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Direct Ex Vivo Analysis of Human CD4+ Memory T Cell Activation Requirements at the Single Clonotype Level

Arlene D. Bitmansour,‡ Daniel C. Douek, Vernon C. Maino,§ and Louis J. Picker

CD4+ memory T cells continuously integrate signals transmitted through the TCR and costimulatory molecules, only responding when the intensity of such signals exceeds an intrinsic activation threshold. Recent data suggest that these activation thresholds can be regulated independently of TCR specificity, and that threshold tuning may constitute a major mechanism for controlling T cell effector activity. In this work we take advantage of the profound clonotypic hierarchies of the large human CD4+ T cell response to CMV to study activation thresholds of fresh (unexpanded) memory T cells at the clonotypic level. We identified dominant responses to CMV matrix determinants mediated by single TCRB sequences within particular TCR-β/δ families. The specific response characteristics of these single, Ag-specific, TCRB-defined clonotypes could be unequivocally determined in fresh PBMC preparations by cytokine flow cytometry with gating on the appropriate Vβ family. These analyses revealed 1) optimal peptides capable of eliciting specific responses by themselves at doses as low as 2 pg/ml, with each log increase in dose eliciting ever-increasing frequencies of responding cells over a 4- to 5-log range; 2) significant augmentation of response frequencies at all submaximal peptide doses by CD28- and CD49d-mediated costimulation; 3) differential dose response and costimulatory characteristics for IFN-γ and IL-2 responses; and 4) no association of activation requirements with the CD27-defined CD4+ T cell memory differentiation pathway. Taken together these data confirm the possibility that peripheral T cells display variable, perhaps tunable, activation thresholds (3–7). If true, this additional form of functional heterogeneity could play a critical role in governing the initiation, tempo, and outcome of immune effector responses, at once greatly increasing both the complexity of such responses and the capacity for their fine regulation. For example, T cells with low activation thresholds might be expected to be functionally recruited early into effector responses at low Ag densities or low levels of APC activity, whereas T cells with high thresholds might be functionally recruited into such responses late, if at all. Indeed, anergy would represent one end of the threshold spectrum, a situation in which the activation threshold of a T cell for a given functional response is so high that physiologically attainable stimuli fail to achieve triggering.

Memory T cells of the CD4 lineage coordinate immune responses against viruses and other pathogens via the Ag-induced secretion of potent effector cytokines. The efficacy of these responses is thought to depend on both the overall number of pathogen-specific memory T cells and the particular array of cytokines that these cells are programmed to secrete (1, 2). Recently, theoretical considerations, as well as studies of TCR-transgenic mice and in vitro propagated T cell clones, have suggested the possibility that peripheral T cells display variable, perhaps tunable, activation thresholds (3–7). If true, this additional form of functional heterogeneity could play a critical role in governing the initiation, tempo, and outcome of immune effector responses, at once greatly increasing both the complexity of such responses and the capacity for their fine regulation. For example, T cells with low activation thresholds might be expected to be functionally recruited early into effector responses at low Ag densities or low levels of APC activity, whereas T cells with high thresholds might be functionally recruited into such responses late, if at all. Indeed, anergy would represent one end of the threshold spectrum, a situation in which the activation threshold of a T cell for a given functional response is so high that physiologically attainable stimuli fail to achieve triggering.

Assessment of T cell responsiveness has been greatly facilitated by the development of cytokine flow cytometry (CFC), a technique that allows precise determination of the frequency of T cells reacting to a given Ag under varying conditions (8–10). This assay determines response frequencies within 6 h of Ag exposure without any amplification and before significant cell death. Moreover, it includes the secretion inhibitor brefeldin A, which prevents induction of potentially modulatory secreted cytokines and cell surface signaling molecules by T cells or APC, and thus prevents secondary regulatory events. Because of these considerations, CFC focuses on the ability of an individual T cell to be initially triggered by TCR-derived and costimulatory signals and thus provides a unique tool for the study of T cell triggering thresholds.

Using this assay, we have previously reported that exogenous costimulation in the form of CD28 and CD49d mAbs increased in stepwise fashion the observed frequencies of human CMV-responsive CD4+ memory T cells by up to 3-fold but had no stimulatory effect by themselves (8). Thus, the overall cohort of CMV-specific CD4+ memory T cells could be divided into subsets requiring no, low, or high levels of costimulation to achieve triggering. Because co-stimuli effectively act to lower activation thresholds by lowering the intensity of TCR-mediated signaling required to achieve triggering (11–14), these distinctly different activation requirements suggested heterogeneity of triggering thresholds among the CMV-specific CD4+ memory T cell population. Such triggering differences may be the consequence of intrinsic, TCR-independent differences in activation set points (4), or, given that these CMV-specific T cell cohorts were polyclonal, may simply reflect a spectrum of TCR avidities among the various clonotypes involved in the response (15). The observation that exogenous costimulation

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increased the frequency of responding cells at any given level of TCR signaling strength, as measured by TCR down-regulation (11, 16), suggested that regulation of memory T cell thresholds may operate independently of TCR specificity (8).

In this work we sought to more definitively address this issue by the analysis of CD4+ memory T cell activation thresholds at the clonotypic level, eliminating, to the greatest extent possible, TCR specificity as a variable in these analyses. Historically, this kind of analysis would require the use of in vitro-derived T cell clones; however, recent studies have demonstrated that the triggering thresholds of such clones are altered by the repeated stimulations inherent in the cloning process (5). Therefore, we developed an alternative approach based on our recent demonstration of profound clonotypic hierarchies among the large populations of CMV-specific CD4+ memory T cells that exist in CMV-exposed individuals—hierarchies that include individual CMV-specific, TCRB-defined clonotypes as large as 4% of total CD4+ T cells (17). We determined that, in some instances, these large single clonotypes are the only responding T cells within a particular TCR-Vβ family, indicating that the response characteristics of these single clonotypes can be directly and unequivocally determined in fresh PBMC by multiparameter FPC with gating on the TCR-Vβ family of interest. We then used this approach to study in detail single CD4+ memory T cell clonotype responses directed at defined epitopes within the 65-kDa CMV internal matrix phosphoprotein (pp65), focusing on the ability of these clonotypes to produce IFN-γ and IL-2 after variable levels of TCR triggering, with or without exogenous costimulation. The results confirm a spectrum of triggering thresholds within single TCRB-defined clonotypes, consistent with TCR-independent threshold regulation, and, moreover, demonstrate that intrinsic differences in the set points for IFN-γ and IL-2 yield a situation in which, depending on epitope dose and costimulation availability, single CD4+ memory T cell clonotypes can give rise to qualitatively distinct functional responses.

Materials and Methods
Cell preparation and Ag stimulation
PBMC were isolated from heparinized or citrated venous blood by density gradient sedimentation using Ficoll-Hypaque (Histopaque-1077; Sigma-Aldrich, St. Louis, MO) at 1 x 10^7 cells per milliliter of complete medium (1–10 ml per tube) with appropriately tiered whole CMV viral preparations (ranging from 40 μg/ml), CMV pp65 peptide(s), or no Ag as a negative control (previously shown to be empty of mock virus preparations (9)), with or without the costimulatory mAbs CD28 and CD49d (0.5 μg/ml), CMV pp65 peptides (consecutive 15 mers shifted by 4 aa spanning the whole molecule; consecutive 12 mers or 9 mers shifted by 1 aa spanning regions of interest) were custom synthesized by Dr. D. Stoll (National and Medical Sciences Institute of the University of Tuebingen, Tuebingen, Germany) based on the pp65 sequence of CMV strain AD169. Peptide sequences were confirmed by electrospray mass spectrometry. Optional 15 mers and all 12 mers and 9 mers were subjected to HPLC purification (resulting in an average purity of 95%), mAbs SK3 (CD4; PerCP, CD4 (FITC), TCR-Vβ (AP)), L78 (CD69; PE, PerCP, AP), L25.3 (CD49d; unconjugated), L25.3 (CD49d; unconjugated), 25723.11 (anti-IFN-γ, FITC, AP), 5344.111 (anti-IL-2; FITC, PE, AP), IgG1 and IgG2 isotype-matched controls, and streptavidin-AP were obtained from BD Biosciences. TCR-Vβ2 and -Vβ17 mAbs (PE and biotin) were obtained from Coulter/Immunotech (Hialeah, FL). The anti-IFN-γ mAb (PE) used for surface IFN-γ staining was obtained from Miltenyi Biotec. TCR-conjugated anti-mouse IgM Abs obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

RT-PCR spectratyping and clonotype characterization
Clonotypic characterization was performed as reported (17). Briefly, RNA was isolated from sorted T cells by TRIzol reagent (Invitrogen) or Oligotex (Carl Roth, Karlsruhe, Germany) based on the pp65 sequence of CMV strain AD169. Peptide sequences were confirmed by electrospray mass spectrometry. Optional 15 mers and all 12 mers and 9 mers were subjected to HPLC purification (resulting in an average purity of 95%), mAbs SK3 (CD4; PerCP, CD4 (FITC), TCR-Vβ (AP)), L78 (CD69; PE, PerCP, AP), L25.3 (CD49d; unconjugated), L25.3 (CD49d; unconjugated), 25723.11 (anti-IFN-γ, FITC, AP), 5344.111 (anti-IL-2; FITC, PE, AP), IgG1 and IgG2 isotype-matched controls, and streptavidin-AP were obtained from BD Biosciences. TCR-Vβ2 and -Vβ17 mAbs (PE and biotin) were obtained from Coulter/Immunotech (Hialeah, FL). The anti-IFN-γ mAb (PE) used for surface IFN-γ staining was obtained from Miltenyi Biotec. TCR-conjugated anti-mouse IgM Abs obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

RT-PCR spectratyping and clonotype characterization
Clonotypic characterization was performed as reported (17). Briefly, RNA was isolated from sorted T cells by TRIzol reagent (Invitrogen) or Oligotex Direct mRNA Mini kit (Qiagen, Valencia, CA) per the manufacturer’s instructions. RT-PCR mix (RT-PCR buffer with 1.5 mM MgCl2; Roche Molecular Biochemicals), 0.2 mM dNTP mix (Roche Molecular Biochemicals), 0.5 mM dNTP mix (BD Biosciences), 0.2 μM of primer, 0.5 μM of reverse primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward primer, 0.5 μM of forward prime
eluted DNA sample was used in separate PCR using the appropriate Vβ- and Cβ- or Vα- and Co-specific primers. The PCR products were purified on a 2% agarose gel. Each band was cut out of the agarose gel, DNA was extracted (Concert Matrix Gel Extraction System; Invitrogen) and cloned into pGEM vector (Promega, Madison WI), and JM109 High Efficiency Competent Cells (Promega) were transformed. White colonies were picked, and plasmid DNA was isolated (Promega) and submitted for sequencing. Analysis of sequence data was performed using MacVector software (Oxford Molecular, Madison, WI).

Clonotype-specific qPCR

Clonotype-specific primer pairs and probes were designed for qPCR such that the primers and probes span the TCRB VDJ region (Table I), as described (18). The standard series for each clonotype was made up from the plasmid DNA originally used to sequence the clonotype. Sorted CMV-reactive and nonreactive CD4+ T cells were aliquoted in microfuge tubes and pelleted by centrifugation at 13,000 × g for 3 min. A total of 50 μl of 10 mM Tris-HCl (pH 7.4) containing PCR Grade Proteinase K (50 μg/ml; Roche Molecular Biochemicals) was added to the cell pellets and the lysate was incubated for 4 h at 56°C. The Proteinase K was then inactivated at 95°C for 10 min. For qPCR, 5 μl of cell lysate or clonotype standard was combined with qPCR mix containing PCR buffer (20 mM Tris-HCl (pH 8) and 50 mM KCl), 0.2 mM dNTP mix (Invitrogen), 1.5 mM MgCl2, 0.5 μM 5’ primer, 0.5 μM 3’ primer, 250 nM probe, and 2.5 U Platinum Taq DNA Polymerase (Invitrogen). The PCR protocol included denaturation at 94°C for 0.25 min, annealing and extension at 60°C for 1 min. qPCR data were analyzed using ABI 7700 Sequence Detection System software (version 1.6.3; Applied Biosystems, Foster City, CA).

Results

Identification of single clonotype CD4+ memory T cell responses to CMV pp65 epitopes

CMV-specific T cells are maintained at a high frequency in CMV-exposed humans, averaging ~2% of CD4+ T cells (~4% of CD4+ memory cells) in normal subjects (8, 9). Frequencies >5% are not uncommon, especially in HIV-1-infected subjects (9, 10, 17, 19). These CMV-specific CD4+ T cell populations are polyclonal but display a strikingly hierarchical clonotypic content; in general, one or two TCRB-defined clonotypes dominate the CMV-responsive population and single clonotypes may comprise up to half of the total response and as many as 4% of total CD4+ T cells (17). In some instances, these dominant clonotypes could be shown to be the only CMV-specific clonotypes within a particular TCR-Vβ family, and in a proportion of these instances the precise epitope specificity of the dominant clonotype-bearing T cells can be identified (17).

Table I. PCR primer sequences

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectratyping primers</td>
<td></td>
</tr>
<tr>
<td>5’ Primer</td>
<td></td>
</tr>
<tr>
<td>BV2</td>
<td>ATACGAGCAAGGCGTCTGAGAAG</td>
</tr>
<tr>
<td>BV17</td>
<td>ACTTGACATCGGCCCTTACAAA</td>
</tr>
<tr>
<td>AV 1-29</td>
<td>See Ref. 51</td>
</tr>
<tr>
<td>3’ Primer</td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>TGTGCACCTTCCCTCCCATTCA</td>
</tr>
<tr>
<td>AC</td>
<td>GTGCTCGACGGGCAACAGCACTGTT</td>
</tr>
<tr>
<td>qPCR Primers and probes</td>
<td></td>
</tr>
<tr>
<td>5’ Primer</td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>AAAACAGCTCCCGGGGTGTT</td>
</tr>
<tr>
<td>Subject 2</td>
<td>TCGAGCTTCCATCACCAAAATG</td>
</tr>
<tr>
<td>3’ Primer</td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>CACATCCGAAGACACAGCTT</td>
</tr>
<tr>
<td>Subject 2</td>
<td>GAACCCGACAGCTTCTTCTACCTCG</td>
</tr>
<tr>
<td>Probe</td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>CTCTGTCCCACTGACAGCAGATGAG</td>
</tr>
<tr>
<td>Subject 2</td>
<td>AGGCCCCCTGCTCTTTGTACT</td>
</tr>
</tbody>
</table>

This situation leads to a unique opportunity in which functional analysis of single CMV epitope-specific clonotypes can be performed on freshly isolated PBMC using multiparameter CFC with gating on CD4+ expression and on the TCR-Vβ family containing the clonotype of interest. Fig. 1 demonstrates the characterization of two such single clonotype responses. Initial characterization of subject 1 has been previously reported (subject 4 in Ref. 17) but is included in this work for comparative purposes. Among peripheral blood CD4+ T cells, this high responder subject manifested a 7.6% IFN-γ response to whole CMV preparations (Fig. 1A, upper panels). Of this, ~22% (1.7% of CD4+ T cells) is attributable to a single epitope within CMV pp65 (pp65509-523), and ~60% of this pp65509-523-specific population is contained within the TCR-Vβ2 subset (1% of CD4+ T cells). Similarly, subject 2 demonstrated a 3.3% population of CD4+ T cells capable of IFN-γ production in response to whole CMV, and a 0.7% population specific for pp65545-549, the vast majority of which (>90%) were included in the TCR-Vβ17 subset (Fig. 1A, lower panels). In both subjects, a proportion of the IFN-γ-producing responding cells also made IL-2, but IL-2 production was not observed in the absence of IFN-γ (Fig. 1A, right panels).

The clonotypic composition of these TCR-Vβ2 (subject 1) and -Vβ17 (subject 2) responses was determined by stimulation of PBMC with the appropriate 15-mer epitope, followed by surface IFN-γ staining and sorting of the CD4+ T cells into responsive (CD69 IFN-γ+) vs nonresponsive (CD69 IFN-γ-) populations. These populations were then subjected to RT-PCR amplification of TCRBV2 and -BV17 template, respectively, cloning of the amplified PCR product, and then sequence analysis of the clones (17). As shown in Fig. 1B, spectratyping analysis of TCRBV products from sorted peptide-responsive T cells revealed a dominant BV2 band for subject 1 and a dominant BV17 doublet for subject 2. Sorted nonresponsive CD4+ T cells revealed a smear of BV2 products in subject 1 and a gaussian distribution of BV17 bands (separated by 3 bp) in subject 2 (both patterns associated with clonotypic diversity). Sequence analysis of the dominant band/doublet from the sorted peptide-responsive cells (bands A and C) revealed a single complementarity-determining region-3 (CDR3) sequence for both subjects (the doublet representing a 3’ dA addition by Taq polymerase (17)). In contrast to the single sequences found in the analysis of the pp65 epitope-responsive cells, sequence analysis of the corresponding regions of the gels from non-peptide-responsive cells revealed diverse, nonoverlapping sequences, consistent with the expected polyclonal nature of these non-pp65-specific CD4+ T cells.

We next sought to define TCRA expression by these TCRB-defined clonotypes. To achieve this goal, we again stimulated PBMC from subjects 1 and 2 with their appropriate, optimal 15-mer peptide epitopes, but this time we sorted CD4+ T cells on the basis of both Ag responsiveness (e.g., surface IFN-γ+) and TCR-Vβ2 and -Vβ17 expression, respectively, so as to isolate a pure population of clonotype+ T cells (Fig. 2A). Sorted cells were then analyzed by RT-PCR spectratyping for both the original TCRBV2 or -BV17 clonotypes and 29 TRCA families, followed by sequence analysis of any identified bands (Fig. 2, B and C). Both these sorts (bands A and D) revealed the same clonal TCRB CDR3 sequences identified in the original sorts shown in Fig. 1, confirming the reproducibility of the TCRB clonotype analysis. In subject 2, the single TCRBV17/BV1.5 sequence was associated with a single TCRAV2/AU13 sequence (from band E), verifying the clonality of this response and establishing TCR homogeneity within this clonotype. In subject 1, two major TCRAV bands (AV1 (band B) and AV16 (band C)) were observed, both of which contained single sequences. This finding strongly suggests that this TCRBV2/
analyzed A total of 50,000 events, gated on CD4+ and CD4 (FITC), as previously described (17). RT-PCR spectratyping analysis revealed an indistinct smear of bands (subject 1) or a gaussian distribution of bands (subject 2), whereas the nonresponsive populations revealed distinct, nonrepeated TCR sequences. Given the clonality of the CDR3 sequence in subject 2, the bands of the dominant doublet revealed clonal TCR CDR3 analysis of the responsive population yields a single dominant band in subject 1. The percentages of events within these gated populations are shown for the designated regions or quadrants. As would be expected (11, 12), maximal TCR down-regulation in both subjects (pp65511-12-mer peptide elicited both maximal responder frequencies and maximal CNS A to a portion of the PCR product.

Characterization of the fine specificity of the pp65 epitope-specific BV2/BJ2.2 and BV17/BJ1.5 single clonotype responses The 15-mer peptides used for characterization of the single clonotype responses were selected from a series of consecutive pp65 peptides overlapping by 11 aa (17). To further investigate the fine specificity of these clonotypes, we synthesized and purified a series of consecutive 12-mer peptides (shifted by 1 aa) encompassing the sequence of the original 15-mer peptide, and then assessed the stimulatory efficiency of these 12 mers (at 2 μg/ml) in the standard CFC IFN-γ assay without costimulatory mAbs. Efficiency was assessed by both the frequency of responding cells and the degree of TCR down-regulation on the responding population (11, 16). As shown in Fig. 3, a single 12-mer peptide elicited both maximal responder frequencies and maximal TCR down-regulation in both subjects (pp65511-12 for subject 1; pp65517 for subject 2), although the enhancement from the immediately adjacent 12 mers was not large. Indeed, five consecutive 12-mer peptides for subject 1 and four for subject 2 provided responding frequencies >70% of the optimal 15 mer. Because the class II MHC binding cleft is thought to encompass nonapeptides (22, 23), these high-response 12 mers potentially define such a core epitope sequence. To assess this possibility, candidate 9 mers were synthesized and similarly assessed (Fig. 3). For both the BV2/BJ2.2 and BV17/BJ1.5 clonotypes, a single optimal 9-mer structure capable of specific stimulation was identified. At 2 μg/ml, these optimal 9 mers did not achieve maximal response frequencies, but their ability to stimulate was specific for the appropriate clonotype, and was reproducible (data not shown).

Single CD4+ T cell clonotypes display broad heterogeneity in their activation thresholds

T cell triggering thresholds can be defined as the quantity of TCR-mediated signals required to elicit a defined response (e.g., functional expression of a particular cytokine gene) in a given Ag-specific cell. TCR signaling strength is a function of both the
functional avidity of the agonist (MHC-peptide complex) and the number of such agonists available on APC, and can be manipulated experimentally by dose response analysis and the use of optimal vs suboptimal ligands. Thresholds can also be experimentally dissected by modulation of costimulation. Costimulation is thought to facilitate T cell responses by lowering triggering thresholds, i.e., the number of effective TCR engagements required to achieve the desired response (11–14). Thus, at a given agonist dose, the ability of costimulatory augmentation to reproducibly increase response frequencies in a given T cell population implies the existence of threshold heterogeneity, a low threshold component able to respond without costimulation and a high threshold component requiring additional costimulation for triggering.

With these concepts in mind, we sought to explore the triggering characteristics of the single pp65 epitope-specific CD4+ T cell clonotypes described above. We analyzed IFN-γ and IL-2 responses to optimal 15- and 12-mer peptides, suboptimal 12-mer peptides, and the core 9-mer peptide at 10-fold dose intervals ranging from 2 μg/ml to 0.2 pg/ml (as appropriate to cover the response range for each agonist). All responses were assessed with and without exogenous costimulation in the form of CD28 and CD49d mAbs (8). Figs. 4 and 5 show representative data from these analyses. Several points are noteworthy. First, for IFN-γ production, the higher doses of optimal peptide with costimulation define a response frequency plateau, which we operationally define as the maximum response for each clonotype (~6% of Vβ2+CD4+ cells).
for subject 1; ~14% of Vβ17+CD4+ cells for subject 2; Fig. 4). Second, in the absence of exogenous costimulation, as little as 2–20 pg of optimal peptide are required for the appearance of detectable specific IFN-γ responses in these two clonotypes, and each 10-fold increase in optimal peptide concentration over this minimum amount results in a reproducible, incremental increase in responder frequencies. Third, without exogenous costimulation, maximal IFN-γ response frequencies are not achieved until optimal peptide concentration is increased 10,000-fold or more. Suboptimal 12 mers elicit similar dose response patterns for the IFN-γ response but, without exogenous costimulation, fail to achieve plateau frequencies at the highest peptide concentration tested (2 μg/ml). The core 9 mer is further shifted in its dose response, achieving (without additional costimulation) only 50–67% of maximal frequencies at 2 μg/ml (Fig. 5). Fourth, IL-2 synthesis shows similar broad dose responses with consistent, incremental increases with each log increase in agonist concentration, but without exogenous costimulation fails to reach maximal responder frequencies at the highest concentration tested. Finally, at all subplateau agonist doses for IFN-γ and all tested doses for IL-2, the addition of costimulatory mAbs significantly increased (up to 2- to 3-fold for 15 and 12 mers, depending on peptide dose) single clonotype responder frequencies (Fig. 4). Interestingly, this effect was most pronounced for 9-mer responses (3- to 6-fold), and for both IFN-γ and IL-2 responses the combination of high-dose (2 μg/ml) core 9 mer and CD28 plus CD49d costimulation resulted in responder frequencies at or closely approaching the maximum frequencies observed with optimal 12- or 15-mer peptides (Fig. 5). Taken together, these results indicate a profound triggering heterogeneity within these single clonotype responses, varying from cells capable of responding to subnanogram per milliliter agonist concentration alone to cells requiring ≥10,000-fold higher agonist concentrations and augmented costimulation for triggering.

**Dissimilar triggering thresholds for IFN-γ and IL-2 results in markedly different functional responses depending on epitope dose and costimulatory availability**

Previous work with CD4+ T cell clones has suggested that triggering thresholds for IFN-γ and IL-2 synthesis reproducibly differ with IL-2 synthesis, generally requiring more TCR and/or costimulatory signals (12, 13). Our observation that costimulation increases IL-2, but not IFN-γ, responder frequencies at the highest peptide concentrations tested is in agreement with this concept. To examine this issue further and to determine its potential importance among physiologic (e.g., non-in vitro cloned) CD4+ T cell populations at the clonotypic level, we directly compared the fraction of IL-2-producing cells of total responders (e.g., IFN-γ producers; under all conditions examined, essentially all IL-2 synthesis derives from T cells that also produce IFN-γ; Fig. 1 and data not shown) as a function of agonist dose and costimulation (Fig. 6A). If activation requirements for IFN-γ and IL-2 production were equivalent, this ratio would remain a constant throughout the dose response range and regardless of costimulation intensity. However, as shown in the figure for the optimal 12-mer responses of both subjects, this is not the case. In the absence of CD28 and CD49d costimulation, the fraction of IFN-γ-producing cells that also produce IL-2 doubles from low-dose to high-dose responses. These differences are even more pronounced in the presence of exogenous costimulation with ratios increasing 4- to 5-fold from low to high peptide doses. As illustrated in Fig. 6B, these differences can have a dramatic effect on the functional response of a single clonotype, depending on activation conditions. In this representative example, examining the response to a suboptimal 12 mer with CD28...
plus CD49d costimulation, high-dose peptide (2 μg/ml) results in a maximal IFN-γ response (~14% of Vβ2+CD4+ T cells in this subject) with a high fraction (62%) of these responders also making IL-2. At a 1,000-fold less peptide (0.002 μg/ml), the IFN-γ responder frequency is nearly identical (albeit at somewhat reduced average IFN-γ content per responding cell), but the fraction of these cells producing IL-2 has diminished 3-fold (to 20.7%). Thus, the combination of threshold heterogeneity (i.e., the spectrum of thresholds present in single clonotype population) and threshold differences between different functional responses (e.g., IFN-γ vs IL-2) leads to a complex situation in which the functional output of even a clonally homogeneous memory population cannot be predicted without detailed knowledge of agonist concentration and costimulation availability.

**CD27-defined, CD4+ T cell memory differentiation stage does not correlate with triggering threshold heterogeneity**

T cell signaling capabilities are thought to undergo regulated changes during T cell differentiation in concert with other T cell functions (24–26). In this regard, evolving concepts of memory T cell development have increasingly focused on a more or less linear differentiation pathway based on acquisition of the capability to manifest immediate, specialized anti-pathogen effector activity in tertiary (e.g., extralymphoid) sites. Memory cells that possess this capability (so-called effector memory cells) can be distinguished from less-differentiated memory cells that recirculate predominantly through secondary lymphoid tissues, and are proposed to serve as a precursor/reserve and perhaps immunoregulatory population (central memory cells). Functional correlates of this differentiation along the central to effector memory pathway are thought to include not only enhanced cytotoxic capability, polarized cytokine synthesis (e.g., Th1 vs Th2), and predominant homing to and localization within extralymphoid tissues (including tissue-specific homing populations), but also reduced triggering requirements (24–26). Therefore, we sought to investigate the possibility that the threshold heterogeneity observed in these single clonotypes simply reflects relative progression down this differentiation pathway, or, in other words, that the high and low threshold components of the response might correlate with central memory vs effector memory phenotype, respectively.

The phenotypic markers used to delineate these populations vary among investigators and depending on whether the CD4 or CD8 lineage is being studied; however, in general, expression patterns of CD27 and/or CCR7 have been found to be the most useful for delineating memory differentiation among human CD4+ T cells (25, 27–31). The most-differentiated effector memory population has been characterized as CD27+CCR7−, and the archetype central memory population has been characterized as having the reciprocal phenotype; small CD27+CCR7− and CD27−CCR7+ memory populations are thought to represent intermediate stages (Fig. 7, left panels). Because CD27 allows both robust delineation of the relevant subsets and high stability with short-term activation (data not shown), we investigated the CD27 phenotype of single or without (dashed line) exogenous costimulation (0.5 μg/ml each of CD28 and CD49d mAbs) for 6 h in the presence of the secretion inhibitor brefeldin A for the final 5 h, and then examined for their correlated expression of cell surface TCR-Vβ2 or Vβ17 and intracellular IFN-γ, IL-2, and CD4. The percentage responding for IFN-γ and IL-2 among the CD4+ TCR-Vβ2− small lymphocyte subset of subject 1 are shown in A; the percentage responding for IFN-γ and IL-2 among the CD4+TCR-Vβ17− small lymphocyte subset of subject 2 are shown in B. The results shown are representative of two to four independent experiments for each subject.

**FIGURE 4.** Dose response analysis of optimal and suboptimal epitopes in the presence or absence of exogenous costimulation demonstrates triggering threshold heterogeneity within single CD4+ memory T cell clonotypes. PBMC from subjects 1 and 2 were stimulated with serial 10-fold dilutions (starting at 2 μg/ml) of optimal 15-mer, optimal 12-mer, and representative suboptimal 12-mer epitopes (see Fig. 3) with (solid line, □) or without (dashed line, □) exogenous costimulation (0.5 μg/ml each of CD28 and CD49d mAbs) for 6 h in the presence of the secretion inhibitor brefeldin A for the final 5 h, and then examined for their correlated expression of cell surface TCR-Vβ2 or Vβ17 and intracellular IFN-γ, IL-2, and CD4. The percentage responding for IFN-γ and IL-2 among the CD4+ TCR-Vβ2− small lymphocyte subset of subject 1 are shown in A; the percentage responding for IFN-γ and IL-2 among the CD4+TCR-Vβ17− small lymphocyte subset of subject 2 are shown in B. The results shown are representative of two to four independent experiments for each subject.
clonotype T cells responding to low- vs high-dose agonist concentrations, with and without costimulation (Fig. 7, right panels). As illustrated in Fig. 7, 90% or more of these single clonotype responses were contained within the CD27+ subset at all concentrations of peptide and both with and without costimulation. A small fraction of the responding cells were CD27/−, and the response pattern of these cells appeared to parallel that of the much larger CD27-responding subset, i.e., showing both high and low threshold components. Essentially identical results were observed for optimal 12- and 15-mer responses and for IL-2 production in this subject, as well as all of the analogous responses in subject 2 (data not shown). Taken together, these data indicate that the triggering heterogeneity we have observed in these single clonotypes does not correlate with and therefore cannot be explained by the CD27-defined, CD4+ memory T cell differentiation pathway.

Discussion

The basic unit of T cell immunity is the TCR-defined clonotype, and cellular immune responses, whether protective or pathogenic, are ultimately a function of the specificity, frequency, and function of these fundamental units (17). Given the enormous influence of TCR recognition properties on T cell development, differentiation, homeostasis, and function, it is clearly imperative to study immune physiology at the clonotypic level, thereby allowing TCR-dependent and independent mechanisms to be studied in a controlled fashion. The advent of TCR transgenics has permitted such analysis in mice (7, 26, 32, 33), but direct evaluation of single clonotype function within unmanipulated, normal human T cell populations has heretofore not been possible. In this report, we demonstrate a novel approach to this problem, taking advantage of the multiparameter capabilities of CFC and the natural clonotypic hierarchy of the large human CD4+ T cell response to CMV. By identifying individual CMV-specific clonotypes that are isolated within mAb-definable TCR-Vβ families, we were able to specifically study the functional response of such clonotypes by CFC after gating on the appropriate Vβ family. We then used this approach to analyze the triggering requirements of fresh (uncloned) human T cells at the single clonotype level.

Two single clonotype responses to different CMV pp65 epitopes in different subjects were studied with essentially identical results. In both responses, we identified optimal 15- and 12-mer antigenic epitopes, a series of suboptimal 12-mer epitopes, and a single effective core 9-mer epitope, the latter comprising a sequence common to all stimulatory peptides. The specific responses elicited by these core 9 mers clearly demonstrate the stimulatory capabilities of short peptides for CD4+ T cells. However, unlike class I-restricted responses where 8- to 10-mer epitopes are optimal (34, 35), and in keeping with the demonstrated importance of flanking sequences in CD4+ T cell recognition (36–38), the core 9 mers identified here were not optimal ligands—they could only generate maximal (plateau) or near maximal IFN-γ response frequencies at high epitope concentrations (0.2–2 µg/ml) in the presence of exogenous costimulation. In addition, the response to these 9 mers fell off quickly with successive log decreases in peptide dose. In contrast, optimal 12- and 15-mer peptides were essentially equivalent.
in their ability to generate maximal IFN-γ response frequencies, efficient TCR down-regulation, and robust dose-response curves (stimulatory down to the 2-pg level for IFN-γ synthesis without costimulation). We also determined that the three or four 12 mers adjacent to the optimal structure (e.g., 12 mers successively shifted 1 or 2 aa in sequence from the optimal 12 mer in either direction) showed varying degrees of stimulatory potency as assessed by these same criteria, ranging from slightly submaximal to approximately the same (relatively low) stimulatory capacity of the core 9 mer.

Together, these various structures provided a wide range of stimulatory potencies useful for the analysis of triggering thresholds within these single clonotype responses. Two general themes were apparent with the extensive dose response analysis (± costimulation) of these peptides. First, in the absence of costimulation, we observed that, even with optimal 12/15 mers, a 4-log or more increase in peptide concentration was required to go from the first appearance of responding cells to plateau frequencies. Partial responses to suboptimal 12 mers at the opposite structural ends of the stimulatory spectrum (e.g., 12 mers with the core 9 mer abutting the C terminus vs the N terminus of the 12 mer) were not additive (data not shown), indicating that the same cells respond to low potency stimuli, regardless of the precise stimulatory structure. The reproducible, stepwise increase in response frequencies with each 10-fold increase in ligand concentration strongly suggests the existence of significant triggering heterogeneity within these single clonotype populations. This possibility is further supported by the second key observation: for each subplateau response, provision of exogenous costimulation dramatically increased response frequencies. As discussed above, costimulation elicits no overt response on its own, but rather, effectively acts to lower TCR signaling requirements (e.g., the triggering threshold) for a given response; thus, the difference between those cells that respond at any given epitope dose without costimulation and those that require costimulation is likely one of threshold—lower (easier to activate) for the former and higher (harder to activate) for the latter. Because costimulatory augmentation occurred at all subplateau responses, the high threshold vs low threshold dichotomy does not appear to be binary, but rather, may reflect a broad threshold continuum.

Implicit in the term “threshold” is the concept that the observed triggering differences must originate in intrinsic differences in the signaling apparatus of these clonotypic T cells. However, it is formally possible that the T cell signaling efficiency in these responses is identical and that the observed triggering heterogeneity is due to costimulatory heterogeneity within the APC population. In this scenario, the overall APC population would include a spectrum of constitutive costimulatory capabilities; at one end of the spectrum costimulatory molecules would be absent or low, at the other end they would be high. Potentially responsive T cells would stochastically encounter an epitope-bearing APC in the first minutes after Ag loading and then would form a stable (e.g., semipermanent) interaction with that APC. T cells interacting with high costimulatory APC would be triggered in the absence of exogenous costimulation; those interacting with putative costimulatory effect of Ag dose on functional phenotypes of single clonotype responses is illustrated. PBMC from subject 1 were stimulated with 2 or 0.002 μg/ml of a suboptimal 12-mer peptide (note that this is a different peptide from that used in A) and analyzed as shown in Fig. 1. A total of 10,000 events, gated on CD4+ TCR-Vβ3 small lymphocytes, are shown with the events in the total responding region (IFN-γ+) enlarged and colored black and the events in the nonresponding region colored gray. The percentage of IFN-γ+ (total responding) and the percentage of these cells that are IL-2+ vs IL-2− (+/−) within the gated populations are provided in the left and right profiles, respectively.
deficient APC would not be triggered unless costimulatory mAbs were provided. One would also have to hypothesize that the putative high costimulatory APC would be limiting and/or immediately co-opted by T cells such that they would not be available for interaction with T cells that manage to detach from nonstimulatory APC. Several lines of evidence strongly argue against this explanation of our data. First, T cell-APC interactions have been shown to be highly dynamic, with T cells rapidly shifting from one APC to another that would offer a higher level of stimulation (39, 40).

Second, we have performed APC depletion and add-back experiments showing that, within normal PBMC, dendritic cells and the far more numerous monocytes (but not B cells) contribute essentially equally to APC function in the support of CD4+ T cell superantigen responses (as measured by CFC), and, most importantly, that the responses supported by both these APC populations are equivalently enhanced by exogenous costimulation (L. J. Picker, unpublished observations). Moreover, increasing the APC:T cell ratio in these experiments did not change the level of response frequency enhancement provided by exogenous costimulation (L. J. Picker, unpublished observation), indicating that APC (including a putative “high costimulatory” APC subset) are not limiting in these assays.

These considerations strongly suggest that it is indeed intrinsic differences in T cell triggering requirements that account for the signaling heterogeneity observed in this study, and not differences in APC. Such triggering requirement heterogeneity is potentially mediated by differences in TCR fine specificity, or by differences in the complex matrix of interacting signaling and regulatory molecules that collectively constitute the downstream TCR signaling apparatus. The fact that both sequence and qPCR analysis demonstrated that the responses examined here were mediated by T cells expressing a single TCRB CDR3 sequence and either no CD27 or were stimulated with serial 10-fold dilutions (starting at 2 μg/ml) of the suboptimal pp65513-524 peptide with or without exogenous costimulation (CD28 and CD49d), as described in Fig. 4, and then examined for their correlated expression of cell surface TCR-Vβ2 and CD27 and intracellular IFN-γ and CD4. A total of 5000 events are shown in each profile, gated on CD4+ TCR-Vβ2+ T cells. In the cell surface stain, the cell clusters corresponding to the naive and memory subsets, and the CD27- and CCR7-defined central memory to effector memory differentiation pathways are designated (25, 27–30). In the panels showing the functional response of these cells, events corresponding to (IFN-γ+) responding cells are enlarged and colored black and nonresponding cells are colored grey. The percentage responding with IFN-γ production within the CD27 and CD27- subsets (delineated by a horizontal line) are indicated in the upper right corner of each profile (CD27+CD27-). Similar dose response patterns were observed for responses elicited by optimal 12mers and 9mers and for IL-2 responses to all of these peptide stimuli (data not shown).

**FIGURE 7.** The threshold heterogeneity of these pp65-specific clonotypes is independent of the CD27-defined memory T cell differentiation stage. PBMC from subject 1 were examined immediately for cell surface expression of CD4, TCR-Vβ2, CD27, and CD95 or were stimulated with serial 10-fold dilutions (starting at 2 μg/ml) of the suboptimal pp65513-524 peptide with or without exogenous costimulation (CD28 and CD49d), as described in Fig. 4, and then examined for their correlated expression of cell surface TCR-Vβ2 and CD27 and intracellular IFN-γ and CD4. A total of 5000 events are shown in each profile, gated on CD4+ TCR-Vβ2+ T cells. In the cell surface stain, the cell clusters corresponding to the naive and memory subsets, and the CD27- and CCR7-defined central memory to effector memory differentiation pathways are designated (25, 27–30). In the panels showing the functional response of these cells, events corresponding to (IFN-γ+) responding cells are enlarged and colored black and nonresponding cells are colored grey. The percentage responding with IFN-γ production within the CD27 and CD27- subsets (delineated by a horizontal line) are indicated in the upper right corner of each profile (CD27+CD27-). Similar dose response patterns were observed for responses elicited by optimal 12mers and 9mers and for IL-2 responses to all of these peptide stimuli (data not shown).
cell-state changes do occur among memory T cells and contribute to threshold regulation.

TCR threshold regulation is also thought to occur outside of differentiation pathways in response to environmental signals, as conceptualized in the tunable activation threshold (TAT) hypothesis of Grossman and Paul (3, 41). In the TAT hypothesis, T cell activation thresholds are controlled by the relative amount and/or activity of independently regulated excitation factors (e.g., protein tyrosine kinases or other molecules that promote downstream signaling) and de-excitation factors (e.g., phosphatases or other molecules that inhibit downstream signaling). The level/activity of these factors change in response to the stimuli that T cells repeatedly receive as a function of their constant monitoring of dendritic cells and perhaps other APC for potential ligands (42, 43). The TAT hypothesis further holds that such stimuli, even when below the threshold of any recognizable T cell response, induce changes in the levels of both excitation and de-excitation factors, but with inherently different kinetics (either fast or slow for excitation factors, depending on the nature of the stimulus, and usually slow for de-excitation factors). This regulation alters the balance of these factors such that a T cell’s excitability—its triggering threshold—is an integration of a cell’s recent signaling history, specifically the number, intensity, and quality of both subthreshold and suprathreshold signals, and the elapsed time since both signals. The cyclic changes in the triggering thresholds of T cell clones associated with elapsed time from restimulation (5), and the changes in triggering thresholds associated with T cell clone growth on hypo- or hyperstimulating ligands (4) are likely in vitro manifestations of such threshold tuning mechanisms.

How such integration of sub- and suprathreshold signals might lead to the spectrum of triggering thresholds observed for the normal, CMV-specific clonotypes studied here remains speculative. These clonotypes reflect a steady state memory response (likely decades removed from primary infection) directed at a persistent virus, and thus may receive more or less continuous subthreshold signaling from CMV-irrelevant ligands as well as periodic, suprathreshold signaling from their actual CMV-derived antigenic targets. The potential for the latter would be absent or greatly reduced for memory clonotypes directed at nonpersistent Ags, and thus it would be of interest to determine whether, as a general rule, threshold “spectra” are different for such responses. In this regard, it would also be of interest to determine whether overt re-exposure(s) to specific Ag in vivo materially affects the threshold spectra of an established memory population of both types, and, if so, for how long.

In addition to threshold tuning, the response heterogeneity observed here may reflect, at least in part, the well-recognized “stochastic” nature of activation at the single cell level, presumably due to random fluctuations in the amount and activity of relevant cellular constituents (illustrated in Ref. 44). However, such stochastic effects would likely give rise to relatively narrow gaussian distributions of responding cell frequencies observed within single in vitro-derived clones (e.g., the one- to two-order differences of peptide concentrations defining the response range in a recent report (5)), as compared with the over four orders of magnitude response range observed in the present study among normal T cells studied immediately ex vivo.

Regardless of its mechanism(s) of origin, the threshold heterogeneity demonstrated here among physiologic memory CD4+ memory T cells has important implications for the participation of these cells in effector responses in vivo. This heterogeneity insures that such responses will proceed in a graded or measured fashion even when the responding population has limited clonotypic complexity (as is often the case (17)). A narrow threshold spectrum would regulate the response like an on/off switch. In contrast, with the broad threshold spectrum demonstrated here, low-level Ag exposure with limited associated inflammatory response (keeping APC from maturation/activation and consequently with relatively low costimulatory potential) would support immediate activation of only a small portion of the overall memory cohort, the low threshold fraction. Such a limited response may be able to control the incipient infection with a minimum of “collateral” immunopathologic damage. If the infection is more intense at the outset or progresses despite this initial T cell activation, higher Ag loads and inflammation-associated enhancement of APC costimulatory capabilities would progressively recruit higher threshold fractions of the memory cohort until, at some point, the whole population is triggered. This mechanism would allow the system to maintain high-frequency, low-TCR complexity responses without the necessity of triggering over-large (and potentially hazardous) memory/effector cohorts in response to low-level stimuli. Such a system thus provides for containment of focal reactivation of CMV with low level responses, as well as control of potentially larger viral insults introduced from the outside with strong responses, the latter without the necessity of (and delays associated with) cellular expansion.

The nonidentical threshold spectra of different T cell effector responses (e.g., IFN-γ vs IL-2 production) adds yet another layer of regulation (and complexity) to the system. As has been reported by others (12, 13), our data indicate that the threshold spectrum of IL-2 gene expression is overall higher than that of IFN-γ or, in other words, IL-2 production requires higher ligand concentrations and/or costimulation than IFN-γ production. Thus, IL-2 production is low relative to IFN-γ production in the theoretical early/low-level infection mentioned above, perhaps reflecting a higher priority for effector activity (IFN-γ) over effector cell expansion (IL-2) in this situation. In contrast, if the infection progresses, higher Ag concentrations and APC costimulatory potential would result in relatively increased IL-2 production by responding T cells, providing an environment conducive to rapid effector cell expansion. While such threshold-dependent changes in T cell function make a certain teleological sense, they greatly complicate our attempts to understand the basis of protective immune responses and to determine correlates of protection. T cell clone data indicate that IFN-γ and IL-2 synthesis are not unique: all potential T cell responses, including production of other cytokines (IL-4, TNF-α), cytotoxicity, proliferation, and cell surface molecule induction (i.e., CD40 ligand), exhibit distinct, hierarchical triggering thresholds (12, 45–47). When these threshold hierarchies are combined with the intraclonotype threshold heterogeneity described here, a fundamental issue becomes manifest: the same T cells can exhibit qualitatively different responses depending on ligand and costimulatory availability, and thus T cell function must be interpreted in the context in which it occurs (48, 49).

In conclusion, we have demonstrated the feasibility of analyzing fresh human memory T cells at the level of single TCRB-defined clonotypes. These analyses have revealed that the triggering threshold heterogeneity we previously reported to occur in the setting of polyclonal T cell responses (8) also exists at the level of single clonotypes and appears unrelated to currently understood memory T cell differentiation pathways. Taken together, these data support evolving concepts of peripheral T cell threshold tuning and demonstrate the potentially profound importance of such tuning in both regulating the operation of antimicrobial T cell responses in vivo and complicating our efforts to understand the basis of T cell-mediated protection in clinical settings. T cell threshold tuning has long been recognized as an important aspect of pathologic T cell function in the setting of autoimmunity but has rarely been considered in the context of physiologic T cell function during infection with chronic pathogens. Our data suggest that activation
threshold tuning might be added to the list of memory/effector T cell frequency, fine specificity, clonotypic complexity, and functional differentiation as determinants of T cell-mediated protection in this setting and as key parameters to be considered in the development of vaccines against such agents.

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