Early establishment of diverse T cell receptor profiles for influenza-specific CD8$^+$CD62L^{hi} memory T cells

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Single-cell analysis of endogenous, primary CD8$^+$ T cell responses to the influenza D^{b}NP$_{366}$ and D^{b}PA$_{224}$ epitopes indicates that prominent clonotypes bearing ''public'' or ''shared'' T cell receptors (TCRs) subset early into CD62L^{hi} and CD62L^{lo} populations. The CD62L^{lo} effectors divide more and are rapidly eliminated during the contraction phase, whereas stable CD62L^{hi} memory populations persist in the long-term. Reflecting the high frequency of small CD62L^{hi} clones expressing ''private'' TCRs, the TCR diversity range per mouse is generally two times higher within the CD62L^{hi}CD8$^+$D^{b}NP$_{366}^+$ set (1.6 times higher for CD62L^{hi}CD8$^+$D^{b}PA$_{224}^+$) from 8 to >180 days after antigen challenge. Memory CD8$^+$CD62L^{hi} T cell precursors thus segregate from the outset into populations expressing ''best-fit'' and ''suboptimal'' TCR characteristics, with this pattern being maintained stably thereafter. Hence, our analysis suggests that early establishment of influenza-specific memory within the CD8$^+$CD62L^{hi} subset preserves clonal diversity and prevents ''overdominance'' by a few public, or shared, clones.

influenza A virus | T cell receptor repertoire

Memory T cells are heterogenous populations with distinct lymph node homing properties, anatomical locations, and functions (1). Current thinking is focused on the idea that both human and mouse memory T cells can be classified into distinct central memory (T_{CM}) and effector memory (T_{EM}) sets (1–5). Whereas the T_{CM} lymphocytes are CD62L^{hi}, which means that these T cells transit directly from blood to lymph nodes via the high endothelial venules (6, 7), the more "activated" CD62L^{lo} T_{EM} set accesses the nodes only via afferent lymph and is widely dispersed in a broad range of somatic tissues (8, 9). The CD62L "gating" function does not operate in the spleen, which tends to reflect the CD8$^+$ T cell phenotypes found in blood. The T_{CM} cells also express much higher levels of the IL-7R (10) known to be important for selection of long-term memory (11).

Much of the debate in this field focuses on how the CD62L^{hi} T_{CM} and CD62L^{lo} T_{EM} subsets relate to each other (12, 13). Most would accept that a proportion of the T_{CM} precursors must have the capacity to become fully functional effector and/or T_{EM} cells after secondary challenge (4, 5, 14). Some studies support the view that a $T_{EM} \rightarrow T_{CM}$ transition is possible, both during the acute response and in long-term memory (4, 14). Another idea is that diverse T_{CM} populations represent a range of partially differentiated phenotypes that, reflecting a more limited and varied "signaling experience" during the antigen-driven phase, have not achieved T_{EM} status (15, 16). Others suggest that the T_{EM} and T_{CM} subsets segregate immediately into different lineages from the time of primary antigen challenge (17). At least some of these models can be accommodated within the one conceptual framework, although there is no general agreement on mechanism.

The more informative studies have focused on the analysis of defined clonotypes, with most of the mouse experiments using adoptively transferred T cell receptor (TCR) transgenic T cells (4, 17, 18). This is fine for many purposes, but, because the recipients are generally given reasonably large numbers of lymphocytes that all express the same TCR, the only relationship that can be inferred in the absence of further FACS separation and adoptive transfer is between the pool of naïve precursors and the progeny T_{CM} or T_{EM} populations. An alternative approach is to exploit 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. Very few such studies have been done. In humans, a very 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 (19). These profiles could reflect single or multiple challenges, because people are subject to repeated influenza virus infections. A separate set of mouse experiments used adoptively transferred TCRβ transgenic lymphocytes, then analyzed H-Y-specific CD62L^{hi} and CD62L^{lo} memory T cell clonotypes defined by sequence variation in the complementarity-determining region (CDR) 3α (14). The conclusion was that two-thirds of the T_{CM} and T_{EM} clones isolated at 6–10 weeks after priming shared a common naïve precursor.

The present analysis builds on our earlier, single-cell dissection of TCR CDR3β profiles for influenza virus-specific CD8$^+$ T cells (9, 20–22). Based on the Jβ usage and amino acid sequence of the CDR3β loop, we previously found diverse and "private" (specific for individual mice) TCRβ sequences for CD8$^+$ T cells specific for the D^{b}PA$_{224}$ (acid polymerase) (9) and a very restricted and predominantly "public" (found in at least eight different individuals) or "shared" (shared between two different individuals) repertoire for the D^{b}NP$_{366}$ (nucleoprotein) (20). In this study, partitioning into tetramer$^+$ CD62L^{hi} and CD62L^{lo} subsets specific for CD8$^+$D^{b}NP$_{366}^+$Vβ8.3$^+$ and CD8$^+$D^{b}PA$_{224}^+$Vβ7$^+$ cells has been analyzed from the early phase of the response [day 8 (d8)] through to very long-term memory (d690). This detailed (>2,000 sequences from 13 mice) dissection indicates that a substantial component of the more diverse CD62L^{hi} T_{CM} pool is composed of low-frequency clones expressing nonconsensus TCRs.

Results

Naïve B6 mice were infected intranasally with the HKx31 (H3N2) influenza A virus and sampled for FACS phenotyping and/or single-cell CDR3β repertoire analysis 6–575 days later. The analysis focuses on the spleen, reflecting the ease of recovery for the relatively low-frequency, "primary" CD8$^+$ memory T cells from this large lymphoid organ and absence of the gating by high endothelial venules that selectively enriches CD62L^{hi} T cells in the lymph nodes (6, 7).

Conflict of interest statement: No conflicts declared.

Abbreviations: TCR, T cell receptor; d*n*, day *n*; CDR, complementarity-determining region; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; DC, dendritic cell.

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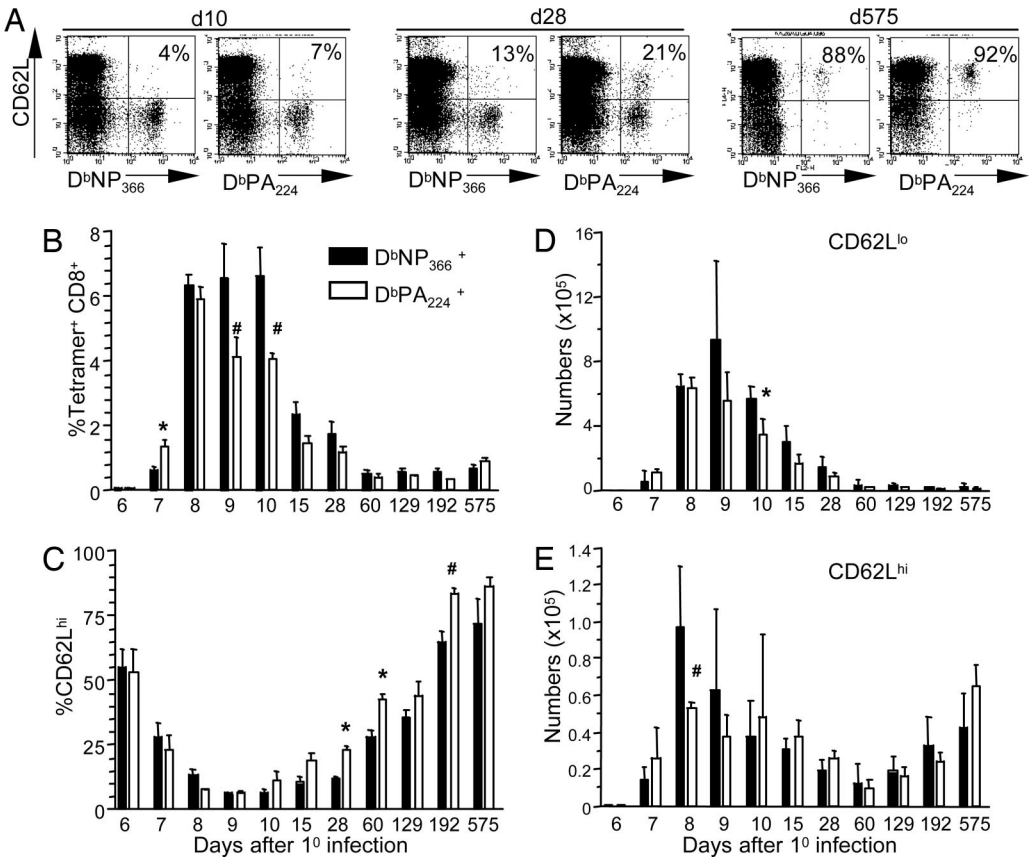

Fig. 1. Profiles of CD62L staining for $CD8^+D^bNP_{366}^+$ and $CD8^+D^bPA_{224}^+$ T cells in a long time course after primary influenza A virus infection. (*A*) Representative FACS plots (gated on the $CD8^+$ population) from the acute (d10), early memory (d28), or long-term memory (d575) phases after primary intranasal challenge of naïve B6 mice with the HKx31 influenza A virus. (*B*) Frequency values for $D^bNP_{366}^+$ (black bars) and $D^bPA_{224}^+$ (white bars) T cells within the total $CD8^+$ set. (*C*) Relative proportion of $CD62L^{hi}$ T cells within the tetramer$^+$ populations. (*D* and *E*) Numbers of $CD62L^{lo}$ (*D*) and $CD62L^{hi}$ (*E*) $CD8^+D^bNP_{366}^+$ and $CD8^+D^bPA_{224}^+$ T cells, calculated from the total cell counts (data not shown), and the percentage values. Lymphocytes were obtained from the spleens of individual mice ($n = 3$–9) at 6–575 days after infection. Enriched $CD8^+$ T cells were stained with the D^bNP_{366} or D^bPA_{224} tetramers conjugated to streptavidin-phycoerythrin, anti-CD8α-peridinin chlorophyll protein, and anti-CD62L-allophycocyanin. Statistically significant differences (*, $P < 0.01$; #, $P < 0.05$) are shown for the $CD8^+D^bNP_{366}^+CD8^+$ and $CD8^+D^bPA_{224}^+$ T cells at the various time points.

Profiles of CD62L Expression from the Acute Response to Long-Term Memory. An inverse correlation between $CD62L^{hi}$ phenotype and epitope-specific $CD8^+$ T cell prevalence is immediately apparent when CD62L staining characteristics are measured for all D^bNP_{366}- and D^bPA_{224}-specific tetramer$^+CD8^+$ T cells over a very long time course (Fig. 1 *A*–*C*). Approximately 50% of the D^bNP_{366}- and D^bPA_{224}-specific $CD8^+$ T cells are $CD62L^{hi}$ on d6, the earliest stage that these virus-specific $CD8^+$ T cells are consistently detected. The $CD62L^{hi}$ component of both responses then falls to <10% at the peak on d9 and does not return to a >50% level until after d129 (Fig. 1 *B* and *C*). The CD62L expression profiles are similar, although not identical, for the D^bNP_{366}- and D^bPA_{224}-specific populations.

The massive contraction of $CD8^+D^bNP_{366}^+$ and $CD8^+D^bPA_{224}^+$ T cell numbers that follows the antigen-driven phase of the response (Fig. 1*B*) occurs predominantly in the $CD62L^{lo}$ set (Fig. 1 *D* and *E*), which presumably includes both effector cells and T_{EM} precursors. Between d8 and d129, the average decreases were 28 times for $CD8^+D^bNP_{366}^+CD62L^{lo}$, 29 times for $CD8^+D^bPA_{224}^+CD62L^{lo}$, 2.3 times for $CD8^+D^bNP_{366}^+CD62L^{hi}$, and 3.4 times for $CD8^+D^bPA_{224}^+CD62L^{hi}$. Given that the T cell "diaspora" to other sites is already well established at the acute phase of the infectious process (8), it is likely that much of the $CD62L^{lo}$ population dies rather than localizes to other somatic tissues. Also, it seems that relatively few of the $CD62L^{lo}$ T cells (Fig. 1*D*) regress to a "survivor" $CD62L^{hi}$ state, although such conversion could contribute to the increase in $CD62L^{hi}$ numbers between d60 and d575 for both antigen-specific populations (Fig. 1*E*).

The $CD8^+D^bNP_{366}^+$ and $CD8^+D^bPA_{224}^+$ T cell counts peak between d8 and d10 after intranasal challenge (Fig. 1*B*). Although the $CD8^+D^bPA_{224}^+$ set is more prominent initially (d7), the $CD8^+D^bNP_{366}^+$ population achieves a 1.5-times-higher maximum between d9 and d15 (Fig. 1*B*). This differential kinetics has been interpreted previously as indicating that a larger, naïve TCR repertoire specific for D^bPA_{224} allows that response to emerge earlier (23). However, the much more abundant viral nucleoprotein induces higher-level expression of the D^bNP_{366} epitope, which in turn drives greater proliferation of the $CD8^+D^bNP_{366}^+$ T cells so that they ultimately dominate in the numerical sense (23). Conversely, the $CD8^+D^bPA_{224}^+$ population is more likely to remain $CD62L^{hi}$ from d10 through to d575 (Fig. 1*C*), indicating again that much of the phenotypic character of long-term memory is established during the antigen-driven phase of the response and correlates with magnitude. It is important to note that tetramer$^+CD8^+$ T cells displayed either $CD44^{hi}$ or $CD44^{int}$ phenotype (data not shown).

Clonotype Diversity and CD62L Phenotype. The predominant $V\beta8.3^+CD8^+D^bNP_{366}^+$ and $V\beta7^+CD8^+D^bPA_{224}^+$ T cell populations were sorted under stringent conditions as single $CD62L^{hi}$ or $CD62L^{lo}$ T cells, and then the extent of TCR diversity was assessed subsequent to cDNA expansion by RT-PCR and CDR3β sequencing. Previous experiments have established that, whereas the $V\beta8.3^+CD8^+D^bNP_{366}^+$ response is characterized by a substantial proportion of TCRs that are public for all mice (20, 22), the $V\beta7^+CD8^+D^bPA_{224}^+$ response is more diverse (9, 21). Although "recurrent" $D^bPA_{224}^+$-specific sequences can be found in several different individuals, most of the TCR signatures are private to one mouse. These private clonotypes are sometimes represented by only a single cell and are more likely to express TCRs that are "non-consensus" for CDR3β length, sequence, and Jβ usage.

The CDR3β profiles detected for the $CD62L^{lo}$ T cells were generally similar to those described previously for the total $V\beta8.3^+CD8^+D^bNP_{366}^+$ and $V\beta7^+CD8^+D^bPA_{224}^+$ populations, but, somewhat to our surprise, we found evidence of much greater TCR diversity in (particularly) the $CD62L^{hi}$

Table 1. Frequency of TCRβ public/recurrent and unique amino acid sequences in the CD62L^{lo} and CD62L^{hi} sets of D^{b}NP$_{366}$Vβ8.3$^+$CD8$^+$ T cells

CDR3β region	Jβ	Amino acids	d8		d11		d28		d180	
			lo	hi	lo	hi	lo	hi	lo	hi
Public/recurrent										
SGGSNTGQL	2.2	9	1.3	1.3		2.2	57	5.3	56	58
SGGANTGQL	2.2	9	75	56		4.3	3.3	7.1	4.8	9.3
SGGGNTGQL	2.2	9	5	9.2	1.7	2.2	38	43	6.4	9.3
RGGANTGQL	2.2	9			6.9			1.8		2.3
RGGGNTGQL	2.2	9	15	27						
KGGANTGQL	2.2	9		1.3						
KGGGNTGQL	2.2	9						27		
KGGSNTGQL	2.2	9			10.4	6.5				
SARTANTEV	1.1	9			81	72				2.3
Unique										
KGGAGTGQL	2.2	9	3.7							
SARANTGQL	1.1	9						1.8		
SAGTAEV	1.1	7					1.6			
SARVDNQAP	1.5	9						1.8		
SCAGSNTGQL	1.5	10						1.8		
SDAGGQDTQ	2.5	9				2.2				
SDAGGTEV	1.1	8						1.8		
SDLTGKAGNTL	2.4	11				2.2				
SDPTGGYAEQ	2.1	10		1.3						
SDPGGYNSPL	1.6	10		1.3						
SDRQANTEV	1.1	9			2.2					
SDTGANYAEQ	1.1	10				1.8				
SDVNSPL	1.6	7				2.2				
SDVGGNQDTQ	2.5	10		1.3						
SDVQGPANERL	1.4	11		1.3						
SEGRSSYEQ	2.6	9				2.2				
SEMTGGNQDTQ	2.5	11			2.2					
SEYGGDNSD	1.2	9								2.3
SGGGQTGQL	2.2	9						5.4		
SGGGRTGQL	2.2	9							31	9.3
SGTGGNYAEQ	2.1	10		1.3						
SGRGPNRQL	2.2	9							1.6	
SLGTANNQAP	1.5	10								2.3
SSDKSANSDY	1.2	9						1.8		
NWGGDTGQL	2.2	9								2.3
TDSSGNTL	2.4	8								2.3
Total clones			80	77	58	46	61	56	62	43

A representative mouse is shown for each time point of the primary influenza A response. The percentage of unique clonotypes within TCRβ repertoires of all the mice tested in the primary response (n = 13) is 3% for the CD62L^{lo} set (lo) and 14.6% for the CD62L^{hi} set (hi) of D^{b}NP$_{366}$ Vβ8.3$^+$CD8$^+$ T cells.

Vβ8.3$^+$CD8$^+$D^{b}NP$_{366}^+$ response (Tables 1 and 2). Whereas progeny of the public and recurrent Vβ8.3$^+$CD8$^+$D^{b}NP$_{366}^+$ clonotypes commonly segregate into both CD62L^{lo} and CD62L^{hi} subsets (Table 1), most of the private, nonconsensus TCRs found from d8 to d180 are detected on single CD62L^{hi} T cells (Table 1). Large, private clones are sometimes found, but again these partition into CD62L^{lo} and CD62L^{hi} subsets (e.g., SGGGRTGQL, d180; see Table 1).

This TCRβ analysis is summarized for T cells recovered at all time points through the primary response in Table 2. The CD62L^{hi} Vβ8.3$^+$CD8$^+$D^{b}NP$_{366}^+$ and Vβ7$^+$CD8$^+$D^{b}PA$_{224}^+$ lymphocytes consistently show more diverse profiles of CDR3β length, Jβ usage, and sequence (four times overall for D^{b}NP$_{366}$) than the corresponding CD62L^{lo} subsets (Table 2). Furthermore, whereas the public TCRs are represented on both CD62L^{hi} and CD62L^{lo} T cells equally, the recurrent signatures are at slightly higher prevalence in the CD62L^{hi} population, and the private CD62L^{hi} clonotypes are five times (Vβ8.3$^+$CD8$^+$D^{b}NP$_{366}^+$) or 1.5 times (Vβ7$^+$CD8$^+$D^{b}PA$_{224}^+$) more common. Overall, by enriching on the basis of CD62L phenotype, we found that the CD8$^+$D^{b}NP$_{366}^+$CD62L^{hi} set is twice as diverse as the comparable CD62L^{lo} population from d8 to >d180.

The permissible "wobble" for the nucleotide in the third position means that the extent of TCR diversity calculated from the CDR3β amino acid sequences (Tables 1 and 2) is likely to be an underestimate. Again, when clonotype is defined by nucleotide sequence, it is apparent that shared signatures are more common for the CD62L^{lo} population reflecting the greater diversity of the CD62L^{hi} set (Fig. 2 *A–C*). The patterns for CD62L^{hi} and CD62L^{lo} Vβ8.3$^+$CD8$^+$D^{b}NP$_{366}^+$ (Fig. 2*A*) and Vβ7$^+$CD8$^+$D^{b}PA$_{224}^+$ (Fig. 2*B*) T cells are shown for individual mice, whereas cumulated data sets (Fig. 2*C*) both illustrate clonotypic diversity profiles for the acute (d8–15), short-term (d28), and long-term (>d180) memory phases and allow statistical analysis (d8–690 and d8–28). The differences between the CD62L^{hi} and CD62L^{lo} subsets are less apparent when the findings are expressed as total T cell numbers (Fig. 2*D*), reflecting that the larger populations bearing public

Table 2. TCRβ diversity in CD62L^{lo} and CD62L^{hi} tetramer$^+$ CD8$^+$ T cells

Cells	No. of mice analyzed	No. of TCRs sequenced	Modal CDR3β length, aa	Minor CDR3β length, aa	Predom. Jβ usage*	Minor Jβ usage†	Diff. aa sequences	Public aa sequences	Recurrent aa sequences‡	Unique aa sequences§	Clonotypes per mouse¶
CD8$^+$D^{b}NP$_{366}^+$Vβ8.3$^+$											
CD62L^{lo}	13	609	9	8, 12	2S2	1S1, 2S4	25	3	4	18	6.0
CD62L^{hi}	13	630	9**	6, 7, 8, 10, 11, 12	2S2	1S1, 1S4, 2S1, 2S4, 2S5, 2S6	102	3	7	92	12.9***
CD8$^+$D^b PA$_{224}^+$Vβ7$^+$											
CD62L^{lo}	9	457	6, 7	5, 8, 9, 11	2S6, 1S1, 2S1	1S2, 1S4, 1S5, 2S4, 2S5	96	—	16	80	15.3
CD62L^{hi}	9	470	6, 7**	5, 8, 9, 10, 11	2S6, 1S1	1S2, 1S4, 1S5, 2S1, 2S2, 2S3, 2S4, 2S5	149	—	22	127	25.1***

Data represent pooled results from primary response at different times after influenza A infection. aa, amino acid; Predom., predominant; Diff., different.
**, $P < 0.01$, statistical analysis performed for proportion of clonotypes with ''consensus'' CDR3β length; ***, $P < 0.05$.
*Predominant CDR3β length and Jβ usage defined as >15%.
†Minor CDR3β length and Jβ usage defined as >1% but <15%.
‡Amino acid sequences found in at least two mice.
§Amino acid sequences found in one mouse only.
¶Clonotypes refer to nucleotide sequences.

TCRs are represented in both populations whereas the low-frequency clonotypes are generally CD62L^{hi} (Table 1 and Fig. 2*D*).

Analyzing the data in this way (Fig. 2) also supports the impression from Table 1 that the composition of the CD62L^{hi} and CD62L^{lo} subsets analyzed at the acute (d8–15), early memory (d28), and long-term memory (>d180) phases of these virus-

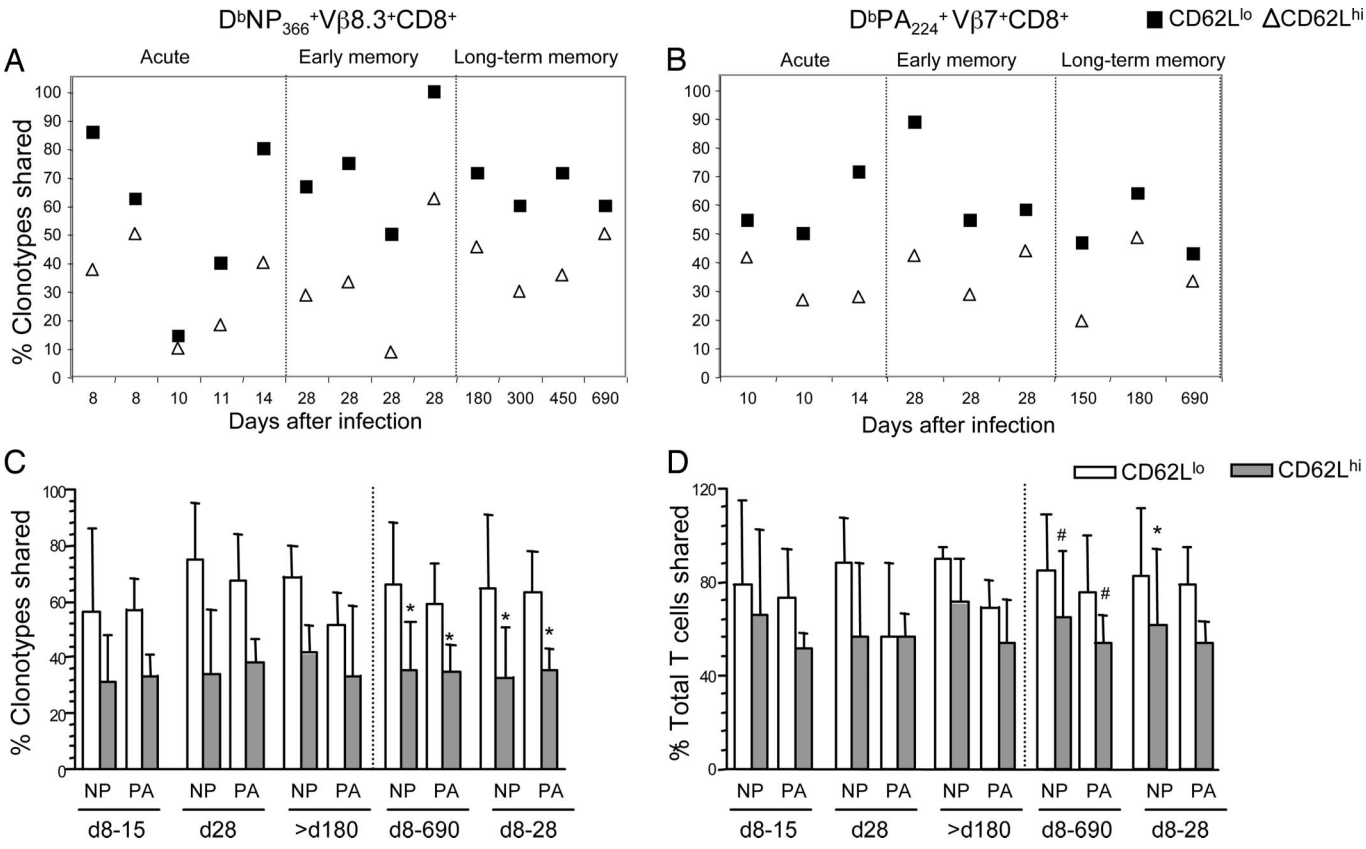

Fig. 2. Prevalence of shared TCRβ signatures for CD62L^{hi} and CD62L^{lo} CD8$^+$D^{b}NP$_{366}^+$ and CD8$^+$ D^{b}PA$_{224}^+$ T cells. Frequency values for clonotypes (defined by nucleotide sequence) that are represented in both the CD62L^{hi} and CD62L^{lo} subsets are shown for CD8$^+$Vβ8.3$^+$D^{b}NP$_{336}^+$ (*A*) and CD8$^+$ Vβ7$^+$D^{b}PA$_{224}^+$ (*B*) T cells from individual mice sampled at different phases after primary infection. (*C*) The results shown in *A* and *B* are expressed as cumulated data (mean ± SD) for statistical analysis (*, $P < 0.01$; #, $P < 0.05$). (*D*) Clone size was taken into account to show the relative distribution of T cell numbers for CDR3β signatures that are represented in both the CD62L^{hi} and CD62L^{lo} subsets. Lymphocytes obtained from spleens of influenza-infected mice were enriched for CD8$^+$ T cells and stained with either the D^{b}NP$_{366}$ or D^{b}PA$_{224}$ tetramers conjugated to streptavidin-phycoerythrin and mAbs against CD8 (allophycocyanin-Cy7), CD62L (allophycocyanin), and Vβ8.3 or Vβ7 (FITC). Single CD62L^{hi} or CD62L^{lo} tetramer$^+$ Vβ^+ CD8$^+$ T cells were sorted into 96-well plates, amplified for CDR3β cDNA, and sequenced.

specific $CD8^+$ T cell responses is remarkably consistent over time. In short, neither the TCR diversity profile nor the separation apparent for the $CD62L^{lo}$ and $CD62L^{hi}$ populations changes substantially from the acute phase through to established memory despite the fact that the proportion of $CD62L^{hi}CD8^+$ T cells increases from the acute time point (≈10%) to long-term memory (≈75%) (Fig. 1). The fact that the percentage of shared clonotypes remains higher in the $CD62L^{lo}$ set from d28 to d180 and beyond (Fig. 2) in turn suggests that $CD62L^{hi}$ memory T cells constitute a relatively stable pool that is not constantly "fed" by conversion from the $CD62L^{lo}$ population. If the latter were a major factor in the maintenance of the $CD62L^{hi}$ set, we would expect to see a progressive transition to a more public repertoire derived from the larger clonotypes. Given that the $V\beta8.3^+CD8^+D^bNP_{366}^+$ and $V\beta7^+CD8^+D^bPA_{224}^+$ TCR repertoires maintain consistent profiles from d8 to >d180 for both the $CD62L^{hi}$ and $CD62L^{lo}$ subsets (Tables 1 and 2 and Fig. 2), it seems reasonable to assume that the characteristics of long-term memory are established during the acute, antigen-driven phase of the response.

Discussion

The fundamental determinant of T cell diversity is, of course, the clonotypic TCR. Given the extreme rarity of more than one nucleotide profile for all except the occasional public TCRs (20), it is at least conceivable that every clonotype we detect in an endogenous T cell response is the progeny of a single T cell precursor. The evidence for this is, of course, indirect, because there is as yet no satisfactory way to measure the extent of any particular naïve TCR repertoire. However, the likelihood is that a unique CDR3β sequence is derived from a distinct clone (21, 22, 24), and perhaps this constitutes a feasible approach for estimating a precursor frequency of antigen-specific $CD8^+$ T cells (K.K., unpublished data).

The present analysis establishes that, irrespective of the stage after primary challenge, the extent of clonal diversity for two concurrent, endogenous $CD8^+$ T cell responses is consistently greater for the $CD62L^{hi}CD8^+$ sets despite numerous qualitative and quantitative differences between these $CD8^+D^bNP_{366}^+$ and $CD8^+D^bPA_{224}^+$ populations (21–24). This finding reflects the skewed distribution of low-frequency clones expressing private TCRs, with the $CD62L^{hi}CD8^+D^bNP_{366}^+$ and $CD62L^{hi}CD8^+D^bPA_{224}^+$ responses being generally more diverse (two times and 1.6 times, respectively) than the $CD62L^{lo}$ sets recovered concurrently from 13 different mice sampled from d8 to >d180 after antigen challenge. Conversely, members of the large public or "repeated" clonotypes are found in both the $CD62L^{hi}$ and $CD62L^{lo}$ populations, suggesting that these T cells that express "best-fit" TCR characteristics have originated from a single, naïve $CD62L^{hi}$ precursor. This long-term dissection of clonotypic diversity indicates that these public clones segregate very early into stable $CD62L^{hi}$ and $CD62L^{lo}$ lineages. Other experiments (K.K., unpublished data) indicate that both populations go through multiple cycles of cell division, but the $CD62L^{lo}$ pool rapidly outgrows the $CD62L^{hi}$ set. Many, but by no means all, of these $CD62L^{lo}$ T cells become terminally differentiated effectors that are progressively eliminated, although some $CD62L^{lo}$ T_{EM} cells can still be detected for 19 months (the latest time point examined) after the cessation of antigen challenge.

Apart from the question of TCR-pMHCI affinity (25, 26), whether a particular T cell from within the one public lineage goes down a $CD62L^{hi}$ or $CD62L^{lo}$ pathway is presumably determined by a spectrum of microenvironmental factors, including cytokine gradients, that determine the nature of the antigenic experience. The dendritic cell (DC)-derived inflammatory cytokines IL-7 and IL-15 (27, 28) are likely to be particularly important in this process. The nature of the underlying stimulatory conditions will also vary with the extent of repeated antigenic exposure (29), pathogen virulence (30), DC maturation (18), and the availability of $CD4^+$ T cell help (31).

It is important to recognize with the influenza model that the antigen load in the lymphoid tissue is low when compared with, say, systemic lymphocytic choriomeningitis infection (32, 33). Production of lytic influenza A viruses that disseminate the infectious process within the host is, because of a requirement for trypsin-like proteases to cleave the viral hemagglutinin molecule, substantially restricted to the superficial epithelium of the respiratory tract (34). There is no viremia, and viral antigen is thought to be carried to the secondary lymphoid tissue by DCs that have been nonproductively infected (make viral proteins but no progeny virus) or have taken up antigen in the lung during the first 36 h after virus challenge (35, 36). The net consequence is, then, that the antigen-presenting cell "environment" that determines the nature of the primary response is likely to be both established early and limited in extent. Furthermore, the DC population may be far from homogeneous, comprising, perhaps, a component of virus-infected DC "migrants" from the respiratory tract, together with other lymphoid tissue "residents" that are expressing influenza epitopes generated by means of some "cross-presentation" mechanism (37). Given that "immature" DCs are more likely to promote the clonal expansion of T cells that remain $CD62L^{hi}$ (18), it would not be surprising if the available DC pool provides a spectrum of antigenic "encounters" that are of varying quality.

The likelihood is high that both naïve precursors and progeny T cells derived during the expansion of the public clonotypes will have a diversity of signaling experiences in different "niches" within the lymphoid tissue (13). Are these effects limited to the first 48 h or so after antigen encounter that are considered to set naïve T cells on an inexorable path of clonal expansion and differentiation, or is this a more protracted process (38, 39)? On the other hand, those T cells that express nonconsensus TCRs will invariably have a "low-end" signaling experience that causes some cycling but induces few (if any) T cells to make the $CD62L^{lo}$ switch. Nonetheless, these nonconsensus $CD62L^{hi}$ T cells endure in the long-term (40), because, once the antigen-driven phase is over, they are not seen as different from any other clonally expanded $CD44^{hi}CD62L^{hi}$ T lymphocyte in the established memory pool (41). Furthermore, these nonconsensus TCRs can be recalled after the secondary challenge, as shown by our longitudinal analyses (9, 20).

Dividing the available memory T cell pool into a $CD62L^{hi}$ component that recirculates from blood to lymph node, the most likely site of "optimal" microenvironmental exposure, and $CD62L^{lo}$ T_{EM} cells that transit through somatic tissues, makes sense for an immune surveillance system. Both sets will, of course, also monitor the spleen. Furthermore, the early establishment of stable T_{CM} and T_{EM} lineages protects the survival of the smaller T_{CM} pool during the process of homeostatic equilibration and effector T cell elimination that follows any immune response. The net result will also be to avoid overdominance by a few high-affinity clonotypes. Others have also suggested early establishment of memory $CD8^+$ and $CD4^+$ T cell populations (42, 43) in the absence of concurrent inflammation.

Why retain a significant T_{CM} component that seems to express "suboptimal" TCRs? Is this simply an illustration of the limits of fine-tuning in evolution, or could it be of some selective value? Perhaps this TCR diversity helps reduce the possibility that mutated pathogens "escape" immune recognition and control (44). However, the contrary view is that throwing off a substantial component of T cells with "minimal-fit" TCRs could also increase the possibility that any given infection might trigger an untoward, crossreactive autoimmune consequence.

Whatever the biological *raison d'etre* (if any) for maintaining these "low-quality" T cells, it is salutary to reflect that, by sorting for a particular subset ($CD62L^{hi}$) within a $tetramer^+CD8^+$ population, earlier estimates (20) of repertoire size (in this case for D^bNP_{366}) have been effectively doubled for each individual ana-

lyzed. Others have also found evidence of greater TCR diversity within the $CD8^+CD62L^{hi}$ set (14, 19), although the question of relative TCR characteristics was not raised. What would happen if the $CD62L^{hi}$ population were further divided by selecting for an additional marker? The possibility that the total T cell response repertoire to any given antigen has a very large, ill defined, suboptimal "tail" may provide some explanation for the unexpected crossreactive immune response profiles that have been detected for apparently unrelated pathogens (45, 46).

Methods

Mice and Viral Infection. Female C57BL/6J (B6, $H2^b$) mice were bred at the University of Melbourne. Naïve B6 mice at 6–8 weeks of age were anesthetized by isoflurane inhalation and infected intranasally with 10^4 plaque-forming units of HKx31 (H3N2) influenza A virus (47) in 30 μl of PBS. All experiments followed guidelines set by the University of Melbourne Animal Ethics Experimentation Committee. Virus stocks were grown in the allantoic cavity of 10-day embryonated hens' eggs and quantified as plaque-forming units on monolayers of Madin–Darby canine kidney cells.

Tissue Sampling and Cell Preparation. Spleens were recovered from mice in the acute (d8 to d15), early memory (d28), or long-term primary memory (>180d) phases after primary HKx31 infection. Spleens were disrupted and enriched for $CD8^+$ T cells by panning on goat anti-mouse IgG and IgM antibody-coated plates (Jackson ImmunoResearch).

Isolation of Single $CD62L^{lo}$ and $CD62L^{hi}$ $CD8^+$ T Cells, RT-PCR, and Sequencing. Enriched lymphocyte populations were stained with the D^bNP_{366} or D^bPA_{224} tetramers conjugated to streptavidin-allophycocyanin (Molecular Probes) for 60 min at room temperature followed by two washes in sort buffer (0.1% BSA in PBS), then stained with anti-CD8α-allophycocyanin-Cy7, anti-CD62L-phycoerythrin, and anti-Vβ8.3-FITC or anti-Vβ7-FITC (Pharmingen) mAbs for 30 min on ice followed by two further washes. Cells were resuspended in 500 μl of sort buffer and transferred to polypropylene FACS tubes (BD Labware, Franklin Lakes, NJ) for subsequent sorting. Lymphocytes were isolated by using a MoFlo sorter (Cytomation, Fort Collins, CO) fitted with a Cyclone single-cell deposition unit. Single $CD62L^{hi}$ or $CD62L^{lo}$ $D^bNP_{336}{}^+V\beta8.3^+CD8^+$ or $D^bPA_{224}{}^+V\beta7^+CD8^+$ T cells were sorted directly into 96-well PCR plates (Eppendorf) containing 5 μl of cDNA reaction mix. Negative controls were interspersed between the samples (1 in 10), and 80 cells were sorted per plate. The cDNA mix contained 0.25 μl of Sensiscript reverse transcriptase, 1× cDNA buffer, 0.5 mM dNTPs (Qiagen, Hilden, Germany), 0.125 μg of oligo $dT_{(15)}$ (Promega), 100 μg/ml gelatin (Roche, Indianapolis), 100 μg/ml tRNA (Roche), 20 units of RNAsin (Invitrogen), and 0.1% Triton-X100 (Sigma). After sorting, plates were incubated at 37°C for 90 min for cDNA synthesis followed by 5 min at 95°C to stop reverse transcriptase activity, and plates were stored at −80°C. The $V\beta8.3^+$ and $V\beta7^+$ transcripts were then amplified and sequenced (9, 20).

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