Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells

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The enormous diversity of the naive T cell repertoire is instrumental in generating an immune response to virtually any foreign antigen that can be processed into peptides that bind to MHC molecules. The low frequency of antigen–specific naive T cells, their high activation threshold, and the constrains of antigen–processing and presentation have hampered analysis of naive repertoires to complex protein antigens. In this study, libraries of polyclonally expanded naive T cells were used to determine frequency and antigen dose–response of human naive CD4+ T cells specific for a variety of antigens and to isolate antigen–specific T cell clones. In the naive repertoire, T cells specific for primary antigens, such as KLH and *Bacillus anthracis* protective antigen, and for recall antigens, such as tetanus toxoid, cytomegalovirus, and *Mycobacterium tuberculosis* purified protein derivative, were detected at frequencies ranging from 5 to 170 cells per 10^6 naive T cells. Antigen concentrations required for half-maximal response (EC50) varied over several orders of magnitude for different naive T cells. In contrast, in the memory repertoire, T cells specific for primary antigens were not detected, whereas T cells specific for recall antigens were detected at high frequencies and displayed EC50 values in the low range of antigen concentrations. The method described may find applications for evaluation of vaccine candidates, for testing antigenicity of therapeutic proteins, drugs, and chemicals, and for generation of antigen–specific T cell clones for adoptive cellular immunotherapy.

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Abbreviations used: APC, allophycocyanin; PA protective antigen; PPD, purified protein derivative; TT, tetanus toxoid.

The naive T cell repertoire is extraordinarily diverse because it contains an enormous number of distinct T cell clones, each represented by only a few cells (Goldrath and Bevan, 1999). Upon antigenic stimulation in secondary lymphoid organs, rare antigen-specific naive T cells undergo clonal expansion and differentiate to effector and memory cells (Jenkins et al., 2001; Kaech and Ahmed, 2001; Sallusto et al., 2004). Thus, the memory T cell repertoire contains a collection of expanded T cell clones that reflect the antigenic experience of the individual. Molecular studies indeed established that the TCR diversity is at least 100-fold lower in the memory compared with the naive repertoire (Arstila et al., 1999).

The identification and characterization of antigen-specific T cells in the naive repertoire is of fundamental relevance to understanding the process of clonal selection and of practical relevance to predicting the immunogenicity of vaccines and therapeutic proteins. Human pep-

tide-specific CD8+ T cells can be directly identified using soluble peptide/MHC class I tetramers (Altman et al., 1996). However, because of the low frequency of naive T cell precursors, this method can be successfully used only in special cases when the frequency is exceptionally high, such as for a Melan-A/MART-1 peptide bound to HLA-A2 (Pittet et al., 1999; Dunbar et al., 2000). Several laboratories have also developed human MHC class II multimers to detect peptide-specific memory CD4+ T cells (Novak et al., 1999; Meyer et al., 2000; Cameron et al., 2002; Lemaitre et al., 2004; Moro et al., 2005), but no data are yet available as to whether these multimers can be used to identify naive CD4+ T cells. More recently, a method based on MHC
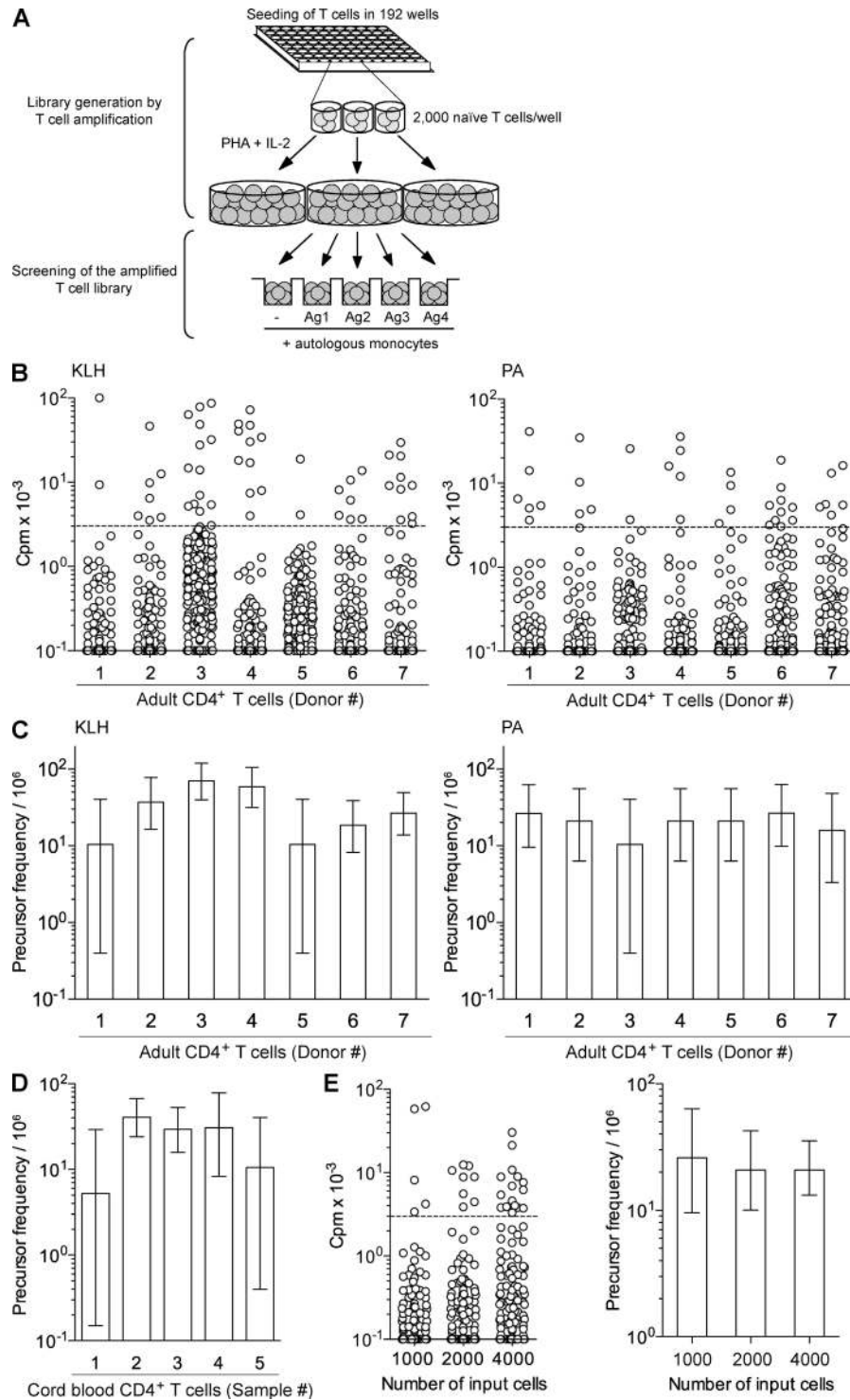
Figure 1.   Amplified T cell libraries as a new tool to measure frequencies of antigen–specific naive T cells in human adult blood and cord blood. (A) Schematic representation of the method. Library generation: naive CD4+ T cells are seeded at 2,000 cells/well in multiple wells containing irradiated allogeneic PBMC, PHA, and IL-2. The individual cultures are expanded into larger wells that make up the library of amplified T cell blasts. Screening of the library: T cell from individual cultures are collected, washed, and tested for their capacity to proliferate in response to various antigens (Ag) in the presence of autologous irradiated monocytes. (B) Libraries consisting of 192 polyclonal cell cultures, each derived from 2×10^3 naive T cells, were prepared from 7 adult donors and screened for their capacity to proliferate in response to KLH or PA (both at 5 μg/ml). After 3 d, proliferation was measured after 16-h pulse with [3H]thymidine. Shown are delta cpm values. Each symbol illustrates one culture out of the 192 screened. In all graphs, background values <100 cpm were set to 100 cpm to represent all data points analyzed in the y-log scale. Dotted lines represent the cut-off value. The specificity of positive cultures was confirmed in a second independent experiment. None of the cultures recognized both antigens (not depicted).

tetramers followed by magnetic bead enrichment was used to identify antigen-specific CD4^{+} and CD8^{+} T cells in naive mice (Hataye et al., 2006; Moon et al., 2007; Obar et al., 2008). Using this method, it has been estimated that the frequency of CD4^{+} T cells specific for epitopes within ovalbumin, I-E α chain, or *Salmonella typhimurium* varies from 20 to 200 cells per mouse, whereas the frequency of CD8^{+} T cells specific for ovalbumin or viral peptides varies from 80 to 1,200 cells per mouse. These figures are consistent with those previously estimated using an indirect method based on adoptive transfer of TCR transgenic T cells (McHeyzer-Williams and Davis, 1995; Butz and Bevan, 1998; Blattman et al., 2002; Stetson et al., 2002; Whitmire et al., 2006).

In spite of several advantages, the MHC multimer technology is not generally applicable in view of the large numbers of MHC alleles and of the requirement of an a priori knowledge of the peptide sequence. In addition, MHC tetramers can assess only one epitope at a time and may identify cells that bind, but do not recognize, the naturally processed antigen. An alternative approach is to prime naive T cells in vitro using antigen-pulsed DCs. Using this method, it has been shown that antigen-specific CD8^{+} T cells can be isolated after consecutive rounds of in vitro antigenic stimulation and enrichments (Ho et al., 2006; Wolfl et al., 2007). However, because of the extensive in vitro selection, this method is not suitable to study the human naive repertoire.

With the aim of developing a method to study the human naive T cell repertoire, we set up a novel in vitro T cell assay that is based on the screening of libraries of polyclonally expanded naive CD4^{+} T cells. The assay was used to determine frequency, antigen dose–response, and epitope specificity of human naive CD4^{+} T cells specific for a variety of antigens, to isolate representative T cell clones from the naive CD4^{+} repertoire, and to compare antigen-specific T cells in the naive and memory T cell compartments.

RESULTS AND DISCUSSION
Identification of antigen–specific T cells using amplified libraries of naive CD4^{+} T cells

The two main obstacles to the identification of antigen-specific naive T cells are their low frequency and low responsiveness to antigenic stimulation. We reasoned that polyclonal activation could be used to amplify each naive T cell into an expanded clone of highly responsive T cell blasts. In the method developed (Fig. 1 A), a sample of human naive CD4^{+} T cells is divided into several replicate cultures, each containing 2,000 cells. The cultures are stimulated by PHA in the presence of irradiated allogeneic feeder cells and IL-2 and expanded for 2 wk to generate a library of T cells amplified by 1,000–5,000-fold. Aliquots from each culture are then tested for their capacity to proliferate in response to a variety of an-

tigens that are processed and presented by autologous monocytes. The culture conditions support the cloning of every naive T cell (Moretta et al., 1983) and allow an even expansion of polyclonal T cells, as revealed by analysis of Vβ expression using antibodies and spectratyping (Fig. S1, A and B).

In a typical experiment, shown in Fig. 1 B, highly purified CD4^{+} naive T cells were isolated from PBMCs of healthy donors (Donor #1–7) by cell sorting according to the expression of CD45RA and CCR7 and the lack of CD45RO and CD25. The cells homogeneously expressed CD62L and CD27, whereas CD31 was expressed on approximately half of the cells (unpublished data), which is consistent with previous reports (Kimmig et al., 2002). Libraries of ∼4 × 10^{5} naive T cells in 192 cultures were generated from each donor. The individual expanded cultures were simultaneously tested for their capacity to proliferate in response to autologous monocytes, either unpulsed or pulsed with KLH or protective antigen (PA) from *Bacillus anthracis*. These antigens were chosen because there was no evidence of prior exposure and could therefore be considered primary antigens. When proliferation was measured by [^{3}H]thymidine incorporation on day 3, all cultures set up in the absence of antigen showed very low counts (cpm < 10[3]), which is consistent with a lack of reactivity to autologous monocytes and human serum components. In contrast, among cultures stimulated with KLH- or PA-pulsed monocytes, a few showed a clear and often strong proliferation (delta cpm values ranging from 3 × 10^{3} to 10[5]; Fig. 1 B).

Cultures were scored positive when the stimulation index was >5 and when the delta value (cpm in response to Ag-pulsed monocytes − cpm in response to unpulsed monocytes) exceeded 3 × 10^{3} cpm. The frequency of antigen-specific naive T cell precursors was calculated on the basis of the fraction of negative cultures and the input of cells per culture and expressed per 10[6] naive CD4^{+} T cells. As shown in Fig. 1 C, frequency of T cell precursors specific for KLH in the 7 donors analyzed varied between 10 and 70 cells per 10[6] naive CD4^{+} T cells, whereas frequency of PA-specific T cell precursors was estimated in the range of 10–26 per 10[6] naive CD4^{+} T cells. We also set up libraries from naive T cells from five different cord blood samples (Sample #1–5). As shown in Fig. 1 D, KLH-specific T cells were detected in all cord blood samples, at frequencies that were comparable to those found in samples from adults (5–40 per 10[6] naive CD4^{+} T cells). The accuracy of precursor frequency measurements was dependent on the absolute number of positive cultures scored, as revealed by the 95% confidence interval (Fig. 1, C and D). Accuracy could be increased either by increasing the size of the library, for instance setting up 384 cultures, or by increasing the number of input T cells per culture (Fig. 1 E). However, for input values that exceeded

(C) Frequencies of naive CD4^{+} T cells specific for KLH and PA in the seven adult donors were calculated from the data shown in B. Bars represent 95% confidence intervals. (D) Frequencies with 95% confidence intervals of naive CD4^{+} T cells specific for KLH in 5 cord blood samples. (E) Libraries of naive CD4^{+} T cells were produced with different input cell numbers (1,000, 2,000, and 4,000 per well) and screened with TT. Shown are delta cpm values (left) and precursor frequencies with 95% confidence intervals (right). Dotted line represents the cut-off value. One representative experiment of two performed.

4,000 cells per culture, the sensitivity of the assay decreased (unpublished data).

Melan-A/MART-1–specific CD8^{+} T cells are present at an unusually high frequency in the naive repertoire of HLA-A2–positive individuals (Pittet et al., 1999), offering the possibility to compare, for this particular antigen, the MHC class I multimer method with the new amplified library method. As shown in

Fig. S2 A, in an HLA-A02–positive individual 0.03% of naive T cells were stained with MHC class I Melan-A/MART-1 pentamers (A*0201/ELAGIGILTV). However, when the pentamers^{+} cells were sorted and cloned, only a fraction of the clones was responsive to the peptide, which is consistent with a significant level of nonspecific staining. Accordingly, the corrected frequency was estimated to be 0.005%. We generated libraries of
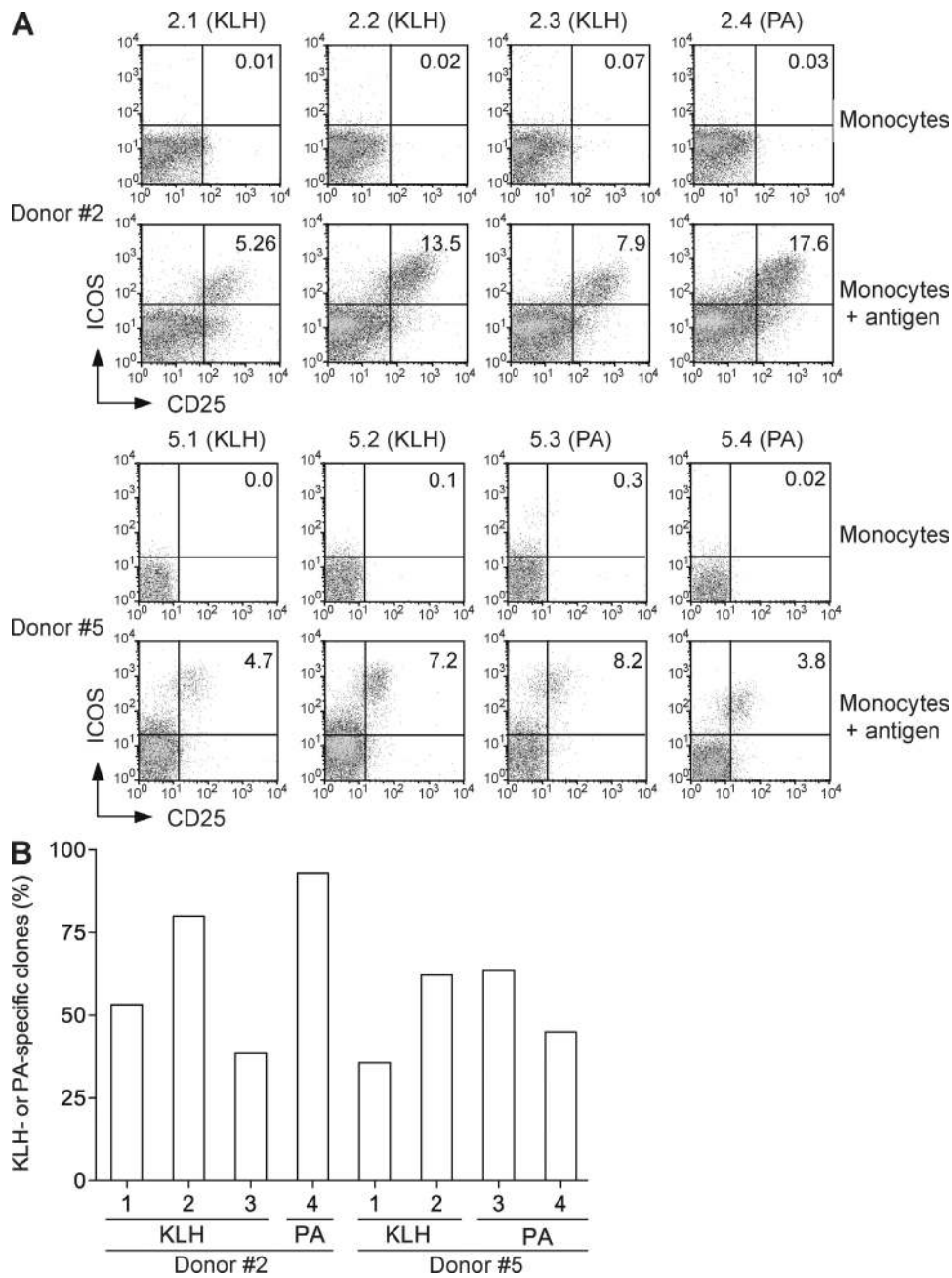

Figure 2. Isolation of KLH– or PA–specific T cell clones. (A) T cell cultures from Donor #2 and Donor #5 that scored positive for KLH- and PA-driven proliferation were cultured for 24 h in medium without IL-2. Cells were then stimulated with autologous monocytes in the presence or absence of KLH or PA. After 5 d, cells were harvested and stained for expression of CD25 and ICOS. Shown is the percentage of CD25^{+} ICOS^{+} T cells in cultures in the absence or presence of antigen. (B) CD25^{+} ICOS^{+} T cells were sorted and cloned by limiting dilution. Shown is the percentage of KLH- or PA-specific T cell clones among the growing clones isolated from each culture. Two representative experiments of six performed.

naive CD8^{+} T cells from the same donor using the procedure depicted in Fig. 1 A, and we screened them for their capacity to proliferate in response to the Melan-A/MART-1 peptide. In this particular donor, the frequency calculated with the library method was 0.004% (Fig. S2 B), a value that is very close to the corrected frequency obtained using pentamers. Melan-A/MART-1–reactive cultures were readily detected in libraries of naive, but not memory, CD8^{+} T cells from five additional HLA-A02 donors (Fig. S2 C and not depicted).

Collectively, the present results indicate that amplified polyclonal libraries of human naive CD4^{+} and CD8^{+} T cells can be interrogated to identify cultures that contain antigen-specific T cell precursors and to establish their frequencies within the naive repertoire. A recent study in mice using MHC multimer technology demonstrated that the frequency of CD4^{+} T cells specific for a given peptide-MHC combination can vary from 20 to 200 cells per mouse (Moon et al., 2007). Our estimates of human naive CD4^{+} T cells specific for whole proteins are not far from these figures if one considers that a mouse contains $\sim$40 $\times$ 10[6] naive CD4^{+} T cells and that large proteins, such as the ones we tested, may contain 10 or more antigenic determinants.

Multiple interrogations of naive CD4^{+} T cell libraries

The polyclonal expansion of naive T cell libraries delivers T cell blasts in numbers sufficient to perform multiple interroga-

tions. When a naive CD4^{+} T cell library was screened with the same antigen at different time points (10 d apart), identical results were recorded, consistent with a high reproducibility of the screening assay (Fig. S1 C). In addition, a library derived from a single donor was analyzed simultaneously for reactivity against KLH, PA, tetanus toxoid (TT), purified protein derivative (PPD) from *M. tuberculosis*, *Dermatophagoides pteronyssinus* major allergen I, and human thyroglobulin. In all cases, whether it was a primary or recall antigen, an environmental allergen or a self-protein, cultures containing specific T cells were readily identified (Fig. S1 D). Naive T cell libraries were also screened with lysates of *Plasmodium falciparum*–infected erythrocytes. In this case, a large fraction of cultures (>40 out of 72 screened) scored positive (unpublished data), indicating that a very high frequency of naive T cells were specific for this complex parasite. These results indicate that naive T cell libraries can be repeatedly interrogated to identify T cells specific for self- and non–self-proteins.

Isolation of T cell clones from amplified T cell libraries

Given the polyclonal nature of the expanded T cell cultures, antigen-specific T cells are expected to occur at low frequencies (1 in 2,000). We therefore established a procedure to isolate specific T cell clones from positive cultures. KLH and PA-reactive T cell cultures were first stimulated with monocytes, which
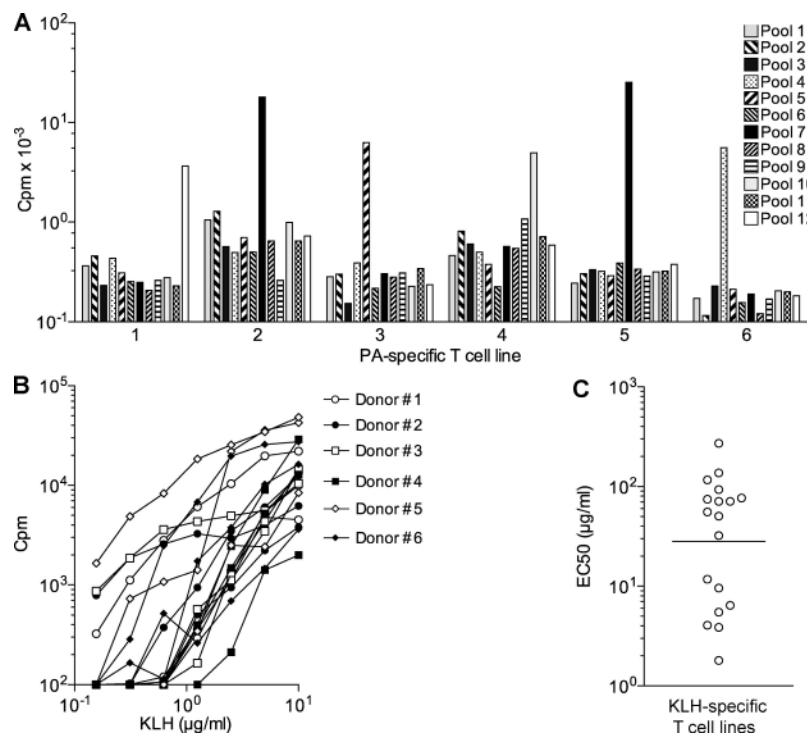

Figure 3. Characterization of antigen–specific naive T cells. (A) Six PA-reactive T cell lines isolated from a single donor were screened for their capacity to proliferate in response to pools of peptides spanning the PA sequence. Shown is the response of each cell line to the 12 pools. The pools were then deconvoluted and the peptide recognized by each cell line was identified (see Table S2). One representative experiment of two performed. (B) 18 KLH-specific T cell lines from six different blood donors were stimulated with increasing concentrations of KLH. (C) EC50 values were calculated as described in the text. Bar indicates median value. The experiment shown in B and C are representative of three different experiments, each performed with 10–18 KLH-specific T cell lines isolated from 3–6 different donors.
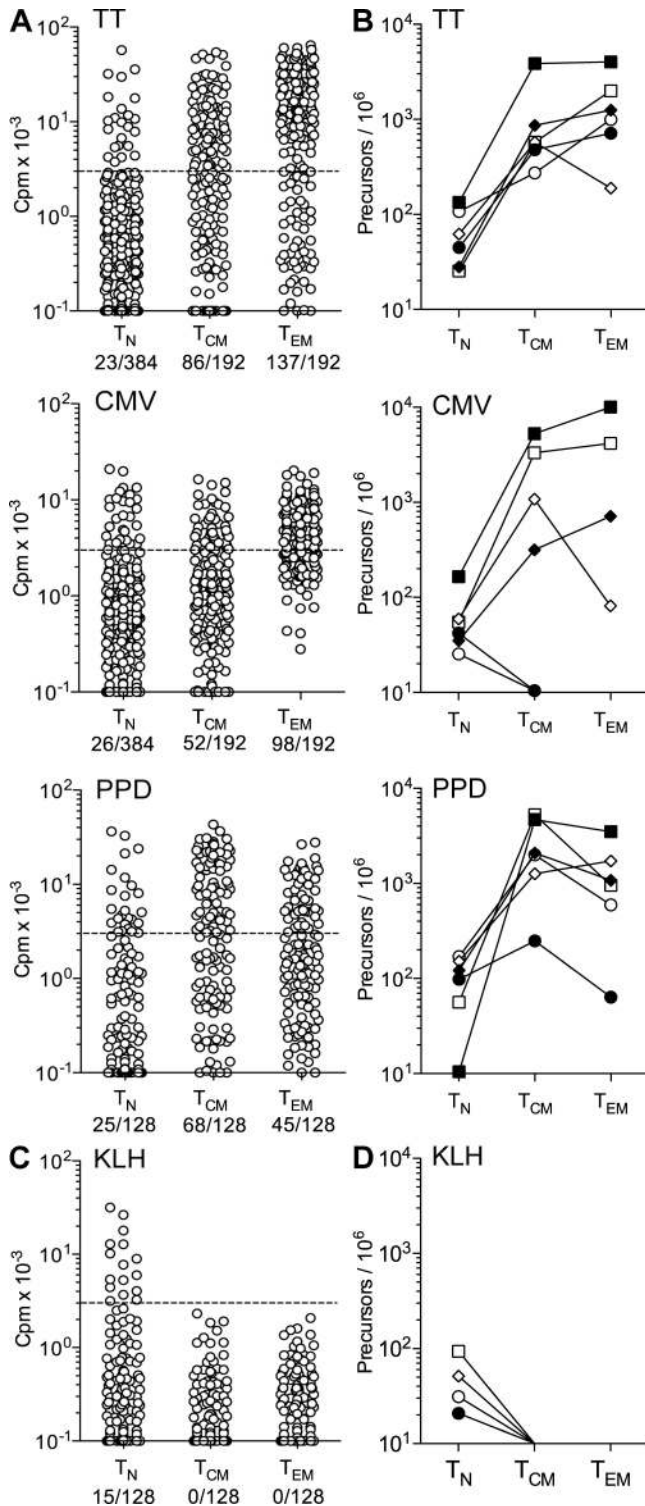
Figure 4. High frequencies of antigen–specific CD4⁺ T cells in memory compartments. (A) Libraries were prepared from naive (T$_N$), central memory (T$_{CM}$), and effector memory (T$_{EM}$) CD4⁺ T cells and screened using the recall antigens TT, CMV, and PPD (each at 5 µg/ml). After 3 d, proliferation was measured after 16-h pulse with [³H]thymidine. Dotted lines represent the cut-off value. Numbers indicate of positive cultures over the total cultures tested. Results are representative of six

were either unpulsed or pulsed with the cognate antigen for 5 d to enrich for specific cells, and then stained with antibodies to CD25 and ICOS. In antigen-stimulated cultures, but not in cultures stimulated with monocytes alone, a sizable proportion of T cells up-regulated ICOS and CD25 (Fig. 2 A). ICOS⁺CD25⁺ T cell blasts were sorted and cloned by limiting dilution. Antigen-specific T cell clones could be identified at frequencies ranging from 25 to 93% of the growing clones (Fig. 2 B). In all cases analyzed, Vβ usage was identical among antigen-specific T cell clones derived from the same polyclonal culture, consistent with the presence of a single antigen-specific T cell precursor in the original culture (Table S1). TCR Vβ CDR3 sequencing performed on T cell clones derived from three additional KLH-reactive T cell lines demonstrated that all clones derived from the same line carried identical Vβ CDR3 sequences (unpublished data). These data demonstrate that using amplified T cell libraries it is possible to isolate, in two culture steps, T cell clones that represent the spectrum of antigen-specific cells present in the human naive T cell repertoire.

Fine specificity and antigen dose–response of specific CD4⁺ naive T cells

We next asked whether the polyclonal cultures containing a single antigen-specific cell could be directly used to analyze fine specificity and antigen dose–response of specific T cells. In a first series of experiments, we selected 6 PA-reactive polyclonal cell cultures derived from a naive T cell library and tested them for their capacity to recognize overlapping 15-mer peptides covering the entire PA sequence. For all cultures, it was possible to identify a peptide pool and, eventually, a single peptide that was specifically recognized (Fig. 3 A and Table S2).

In a second series of experiments, we selected several KLH-reactive polyclonal T cell cultures from six different donors and measured their proliferation to varying concentrations of KLH in the presence of autologous monocytes. As shown in Fig. 3 B, the dose–response curves of individual cultures to KLH showed a typical sigmoid shape. EC50 values, i.e., the concentrations of KLH that induced 50% of maximum proliferation, were calculated from the interpolated curves. For T cells that did not reach a plateau value even at the highest antigen concentration tested, we arbitrarily set a maximum response value of 10⁵ cpm and calculated EC50 values accordingly. As shown in Fig. 3 C, EC50 values varied considerably over a 100-fold range, with some cultures showing low values (EC50

independent experiments performed with six different donors. (B) Frequencies of TT-, CMV-, and PPD-specific T cells in naive and memory compartments in six independent experiments performed with six different donors. Results are presented as precursor frequency per million CD4⁺ T cells. (C and D) Libraries were prepared from naive and memory T cells from four different donors and screened with KLH. Shown are cpm values from a representative experiment performed with a single donor and is representative of four independent experiments performed with four different donors (C) and precursor frequencies for the four different donors (D). Dotted line represents the cut-off value.

< 10 µg/ml) and a few cultures showing much higher values (EC50 > 100 µg/ml).

KLH is a large protein complex, and bias in antigen processing and presentation on different MHC class II molecules may affect the estimates of protein concentrations needed to

achieve 50% proliferative response. We therefore analyzed a panel of CD8$^+$ T cell clones derived from naive T cells specific for the Melan-A/MART-1 peptide presented on the HLA-A02 molecule. The dose-response curves varied widely, with EC50 values ranging from 10^2 to 10^{-2} ng/ml (Fig. S1 D).
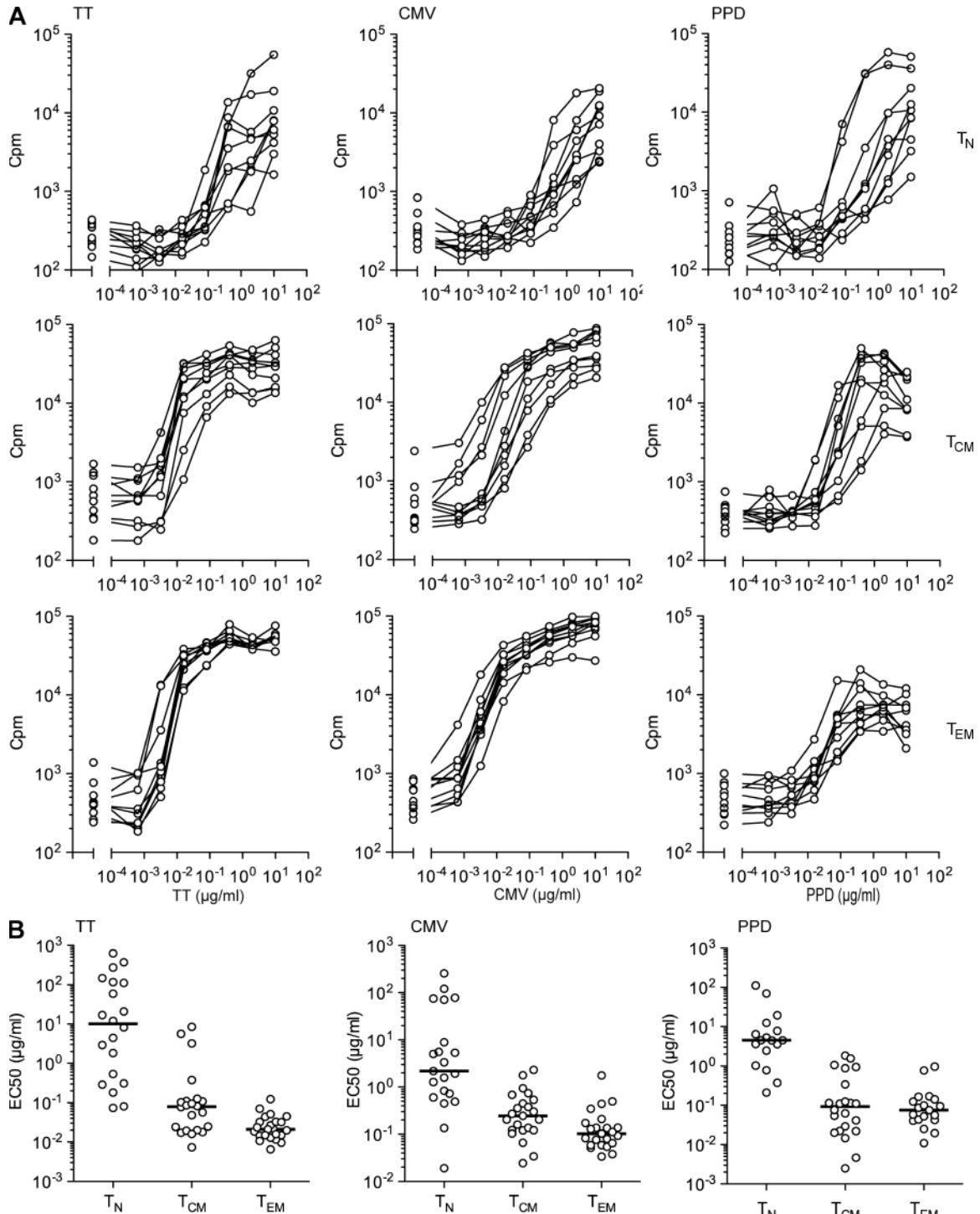

Figure 5. T cells responding to low antigen concentrations are enriched in the memory compartments. (A) Dose-response curves of TT-, CMV-, and PPD-reactive T cell cultures from T_N, T_{CM}, and T_{EM} libraries of a single donor. Values on the left of each plot represent background proliferation in the absence of antigen. Shown are 10 representative cell lines for each library. (B) EC50 values for CMV-, TT-, and PPD-specific T cell cultures derived from different T cell subsets. Bars indicate median values. Data are representative of four independent experiments.

These results indicate that naive CD8$^+$ T cells specific for a single peptide-MHC combination show a broad range of functional avidities. Collectively, these results indicate that the polyclonal cultures that can be readily identified in libraries of amplified naive T cells are sufficiently enriched in antigen-specific cells to allow studies of functional characterization as far as specificity and antigen dose–response.

Comparative analysis of naive and memory CD4$^+$ T cell repertoires

The amplified library method offers the possibility to directly compare the repertoires of naive and memory T cells. With this aim, we prepared libraries of naive (T_N), central memory (T_{CM}; CD4$^+$CD45RA$^-$CCR7$^+$CD25$^-$), and effector memory (T_{EM}; CD4$^+$CD45RA$^-$CCR7$^-$CD25$^-$) T cells. The naive T cell libraries consisted of 384 cultures, each containing 2,000 cells, whereas the T_{CM} and T_{EM} libraries consisted of 192 cultures each containing 1,000 cells. The lower input of T_{CM} and T_{EM} cells was chosen on the basis of preliminary experiments, indicating a high frequency of T cells specific for recall antigens in the memory pools. T_{CM} and T_{EM} cells expanded efficiently upon polyclonal activation, although T_{EM} cells proliferated to a lower extent compared with T_{CM} cells (unpublished data), a finding that is consistent with their highly differentiated state.

The cpm values of individual cultures stimulated with recall antigens (TT, CMV, and PPD) are shown, for one donor, in Fig. 4 A. This analysis was performed on 5 additional donors and frequency data are shown in Fig. 4 B. TT-, CMV-, and PPD-specific T cells were present in the naive repertoire at frequencies ranging from 10 to 170 per 10^6 CD4$^+$ naive T cells; they were present at much higher frequencies (up to >10,000 per 10^6 CD4$^+$ memory T cells for complex antigens such as CMV) in the memory subsets. With a single exception, frequencies of TT- and CMV-specific T cells were higher in T_{EM} compared with T_{CM} compartment, whereas the frequency of PPD-specific T cells was consistently higher in T_{CM} compared with T_{EM} subset. Remarkably, in two CMV-seronegative donors, CMV-specific T cells were detectable in the naive compartment, but not in the memory compartment (Fig. 4 B). This is consistent with the fact that for these donors, CMV, like KLH, was a primary antigen.

Interestingly, in four donors tested, proliferation to KLH was observed only in cultures derived from naive cells, but not in cultures derived from T_{CM} and T_{EM} cells (Fig. 4, C and D). The finding that in nonimmune donors antigen-specific T cells could be detected in the naive but not in the memory compartment is consistent with the high diversity of the former and the much lower diversity of the latter, which represents a collection of clones selected during the individual's immune response. Previous studies on heterologous immunity demonstrated that, in some cases, memory T cells may contain cells specific for primary antigens (Selin et al., 2006). Our analysis suggests that this may be a relatively rare event and that detection of such cells may require screening of large T cell libraries.

T cells responding to low antigen doses are overrepresented in the memory T cell compartments

The aforementioned results demonstrate that antigen-specific proliferating T cells are present at increased frequencies in the T_{CM} and T_{EM} compartments of primed individuals, a finding that is consistent with the notion that naive T cells undergo clonal expansion after antigenic stimulation (Jenkins et al., 2001; Kaech and Ahmed, 2001). To ask whether this process would also result in selection of T cells with high responsiveness to antigenic stimulation, we compared cultures from the naive, T_{CM}, and T_{EM} cell libraries for their capacity to proliferate in response to increasing doses of antigen (Fig. 5 A) and calculated EC50 values (Fig. 5 B). TT-specific naive T cells showed a very broad range of EC50 values ranging from 0.07 to 630 µg/ml (median 10 µg/ml). In contrast, TT-specific memory T cells had much lower EC50 values and showed a narrower distribution compared with naive T cells, with TT-specific T_{CM} cells ranging from 0.007 to 8.5 µg/ml (median 0.08 µg/ml) and TT-specific T_{EM} cells ranging from 0.006 to 0.12 µg/ml (median 0.02 µg/ml). A similar trend with T_{CM} and, even moreso, T_{EM} cells showing lower EC50 values than naive T cells was observed for CMV and PPD, although in these cases the interpretation of the results is complicated by the fact that these antigens contain multiple proteins, each present at different concentrations. Collectively, these results suggest that high avidity T cells are selectively enriched in T_{CM} and T_{EM} memory subsets.

In mice, it has been demonstrated that memory T cells are skewed toward a higher avidity over time, a process that is dependent on the initial strength of antigenic stimulation and on competition for antigen-presenting DCs (Kedl et al., 2000; Williams et al., 2008). The mechanisms that lead to a preferential representation of high avidity T cells in the central and, especially, the effector memory repertoires are not known. One possibility is that only high avidity, but not low avidity, T cells are primed after antigenic stimulation in vivo. The other possibility is that both high- and low-avidity T cells are initially recruited into the immune response and that the pool of T cells subsequently matures in affinity owing to a more prolonged expansion of high-avidity T cell clones, as recently demonstrated for mouse CD8$^+$ T cells (Zehn et al., 2009).

In conclusion, this paper describes a simple and reproducible method that allows a detailed characterization of the spectrum of antigen-specific T cells in the human naive CD4$^+$ T cell repertoire and that can be adapted to study the repertoire of memory T cell subsets. This method offers considerable advantages both in terms of applicability and flexibility. First, it can be used to test antigens of any source and chemical nature and even of great complexity, such as viruses, bacteria, and parasites. Second, the method detects T cells that respond to naturally processed antigens and it is suitable to identify antigenic epitopes within a given protein. Third, it allows detection of antigen-specific T cells over an extremely broad range of functional avidities.

The amplified T cell library method may find broad application for identification of immunogenic sites in vaccines,

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therapeutic proteins, and self-proteins and to predict antigenicity of drugs, chemicals, and cosmetics. When combined with a specific in vitro priming regimen, this method may be suitable to generate effector or regulatory CD4^{+} T cells of known specificities that may be used for adoptive cellular immunotherapies (Hunder et al., 2008). Finally, it can be used to monitor the dynamics of immune responses upon infection or vaccination and to evaluate immunocompetence of the elderly.

MATERIALS AND METHODS

Cell purification and sorting. Buffy coats were obtained from the Basel Swiss Blood Center. Cord blood samples were obtained after informed consent from the San Giovanni Hospital (Bellinzona). Human primary cell protocols were approved by the Federal Office of Public Health (N. A000197/2 to F. Sallusto). CD14^{+} monocytes and CD4^{+} T cells were isolated from PBMC or cord blood samples by positive selection with antibody-coated microbeads (Miltenyi Biotec). CD4^{+} T cell subsets were sorted to 99% purity on a FACSAria (BD) after staining with FITC-labeled anti-CD45RA (ALB11; Immunotech), phycoerythrin-cyanine 5 (PC5)–labeled anti-CD25 (B1.49.9; Immunotech), allophycocyanin (APC)-labeled anti-CD4 (13B8.2; Immunotech), and anti-CCR7 (150503; R&D Systems), followed by staining with biotinylated anti-IgG2a (SouthernBiotech) and streptavidin–Pacific blue (Invitrogen). Naive CD8^{+} T cells were isolated from HLA-A02^{+} donors over 99% purity on a FACSAria as CD45RA^{+}CD62L^{+}CD8^{+} after staining with FITC-labeled anti-CD45RA, PC5-labeled anti-CD62L (DREG56; Immunotech), and APC-labeled anti-CD8 (B9.11; Immunotech), excluding CD4^{+}, CD19^{+} (J3-119), and CD56^{+} (N901) contaminating cells (all PE-conjugated from Immunotech). In some experiments, sorted naive CD8^{+} T cells were stained with r-PE–conjugated Melan-A/MART-1 pentamers (A*0201/ELAGIGILTV; Proimmune).

Amplified T cell libraries. Medium used throughout the experiments was RPMI 1640 supplemented with 2 mM glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate, penicillin (50 U/ml) and streptomycin (50 µg/ml), and 5% human serum (Swiss Red Cross). T cells (from 250 to 2,000 cells/well) were stimulated polyclonally with 1 µg/ml PHA (Remel) in the presence of irradiated (45Gy) allogeneic feeder cells (2.5×10^4 per well) and IL-2 (500 IU/ml) in a 384-well plate format. After 5 d, cells were transferred in 96–well U-bottom plates, pooling 2 wells of the initial plate. 2 d later, cells were split in 48-well format, expanded, and after 3 d transferred in 24–well plates. Library screening was performed at day 14 after initial stimulation by culturing ∼2.5×10^5 T cells/well with autologous monocytes (2.5×10^4), which were either unpulsed or pulsed for 3 h with different antigens including KLH (Calbiochem), PA from *B. anthracis* (LIST), thyroglobulin (Lee Biosolutions, Inc), TT (provided by G. Galli, Novartis Vaccines, Siena, Italy), CMV grade 2 antigen (Microbix Biosystems), PPD from *M. tuberculosis* (Statens Serum Institute), and *Dermatophagoides pteronyssinus* major allergen I (provided by E.L. Roggen, Novozymes, Bagsvaerd, Denmark). Proliferation was measured on day 4 after 16 h incubation with 1 µCi/ml [³H]thymidine (GE Healthcare). Cultures that scored positive in the first screening assay were always reanalyzed to confirm their specificity in a second independent experiment. Precursor frequencies were calculated based on numbers of negative wells according to the Poisson distribution and expressed per million cells (Lefkovits and Waldmann, 1979). The 95% confidence intervals were determined for each dataset according to the modified Wald method (Agresti and Coull, 1998). EC50 values were determined from interpolated dose–response curves. For all calculations, the bottom of the curve was defined as ≥100 cpm and the top was restricted to ≤10^5 cpm. In preliminary experiments, we found that the dose–response curve and EC50 value of individual T cell lines were reproducible in different experiments performed at different time points.

T cell clones. T cell lines were stimulated for 5 d with antigen-pulsed monocytes. After 5 d, cells were stained with antibodies to CD25 and ICOS (biotinylated-ISA-3; eBioscience) followed by staining with streptavidin-

APC (Invitrogen). CD25^{+} ICOS^{+} T cells were sorted and cloned by limiting dilution, as previously described (Messi et al., 2003). Melan-A/MART-1 pentamers^{+} CD8^{+} T cells were sorted directly sorted from PBMC and cloned by single-cell deposition, as previously described (Messi et al., 2003). CD8^{+} T cell clones were stained with PE-labeled Melan-A/MART-1 pentamers and FITC-labeled anti-CD8. The clones were also stimulated with 2.5×10^4 autologous CD14^{+} monocytes after a 3-h pulse with different concentrations of Mart-1/Melan-A peptide.

Vβ analysis. T cell lines and clones were characterized using a TCR Vβ repertoire kit (Beckman Coulter) containing 24 different Vβ antibodies. For Vβ fragment analysis, total RNA was isolated from T cells using the RNeasy kit (QIAGEN). PCR for Vβ analysis of T cell clones was performed using the following as forward primers: Vβ2 5′-TCATCAACCATGCAAGC-CTGACCT-3′, Vβ9 5′-CCTAAATCTCCAGACAAAGCT-3′, and Vβ14 5′-GTCTCTCGAAAAGGAAGAGGAAT-3′; 5′-TGCTGACCCCACT-GTCGACCTCCTCCCATT-3′ was used as a reverse primer. Vβ fragments were amplified using the PCR TCRB Gene Clonality Assay (Invivoscribe) and sequenced.

Online supplemental material. Fig. S1 shows maintenance of the initial TCR Vβ repertoire during polyclonal expansion. It also shows reproducibility of frequency measurements at two time points and the interrogation of a T cell library using multiple antigens in parallel. Fig. S2 shows a comparison of frequency measurements of Melan-A/MART-1 naive CD8^{+} T cells using MHC class I multimers or T cell libraries, as well as EC50 values of different naive Melan-A/MART-1–specific T cell clones. Table S1 shows that the antigen-specific T cell clones isolated from the same T cell culture and shown in Fig. 2 B express the same TCR Vβ chain. Table S2 shows the results of the analysis using overlapping peptides covering the entire PA sequence of the epitope specificity of the PA-specific T cell cultures shown in Fig. 3 A. Online supplemental material is available at http://www.jem .org/cgi/content/full/jem.20090504/DC1.

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