8} selects a more diverse and higher avidity than spectrum of responding TCRs (Messaoudi et al., 2002). As TCR avidity is thought to be associated with functional capacity (Slifka and Whitton, 2001), it is reasonably to think that the nature of the selected TCR repertoire influenced this divergence in resistance profiles

for the B6 wt and K^{bm8} mice. The results suggest that both TCR repertoire diversity and TCR avidity play into this equation.

In the rhesus macaque SIV model (Price et al., 2004), CD8⁺ T cell responses to the SIV TL8 peptide are characterised by a conserved, restricted TCR repertoire and the rapid emergence of viral escape mutants with a point mutation at position 5 of the TL8 peptide. The specific mutation of Ser to Leu in TL8 evades TCR recognition without affecting peptide binding to Mamu-A*01. This is possibly due to the fact that the restricted TL8-Mamu-A*01-specific TCR repertoire lacks the flexibility to recognise the TL8 epitope with minor structural changes, thereby allowing SIV-infected cells to escape immune elimination (Price et al., 2004). Conversely, the diverse TCR repertoire of SIV CM9-specific CD8⁺ T cells is not associated with the emergence of viral escape mutants. Although mutations may occur within the CM9 peptide, the CM-9-Mamu-A*01-specific TCRs have the breadth needed to recognise escape variants, thereby preventing the outgrowth of mutant viruses. Thus, the extent of TCR diversity can play a key role in limiting the emergence of escape mutants.

Apart from TCR diversity affecting the protective capacity of CD8⁺ T cells and the ability to recognise mutated viral epitopes, diverse TCR repertoires provide more scope for cross-reactive CD8⁺ T cell responses. The fact that a diverse TCR repertoire for a specific antigen has a very diverse “tail” consisting of low frequency clonotypes may provide some explanation for unexpected cross-reactive immune response profiles that have been detected for some unrelated pathogens (Kim et al., 2005; Selin and Welsh, 2004).

CONCLUSIONS

Since the advent of tetrameric complexes, a substantial body of research has been done to characterise the TCR repertoires generated in responses to various acute and persistent pathogens. Recently, a combination of TCR repertoire analysis with FACS subsetting of functionally and phenotypically distinct T cell populations has provided a novel way of dissecting specific immune response. Continuation of this approach should allow us to gain more insight into the mechanisms determining both the expression of various phenotypic markers (e.g. CD62L and IL-7R α) and the capacity to generate a diversity of effector molecules (e.g. IFN- γ , TNF- α and IL-2) in both the acute response and long-term memory. A better understanding of how T cell-mediated immunity is established and maintained at the clonal level may, in turn, suggest possibilities for improved vaccine and immunotherapy protocols.

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Figure 1. Different experimental approaches to TCR repertoire analysis

Antigen-specific T cells are sorted either as a bulk population (A, B) or single-cells (C). Following amplification of Vβ (or alternatively Vα) regions by RT-PCR, PCR products are either (A) analysed by spectratyping method, (B) cloned into TOPO vector, followed by sequencing or (C) used for a nested PCR and sequencing.

Figure 2. CDR3β and Jβ characteristics of naive Vβ8.3 and Vβ7 CD8⁺ TCR repertoires

Lymphocytes were obtained from spleens of C57BL/6J mice and stained with mAbs directed to CD8 and either (A, C) Vβ8.3 or (B, D) Vβ7. Vβ8.3⁺CD8⁺ or Vβ7⁺CD8⁺ T cell populations were single-cell sorted into 96-well plates, followed by RT reaction, nested PCRs and sequencing of positive PCR products. The CDR3β sequences were deduced from the known genomic sequence (Arden et al., 1995; Iwamoto et al., 1987; Saito et al., 1984) and the Jβ-regions were assigned according to Gascoigne *et al.* and Malissen *et al.* (Gascoigne et al., 1984; Malissen et al., 1984).

Figure 3. CDR3β and Jβ analysis of immune D^bNP₃₆₆⁺Vβ8.3⁺CD8⁺ and D^bPA₂₂₄⁺Vβ7⁺CD8⁺ T cells at the peak of the primary response

Lymphocytes were obtained from spleens of C57BL/6J mice at 10 days after i.n. infection with the HK X31 influenza A virus (primary response). Cells were enriched for CD8⁺ T cells and stained with either D^bNP₃₆₆ or D^bPA₂₂₄ tetramers, anti-CD8 and anti-Vβ8.3 (for D^bNP₃₆₆⁺CD8⁺ T cells) or anti-Vβ7 (for D^bPA₂₂₄⁺CD8⁺ T cells) mAbs directly conjugated to APC and FITC respectively. The D^bNP₃₆₆⁺Vβ8.3⁺CD8⁺ cells (black bars) and D^bPA₂₂₄⁺Vβ7⁺CD8⁺ cells (white bars) were single-cell sorted into the cDNA buffer using the MoFlo sorter. Comparison of (A) CDR3β and (B) Jβ regions are shown.

Table 1
Clonal diversity of CD62L^{hi} and CD62L^{lo} subsets of D^bPA₂₂₄⁺Vβ7⁺CD8⁺ memory populations on d28 after primary infection with influenza A virus.

CDR3β region	YFG	Jβ	Length	CD62L ^{lo} Frequency	CD62L ^{hi} Frequency
SLGGYEQ	YFG	2S6	7	21/36	1/38
SPGSEV	FFG	1S1	6	4/36	6/38
LGGNYAEQ	FFG	2S1	8	4/36	4/38
SSGGAV	FFG	1S1	6	2/36	1/38
SQGSEV	FFG	1S1	6	1/36	2/38
SLTGEQ	YFG	2S6	6	1/36	1/38
SLAGYEQ	YFG	2S6	7	1/36	1/38
SSVEQ	YFG	2S6	5	1/36	3/38
SAVEQ	YFG	2S6	5	1/36	-
SGGTEV	FFG	1S5	6	-	1/38
SPYSANSY	TFG	1S2	9	-	1/38
SFGDNSNERL	FFG	1S4	10	-	1/38
VQGSNERL	FFG	1S4	8	-	1/38
LGNTGQL	YFG	2S2	7	-	1/38
SLGGNQNTL	YFG	2S4	9	-	1/38
SGGDEQ	YFG	2S6	6	-	1/38
SSGGEQ	YFG	2S6	6	-	2/38
SWGAEQ	YFG	2S6	6	-	8/38
RTGTEQ	YFG	2S6	6	-	1/38
AEGDTQ	YFG	2S5	6	-	1/38
# shared clonotypes (% shared sequences)				8 (97.2%)	8 (50.0%)
# clonotypes in CD62L ^{lo} only				1 (2.8%)	-
# clonotypes in CD62L ^{hi} only				-	11 (50.0%)

* TCR clonotypes for a representative mouse out of 13 individual mice are shown.