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Frequency of Epitope-Specific Naive CD4+ T Cells Correlates with Immunodominance in the Human Memory Repertoire

William W. Kwok,* Venus Tan,* Laurie Gillette,† Christopher T. Littell,† Michele A. Soltis,† Rebecca B. LaFond,* Junbao Yang,* Eddie A. James,* and Jonathan H. DeLong*

The frequency of epitope-specific naive CD4+ T cells in humans has not been extensively examined. In this study, a systematic approach was used to examine the frequency of CD4+ T cells that recognize the protective Ag of Bacillus anthracis in both anthrax vaccine-adsorbed vaccinees and nonvaccinees with HLA-DRB1*01:01 haplotypes. Three epitopes were identified that had distinct degrees of immunodominance in subjects that had received the vaccine. Average naive precursor frequencies of T cells specific for these different epitopes in the human repertoire ranged from 0.2 to 10 per million naive CD4+ T cells, which is comparable to precursor frequencies observed in the murine repertoire. Frequencies of protective Ag-specific T cells were two orders of magnitude higher in immunized subjects than in nonvaccinees. The frequencies of epitope-specific memory CD4+ T cells in vaccinees were directly correlated with the frequencies of precursors in the naive repertoire. At the level of TCR usage, at least one preferred Vβ in the naive repertoire was present in the memory repertoire. These findings implicate naive frequencies as a crucial factor in shaping the epitope specificity of memory CD4+ T cell responses. *The Journal of Immunology, 2012, 188: 2537–2544.*

The frequency of epitope-specific T cells within the naive repertoire is thought to play an important role in shaping the complexity and diversity of the memory repertoire and the specificity of adaptive immune responses to infectious pathogens or vaccinations. As such, knowledge of naive T cell frequencies is an important research goal. Over the past 15 yr, significant discoveries have been made by studies utilizing indirect assays such as adoptive transfer of TCR transgenic T cells in mouse models (1–3) or sequencing the CDR3 regions of Ag-specific T cells (4). The development of MHC-tetramer reagents provided an additional tool capable of detecting Ag-specific T cells in a direct fashion (5, 6). Additionally, the subsequent development of an enrichment protocol for tetramer-positive T cells was necessary to enable the detection of epitope-specific naive T cells, which are typically present at very low frequencies (7, 8). Moon et al. (9) first used this tetramer enrichment approach to examine the frequency of epitope-specific naive CD4+ T cells in mice. Their results suggest that there are 20–200 naive CD4+ T cells for specific antigenic epitopes in the mouse T cell repertoire. Using a similar approach, Obar et al. and Kotturi et al. (10, 11) estimated the number of epitope-specific naive CD8+ T cells to be between 15 and 1200 cells per mouse. Most attempts to estimate the frequencies of epitope-specific naive T cells have been carried out in mice; however, two recent studies in human subjects indicated that the frequency of epitope-specific CD8+ T cells ranges from 0.6 to 500 per million CD8+ T cells (12, 13). Another study examined the frequency of CD45RA+ DRB1*04-restricted influenza A hemagglutinin (HA)306–319-specific T cells in human subjects, and reported an average frequency of 6 per million CD4+ T cells (14). However, most human subjects have been repeatedly exposed to influenza A, and it has been suggested that memory CD4+ T cells can revert to a CD45RA+ phenotype as a consequence of repeated stimulation (15). Therefore, examining influenza A-specific T cell frequencies in adults may not be the most appropriate experimental approach to assess epitope-specific naive T cell frequencies in human subjects.

In the current study, protective Ag (PA) of Bacillus anthracis was chosen as a model Ag for studying the frequency of epitope-specific T cells in naive subjects and vaccinees. PA is a major component of the anthrax vaccine adsorbed (AVA) (16, 17). The vast majority of healthy subjects have had no exposure to this Ag. However, military personnel are typically exposed to this Ag through multiple AVA vaccinations as a precaution against the potential release of anthrax spores in an act of biological warfare (18). With blood samples from AVA vaccinees, the tetramer-guided epitope mapping (TGEM) approach was used to identify CD4+ T cell epitopes within the PA of B. anthracis (19, 20). The PA-specific tetramers were subsequently used to examine the frequency of PA-specific T cells in both healthy vaccinees and nonvaccinees. Direct ex vivo tetramer staining indicated that the average frequency of naive epitope-specific T cells ranged from 0.2 to 10 per million naive CD4+ T cells. The naive frequencies of epitope-specific CD4+ T cells also correlated with the frequency of memory T cells observed in vaccinees.

Materials and Methods

Subject recruitment

AVA vaccinees were recruited with informed consent as part of a study approved by the Institutional Review Boards of both the Benaroya Research Institute and Madigan Army Medical Center. Subjects received the AVA...
vaccine during 2008, 2009, and 2010, and were recruited and sampled within 1 y of receiving a final vaccine dose or annual booster. Unvaccinated control subjects were recruited with informed consent from the Benaroya Research Institute. DRB1* HLA alleles were typed by PCR using Dyanal Unitary SSP kits (Invitrogen, Carlsbad, CA), and those subjects that carried DRB1*01:01 (DR0101) were selected for further study.

PA peptides and class II tetramers
A total of 94 overlapping peptides that cover the entire sequence of PA of *B. anthracis* was synthesized by Mimotopes (Clayton, Australia). Each peptide was 20 aa in length, with a 12-aa overlap. Peptides were divided into 19 pools, with 5 peptides in each pool. Recombinant DR0101 protein was purified from Schneider S2 transfectants using L.243 affinity column chromatography. The protein was subsequently biotinylated with Bir A (Avidita, Auroville, CA), according to the manufacturer’s protocol. Peptide pools at 20 mg/ml were loaded onto biotinylated DR0101 monomer at 1 mg/ml for 48 h at pH 6 in 100 mM sodium phosphate and 0.2% γ- octyl-B-β-glucopyranoside (Sigma-Aldrich, St. Louis, MO). These peptide-loaded monomers were subsequently cross-linked with PE-labeled streptavidin (Invitrogen, Carlsbad, CA) for at least 4 h at room temperature to produce pooled peptide tetramers (21). Single peptide PE-labeled tetramers were produced in similar fashion by loading DR0101 monomer with each individual PA peptide from positive peptide pools. Single peptide allopbyocyanin-labeled tetramers were produced with allopbyocyanin-labeled streptavidin (Invitrogen).

**TGEM**
PBMC were isolated from heparinized blood by Ficoll (GE Healthcare, Chalfont St. Giles, U.K.) underlay. CD4+ T cells were isolated by “no touch” CD4+ magnetic isolation (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD4+ fraction was used as APC by plating 3 × 10^6 APC in 1 ml complete medium (RPMI 1640 supplemented with 10% human pooled serum, 1% pen/strep, and 1% L-glutamine) in 48-well plates. Nonadherent cells were washed away, and each well was seeded with 2.5 × 10^4 CD4+ T cells. Cells in each individual well were stimulated with a peptide pool for 14 d. Cells were washed using fresh T cell medium and 20 U/ml IL-2 (Hemagen, Columbia, MD) as needed. On day 14, one-tenth of the cells (∼75,000) from each well was stained with the corresponding pooled tetramers and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Diego, CA). Cells from tetramer-positive wells were subjected to a second staining using tetramers loaded with individual peptides within the corresponding positive peptide pools (19).

**Direct ex vivo staining of T cells with tetramer**
The anti-PE magnetic bead enrichment protocol was used to detect and determine the frequency of PE tetramer-positive CD4+ T cells. In brief, 30–200 million PBMC in a volume of 200–600 μl were stained with 10 μg/ml PE tetramer at room temperature for 2 h. PA_{713-732} -reactive T cell clones were obtained by single cell sorting (DR0101/PA_{713-732} -tetramer-positive cells), followed by expansion in the presence of PHA and irradiated allogeneic PBMC. RNA was extracted from approximately 1 million T cells of each clone using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, and eluted in 32 μl diethyl pyrocarbonate-treated water (Ambion, Foster City, CA). Reverse transcription was performed with 8 μl total RNA using the SuperScript III First-Strand synthesis kit (Invitrogen) and random hexamers according to the provided protocol. To determine the identity of the TCR Vβ V region for each clone, the synthesized cDNA was used in a multiple PCR assay, as described by Akatsuka et al. (23). The PCR for each clone that contained a major band was purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer’s protocol. After purification, each major PCR product was subjected to full automated sequencing on the ABI Prism 3100 Genetic Analyzer using the Big Dye v3.1 DNA sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed in both directions using the antisense primer from the TCR Vβ C region and the sense primer from the TCR Vβ V region determined by PCR band visualization, as described above. To confirm the TCR Vβ identity of each major band, consensus sequences determined with the Contig Assembly Program were aligned with the known TCR Vβ sequence using the Clustal W multiple alignment program. The products from the multiplex PCR assay also include the CDR3 region.

**Online supplemental material**
Supplemental Fig. 1 shows the use of TGEM for the identification of DR0101-restricted PA-reactive epitopes. Supplemental Table I shows the specificity of PA-reactive T cells isolated from nonvaccinees by proliferation assays. Supplemental Fig. 2 shows control tetramer staining of PBMC from a representative DR0101-negative individual and PBMC that had already been depleted of Ag-specific cells. Supplemental Fig. 3 shows a representative titration experiment to illustrate the limit of detection for the PE-enrichment methodology.

**Results**
**PA-specific memory T cells in AVA vaccines**
TGEM was used to identify HLA-DRA*01:01/DRB1*01:01 (DR0101)-restricted PA-specific CD4+ T cell epitopes using peptide-stimulated PBMC from DR0101 AVA vaccines, as described previously (19, 20). Tetramer staining indicated that PA_{93-142}, PA_{99-142}, PA_{505-524}, and PA_{713-732} contain DR0101-restricted T cell epitopes (Table I, Supplemental Fig. 1). Similar experiments were carried out using samples from two other DR0101 AVA subjects, and identical results were obtained. An in vitro binding assay indicated that these three peptides bound to DR0101 with high affinity (Table I). Given that the PA_{99-142} and PA_{99-142} peptides overlap by 12 aa, these regions are likely to contain a single epitope within overlapping region PA_{99-142}. Indeed, as shown in Table I, these two peptides share the same putative core binding region as determined by the ProPred prediction algorithm (24). Based on these results, all subsequent experiments focused on PA_{99-142}, PA_{505-524}, and PA_{713-732}.

To confirm the relevance of these epitopes, DR0101/PA_{99-142}, PA_{505-524}, and PA_{713-732} tetramers were used to detect PA-specific CD4+ T cells in DR0101 AVA vaccines directly ex vivo through the anti-PE bead enrichment approach (7, 8). Representative data of CD4+ T cells in the sample (10 times the number of CD4+ T cells in the precolumn fraction) and multiplying this number by 1 million (7, 20).

**Peptide-binding competition assay**
Various concentrations of each peptide were incubated in competition with 0.01 μM biotinylated HA_{306-319} peptide in wells coated with HLA-DR0101 protein essentially as previously described (22). After washing, the biotin-HA peptide was labeled using europium-conjugated streptavidin (PerkinElmer) and quantified using a Victor2 D time-resolved fluorometer (PerkinElmer). Peptide-binding curves were simulated by nonlinear regression with Prism software (version 4.03; GraphPad Software) using a sigmoidal dose-response curve. IC50-binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of reference peptide binding.

**TCR Vβ analysis of anthrax-reactive T cells**
PA_{713-732} -reactive T cell clones were obtained by single cell sorting DR0101/PA_{713-732} -tetramer-positive cells, followed by expansion in the presence of PHA and irradiated allogeneic PBMC. RNA was extracted from approximately 1 million T cells of each clone using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, and eluted in 32 μl diethyl pyrocarbonate-treated water (Ambion, Foster City, CA). Reverse transcription was performed with 8 μl total RNA using the SuperScript III First-Strand synthesis kit (Invitrogen) and random hexamers according to the provided protocol. To determine the identity of the TCR Vβ V region for each clone, the synthesized cDNA was used in a multiple PCR assay, as described by Akatsuka et al. (23). The PCR for each clone that contained a major band was purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer’s protocol. After purification, each major PCR product was subjected to full automated sequencing on the ABI Prism 3100 Genetic Analyzer using the Big Dye V3.1 DNA sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed in both directions using the antisense primer from the TCR Vβ C region and the sense primer from the TCR Vβ V region determined by PCR band visualization, as described above. To confirm the TCR Vβ identity of each major band, consensus sequences determined with the Contig Assembly Program were aligned with the known TCR Vβ sequence using the Clustal W multiple alignment program. The products from the multiplex PCR assay also include the CDR3 region.
Table I. PA antigenic epitopes and their binding affinities to DR0101

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Peptide Sequence</th>
<th>IC50</th>
</tr>
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<tbody>
<tr>
<td>PA303–412</td>
<td>IRYVNTGTPAIXNVLPTTSL</td>
<td>ND</td>
</tr>
<tr>
<td>PA401–420</td>
<td>APIYNVLPFTSLVLEKNQTL</td>
<td>IC50 0.39 μM</td>
</tr>
<tr>
<td>PA505–524</td>
<td>NWSEVLFIQETTARIGFNG</td>
<td>IC50 0.22 μM</td>
</tr>
<tr>
<td>PA713–732</td>
<td>KLPFLISNPYKIVVIAVT</td>
<td>IC50 0.20 μM</td>
</tr>
</tbody>
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Putative MHC II binding regions are shown in bold.

from one subject are shown in Fig. 1A. For all three peptide specificities, PA-specific CD4+ T cells exhibited a CD45RA- phenotype, indicating that these were memory T cells. The observed memory phenotype of these cells suggests that the peptides containing the epitopes are likely to be naturally processed. In this vaccinee, PA713–732-reactive CD4+ T cells were observed at a frequency of 1000 per million total CD4+ T cells. PA401–420- and PA505–524-specific CD4+ T cells were observed at lower frequencies, 63 and 22 per million, respectively. These results indicate that PA713–732 was the most immunodominant epitope in this subject, followed by PA401–420 and PA505–524, which could be considered subdominant epitopes (Fig. 1A). As shown in Fig. 1B, the hierarchy of frequencies observed in T cells specific for the three epitopes were similar in all of the DR0101 AVA vaccinees examined. Furthermore, a similar hierarchy was observed when the experiments were repeated for these four DR0101 subjects at a time point 3 mo later (data not shown). Ex vivo staining with Abs against additional surface markers of CD4+ T cells specific for the three epitopes indicated that most of these cells express CCR4, in agreement with a previous report (20), and that the population is heterogeneous for CD27 expression (Fig 1C, data not shown).

PA-specific naive T cells in non-AVA vaccinees

To interrogate the naive repertoire, the anti-PE bead enrichment protocol was applied to determine the frequency and phenotype of PA713–732-reactive CD4+ T cells in non-AVA vaccinees. Initial experiments focused on PA713–732, the most immunodominant CD4+ T cell epitope in vaccinated subjects. Representative staining for one of these unvaccinated subjects is shown in Fig. 2. Tetramer staining in the CD3+ cell population was specific for CD4+ cells, with no nonspecific staining detected in the CD8+ population (Fig. 2A, 2B). The frequency of PA713–732-specific T cells for this subject was determined to be 6 per million total CD4+ T cells. To confirm the specificity of tetramer staining, PBMC from unvaccinated subjects were costained with the PE-conjugated tetramer and with a tetramer that is labeled with a dye not used for enrichment (allophycocyanin). Whereas the observed frequency of PA713–732-specific CD4+ T cells in this particular staining was slightly lower (3 per million total CD4+ T cells), >85% of the PE tetramer-labeled cells were also allophycocyanin tetramer positive (Fig. 2C). In addition, PMBC from non-DR0101 subjects and previously depleted PBMC from DR0101 subjects were stained to further validate the sensitivity and specificity of ex vivo tetramer staining (Supplemental Fig. 2). The surface phenotype of PA713–732-specific T cells in non-AVA vaccinees was also characterized by direct ex vivo staining. Typically, at least 80% of the tetramer-positive cells were CD45RA- because these subjects were neither vaccinated with AVA nor naturally exposed to B. anthracis, the surface phenotype suggests that these epitope-specific CD4+ T cells are naive. In contrast to the anthrax-specific memory T cells from vaccinees (Fig. 1C), PA713–732-specific cells in non-AVA vaccinees were exclusively CD27- positive and predominantly CCR4+ (Fig. 2E, 2F). Ag specificity of PA713–732 tetramer-positive T cells was confirmed by single cell sorting PE tetramer-positive T cells from PBMC of three different subjects. After nonspecific expansion, the clones were stained with DR0101/PA713–732 tetramer. Of 96 T cell clones examined, 90 could be clearly stained by tetramer following expansion (data not shown). Ag specificity for 20 of these clones was...
Further corroborated with proliferation assays using DR0101 PBMC as APCs. Eighteen of 20 clones showed specific proliferation in response to PA713–732 peptide (Supplemental Table I).

In addition to CD4+ T cells specific for PA713–732, PA401–420-specific T cells could also be detected in PBMC from unvaccinated subjects. The specificity of this staining was confirmed by double staining T cells with both PE-conjugated and allophycocyanin-conjugated DR0101/PA401–420 tetramers (Fig. 3A). The frequency of DR0101/PA401–420-specific CD4+ T cells in the representative subject shown was determined to be 1.3 per million total CD4+ T cells. The majority of the tetramer-positive T cells were also CD45RA positive (Fig. 3B). As observed for PA713–732-specific naïve T cells, the vast majority of PA401–420-specific cells were CD27+ and CCR4+ (data not shown). Detecting DR0101/PA505–524-specific T cells was more difficult because the signal was relatively weak and infrequent. Despite their low frequency, DR0101/PA505–524-specific T cells could be double stained with PE-conjugated and allophycocyanin-conjugated tetramers, confirming the specificity of the staining. Based on this staining, the frequency of DR0101/PA505–524-specific T cells of the subject as shown was determined to be 0.2 per million total CD4+ T cells (Fig. 3C). However, this staining is close to the limit of detection for our methodology, which (based on empirical observations) is estimated to be 0.2 per million total CD4+ T cells with 200 million PBMC (see Supplemental Fig. 3). Therefore, these frequencies should be considered to be approximate. Because of the limited number of events, we were unable to determine the surface phenotype of DR0101/PA505–524-specific T cells.

Equivalent ex vivo tetramer-staining experiments were completed for a total of 11 unvaccinated DR0101 subjects. PA713–732-

**FIGURE 3.** Frequencies of DR0101-restricted PA401–420-reactive T cells and PA505–524-reactive T cells in DR0101 nonvaccinees. (A) PBMC from a DR0101 subject were stained with DR0101/PA401–420 PE tetramers, CD4 Ab, and gating Abs (anti-CD3, anti-CD14, and anti-CD19) ex vivo. The frequency of PA401–420-specific T cells was determined to be 1.3 per million total CD4+ T cells. (B) PBMC were stained with DR0101/PA401–420 PE tetramers and CD45RA Ab, and gating Abs ex vivo and were then enriched. The frequency of PA401–420-specific T cells was determined to be 0.5 per million total CD4+ T cells. (C) PBMC from a DR0101 subject were stained with DR0101/PA505–524 PE tetramers and DR0101/PA505–524 allophycocyanin tetramers, and gating Abs ex vivo and were then enriched. The frequency of PA505–524-specific T cells was determined to be 0.2 per million total CD4+ T cells. Each plot represents staining from a different DR0101 subject. For each panel (A–C), we gated on forward and side scattering and then on cells that were Via-Probe+, CD4+, CD14+, and CD19+. Data shown in (A) and (B) are representative of >11 experiments in 11 subjects, and the data shown in (C) are representative of data from 7 different subjects.
and PA$_{401-420}$-specific T cells were detected in every subject examined. Because PA$_{505-524}$ staining required a much larger sample volume for analysis (200 million PBMC), ex vivo tetramer staining was repeated for a total of 7 subjects. For these, detectable staining was observed in only 3 subjects, as staining was below the threshold of detection for the other 4 subjects. As summarized in Fig. 4, PA$_{713-732}$-specific T cells were most frequent, averaging 5 per million total CD4$^+$ T cells or $\sim$10 per million naive CD4$^+$ T cells, followed by PA$_{401-420}$-specific T cells, averaging $\sim$1 per million total CD4$^+$ T cells or $\sim$2 per million naive CD4$^+$ T cells, and PA$_{505-524}$-specific T cells were seen at no more than 0.4 cells per million naive CD4$^+$ T cells. These results suggest a hierarchy of frequencies for these three epitopes in the naive T cell repertoire that corresponded to the relative immunodominance of these specificities in the expanded CD4$^+$ T cell repertoire of DR0101 AVA vaccinees.

TCR V$\beta$ usages of naive and memory PA$_{713-732}$-specific T cells

PA$_{713-732}$-reactive T cell clones were isolated from three unvaccinated subjects by tetramer staining and single cell sorting. The TCR V$\beta$ regions of these naive PA$_{713-732}$-reactive T cells were sequenced, and as described in Materials and Methods (Table II). TCR V$\beta$ sequences indicated that each of the T cell clones has a different clonotype. Although a limited number of clones was sequenced, the data indicated that TCR V$\beta$ 13.6 and V$\beta$ 15 genes were preferentially selected for the recognition of DR0101/PA$_{713-732}$ (seen in 6 of 23 and 5 of 23 clones, respectively) among the naive repertoire of these subjects. Similarly, the TCR V$\beta$ gene usage of memory PA$_{713-732}$-specific CD4$^+$ T cells from three DR0101 AVA vaccinees was examined by co-tetramering PA$_{713-732}$ tetramer-positive T cells with TCR V$\beta$ 13.2 and TCR V$\beta$ 13.6 Abs. TCR V$\beta$ 15 was not included in this analysis because V$\beta$ 15 Ab is not currently available. Ab-staining results are summarized in Table III. Like the naive population, these PA$_{713-732}$-reactive memory T cells in vaccinees were enriched for TCR V$\beta$ 13.6 compared with the total CD4$^+$ T cell population (a paired Student $t$ test indicated a $p$ value of 0.04). Whereas the average percentage of PA$_{713-732}$-reactive memory T cells that used V$\beta$ 13.6 in AVA vaccinees (7%) was lower than the average percentage of V$\beta$ 13.6 PA$_{713-732}$-reactive naive T cells in unvaccinated subjects (26%), this discrepancy could be due in part to differences in the sensitivity of Ab-based measurement versus PCR-based quantitation.

**FIGURE 4.** Frequencies of DR0101-restricted PA-reactive T cells in DR0101 non-AVA-vaccinated subjects. Each symbol within each PA epitope represents staining for a different subject. Frequencies <0.1 per million were below the threshold of detection. The $p$ values were determined by using two-tailed unpaired $t$ tests.

**Discussion**

The primary objective of this study was to examine the frequency of epitope-specific naive CD4$^+$ T cells in the human T cell repertoire.

Because most healthy subjects in the United States have not been exposed to *B. anthracis*, the PA protein of *B. anthracis* provided an ideal model Ag for studying epitope-specific naive CD4$^+$ T cells in the general population. First, the TGEM approach was used to identify DR0101-restricted PA epitopes using samples from military personnel who had received the AVA vaccine. A total of three epitopes was consistently present in the T cell repertoire of these subjects. Among these, PA$_{713-732}$ was identified as the most immunodominant CD4$^+$ T cell epitope, with CD4$^+$ T cells specific for this epitope occurring at markedly higher frequencies (up to 1000 per million total CD4$^+$ T cells). T cell frequencies for the other two specificities (PA$_{401-420}$ and PA$_{505-524}$) were lower (average of 62 and 23 per million total CD4$^+$ T cells, respectively) in the subjects examined. However, all of these frequencies were comparable to those observed for prevalent influenza-specific CD4$^+$ T cells, which typically range from 30 to 1600 per million CD4$^+$ T cells (25). The surface phenotype of PA-specific T cells detected in AVA vaccinees was consistently CD45RA$^-$, CCR4$^+$, and CD27 heterogeneous, suggesting that these are central memory T cells (26). The ex vivo detection of these memory T cells in all subjects tested verifies their importance in the PA-specific response elicited by AVA vaccination.

These same tetramers allowed the visualization of PA-reactive T cells in healthy DR0101 non-AVA-vaccinated subjects. Whereas the majority of PA-reactive T cells in these nonexposed subjects were naive (CD45RA$^-$), $\sim$20% were observed in the CD45RA$^+$ population. One possible explanation for this, based on a study of the murine naive repertoire, is that naive T cells that undergo homeostatic proliferation can acquire a CD45RA$^-$ phenotype (27). A more likely explanation is that existing memory T cells in these unexposed subjects can cross-recognize PA epitopes presented by DR0101. The existence of cross-reactive memory T cells in nonexposed subjects is intriguing, because this implies that such cells could play a protective role, augmenting the naive response. This would suggest that individuals with less developed memory repertoires could have reduced protection against novel pathogens. As shown in Table IV, average naive T cell frequencies for all three epitopes were <10 per million total CD4$^+$ T cells, approximately two orders of magnitude lower than the stable frequencies of memory T cells in vaccinated subjects. It is possible that expansion of PA-specific T cells could have been even more robust if measured soon after vaccination. However, to draw firm conclusions about expansion kinetics, it would be necessary to assay longitudinal samples from subjects at multiple time points before and after immunization. As in subjects exposed to the vaccine, naive T cells specific for the immunodominant PA$_{713-732}$ epitope were most frequent, followed by PA$_{401-420}$ and PA$_{505-524}$.

Average naive precursor frequencies of PA-specific T cells in the human repertoire ranged from 0.2 to 10 per million naive CD4$^+$ T cells. Frequencies as high as 20 per million CD4$^+$ T cells were observed for the three PA epitopes combined. A recent study concluded that low-affinity (tetramer-negative) CD4$^+$ T cells can be prevalent during polyclonal responses, comprising up to half of the effector T cell population (28). Because the proportion of low-affinity naive CD4$^+$ T cells that are of low avidity is not clear, it is possible that tetramers underestimate the overall frequency by failing to detect low-affinity cells. However, Geiger et al. (29) described an approach for examining the diversity of the human naive CD4$^+$ T cell repertoire using polyclonal, expanded naive CD4$^+$ T cells, and determined frequencies of PA-specific T cells to be in the range of 10–26 per million naive CD4$^+$ T cells. Because their assay did not distinguish between T cells that recognize different epitopes, these results probably reflect responses to

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multiple MA peptides restricted by any of the three to eight MHC II HLA alleles within each subject. Therefore, the total frequencies observed in our study and those reported by Geiger et al. are comparable, suggesting the tetramer approach does not underestimate the frequency of epitope-specific CD4\(^+\) T cells.

Our observed frequency estimates for epitope-specific naive CD4\(^+\) T cells in humans are also consistent with those reported in mice. Moon et al. (9, 30) estimated that the number of naive Ag-specific CD4\(^+\) T cells ranged from 20 to 200 cells per mouse. Given that a mouse has ~30 million naive CD4\(^+\) T cells, these estimates correspond to frequencies ranging from 0.7 to 7 specific cells per million. To some degree, this similarity is surprising, as the human TCR repertoire is known to be at least 10 times more diverse than that of mice (31–33). Based on frequency observations in humans, we project that the frequency of most antigenic epitope-specific naive CD4\(^+\) T cells, similar to naive PA401–420-specific cells in mice. Moon et al. (9, 30) estimated that the number of naive Ag-specific CD4\(^+\) T cells ranged from 20 to 200 cells per mouse. Given that a mouse has ~30 million naive CD4\(^+\) T cells, these estimates correspond to frequencies ranging from 0.7 to 7 specific cells per million. To some degree, this similarity is surprising, as the human TCR repertoire is known to be at least 10 times more diverse than that of mice (31–33). Based on frequency observations in humans, we project that the frequency of most antigenic epitope-specific naive CD4\(^+\) T cells, similar to naive PA401–420-specific cells, is ~2 per million naive CD4\(^+\) T cells. We speculate that 10–20 cells per million may represent an upper range limit for epitope-specific naive CD4\(^+\) T cells, similar to naive PA401–420-tetramers in humans, we project that the frequency of most antigenic epitope) compared with CD8\(^+\) T cells may also contribute to the lower magnitude of CD4\(^+\) T cell responses.

However, our previous study demonstrated that PA505–524 is an subdominant in the DR0101-restricted repertoire. Therefore, it might be expected that this epitope is not efficiently processed. This has been partly attributed to the fact that CD4\(^+\) T cells require continuous Ag exposure for expansion, whereas CD8\(^+\) T cells can expand in the absence of Ag (36–38). The observed differences in frequency imply that the lower number of naive CD4\(^+\) T cells (per epitope) compared with CD8\(^+\) T cells may also contribute to the lower magnitude of CD4\(^+\) T cell responses.

Several factors could account for the relative immunodominance of PA-specific CD4\(^+\) T cells. For example, MHC binding and Ag processing can explain some differences. To test this possibility, we examined the binding affinities of the three PA peptides, PA401–420, PA505–524, and PA713–732, to DR0101 using a competitive binding assay. These results indicated that all three peptides bound with similar affinities (Table I). The role of Ag processing is subtle and more difficult to study directly. PA505–524-specific T cells were subdominant in the DR0101-restricted repertoire. Therefore, it might be expected that this epitope is not efficiently processed. However, our previous study demonstrated that PA505–524 is an immunodominant epitope in DR0701 vaccines. This implies that allorecognition rather than nonspecific staining. Therefore, possible differences in tetramer sensitivity should be taken into consideration when tetramers are being used to estimate the frequency of Ag-specific CD4\(^+\) T cells that are present at low frequencies.

Others have estimated the precursor frequency of naive CD8\(^+\) T cells in humans to be between 0.6 and 500 per million (12, 13). Thus, the frequency of naive CD8\(^+\) T cells can be up to 50-fold higher than that of naive CD4\(^+\) T cells for a given antigenic epitope specificity. It is well documented that the magnitude of CD8\(^+\) immune responses is stronger than that of CD4\(^+\) T cells (34, 35). This has been partly attributed to the fact that CD4\(^+\) T cells require continuous Ag exposure for expansion, whereas CD8\(^+\) T cells can expand in the absence of Ag (36–38). The observed differences in frequency imply that the lower number of naive CD4\(^+\) T cells (per epitope) compared with CD8\(^+\) T cells may also contribute to the lower magnitude of CD4\(^+\) T cell responses.

FREQUENCY OF EPITOPE-SPECIFIC NAIVE CD4\(^+\) T CELLS

### Table II. TCR V\(\beta\) sequences from different PA713–732 T cell clones derived from nonvaccinees

<table>
<thead>
<tr>
<th>Individual/Clone No.</th>
<th>TCR V(\beta) Type</th>
<th>TCR V(\beta) Subtype</th>
<th>CDR3 Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>835/8</td>
<td>V3</td>
<td>S1</td>
<td>AAPPRVIGRSTEAFFQGQTLTVV</td>
</tr>
<tr>
<td>835/13</td>
<td>V3</td>
<td>S1</td>
<td>ASTPRGWYTFGSQGTRLTVV</td>
</tr>
<tr>
<td>835/19</td>
<td>V6</td>
<td>S3</td>
<td>ASSPLRSESSYNPLHPNGTRLTVT</td>
</tr>
<tr>
<td>835/18</td>
<td>V11</td>
<td>S1</td>
<td>ASSDRAGEQYQFPQGTRLTVL</td>
</tr>
<tr>
<td>835/4</td>
<td>V13</td>
<td>S6</td>
<td>ASSYSSQGQGLDTQYGQGTRLTVL</td>
</tr>
<tr>
<td>835/5</td>
<td>V13</td>
<td>S2</td>
<td>ASLRDREGNEQFQGQTRLTVL</td>
</tr>
<tr>
<td>835/16</td>
<td>V13</td>
<td>S6</td>
<td>ASSYSSDNQHPFDQGTRLSIL</td>
</tr>
<tr>
<td>835/24</td>
<td>V13</td>
<td>S6</td>
<td>ASSYSSQGQGLTFQGTRLSIL</td>
</tr>
<tr>
<td>835/12</td>
<td>V15</td>
<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
</tr>
<tr>
<td>835/15</td>
<td>V15</td>
<td>S1</td>
<td>ATSDQGSDLQDQFQGTRLTVL</td>
</tr>
<tr>
<td>835/22</td>
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<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
</tr>
<tr>
<td>835/25</td>
<td>V15</td>
<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
</tr>
<tr>
<td>835/23</td>
<td>V23</td>
<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
</tr>
<tr>
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<td>V23</td>
<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
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<tr>
<td>835/20</td>
<td>V23</td>
<td>S1</td>
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</tr>
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<td>S1</td>
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</tr>
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</tr>
<tr>
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<td>S1/2</td>
<td>ASSPRVTEGIFQGQTRLTVL</td>
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<td>S6</td>
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<tr>
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<td>S1</td>
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</tr>
<tr>
<td>765/31</td>
<td>V13</td>
<td>S6</td>
<td>ASSYEYNAGNVQTVFPQGTRLTVL</td>
</tr>
<tr>
<td>765/13</td>
<td>V15</td>
<td>S1</td>
<td>ATSDQRGSDGAVNLTPAGSRNLTVL</td>
</tr>
</tbody>
</table>

aExperiment was performed with PBMC from the same subject 3 mo after the first experiment.

### Table III. V\(\beta\)13.2 and V\(\beta\)13.6 usage in total CD4\(^+\) T cells and DR0101-restricted PA713–732-specific memory T cells

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total CD4(^+) T Cells (%)</th>
<th>PA713–732 T Cells (%)</th>
<th>% V(\beta)13.2</th>
<th>Total CD4(^+) T Cells (%)</th>
<th>PA713–732 T Cells (%)</th>
<th>% V(\beta)13.6</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>14</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>1(^a)</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0.7</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2(^a)</td>
<td>2</td>
<td>0</td>
<td>0.5</td>
<td>3</td>
<td></td>
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<tr>
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<td>2</td>
<td>5</td>
<td>0.5</td>
<td>4</td>
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</table>

Approximate Expansion

<table>
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<tr>
<th>Epitope</th>
<th>Vaccinated</th>
<th>Unvaccinated</th>
<th>Approximate Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA713–732</td>
<td>500 per million(^a)</td>
<td>5 per million</td>
<td>100-fold</td>
</tr>
<tr>
<td>PA401–420</td>
<td>60 per million</td>
<td>1 per million</td>
<td>60-fold</td>
</tr>
<tr>
<td>PA505–524</td>
<td>22 per million</td>
<td>0.1 per million</td>
<td>200-fold</td>
</tr>
</tbody>
</table>

\(^a\)Frequency per million total CD4\(^+\) T cells.
the PA05-524 peptide can be processed and presented effectively in that context (20). Thus, neither MHC binding nor Ag processing appears to account for the hierarchy of immunodominance observed for the three DR0101-restricted PA epitopes in the repertoire of DR0101 AVA vaccinees. La Gruta et al. (39) have suggested the extent of naive T cell recruitment and subsequent clonal expansion is critical in determining immunodominance. These factors are dependent on MHC/peptide loading on the surface of APC, which is, in turn, dependent on Ag processing, affinity of the peptide for MHC, and Ag availability. In the current study, we focused on epitopes within a single Ag. For multiple epitopes derived from a single Ag, Ag availability and naive T cell recruitment can be expected to be similar for epitopes with similar processing efficiency. Thus, binding affinity of the peptides, efficiency of processing, and Ag availability and recruitment fail to adequately explain the relative immunodominance seen for these three PA epitopes. Rather, this hierarchy is best accounted for by differences in the relative frequencies of cells specific for these epitopes in the naive T cell repertoire. Table IV summarizes average naive frequencies (in nonvaccinated subjects) and memory frequencies (in vaccinated subjects) for PA-specific T cells. Although these frequencies were measured in different groups of subjects, these observations provide an estimate of the expansion of naive PA-specific T cells in response to vaccination. The change in frequency between the two groups implies ~60- to 200-fold expansion in response to AVA vaccination (Table IV). These conclusions are supported by similar findings in murine studies (9, 10).

Healthy individuals have ~1 × 10^{11} naive CD4^{+} T lymphocytes (40, 41). Given estimates that the human TCR gene has up to 10^5 possible rearrangements (32, 33), assuming an even distribution, it follows that there are ~1000 naive T cells per TCR clonotype. The average frequency of epitope-specific naive CD4^{+} T cells appears to be ~2 per million. Therefore, each individual should have an average of ~2 × 10^7 naive T cells for a typical epitope, and up to 1 × 10^9 naive cells for immunodominant epitopes. Epitope-specific naive T cells for a specific MHC/peptide complex can be expected to consist of 200–1000 distinct TCR clonotypes for the average and more abundant epitopes. Our limited TCR V{beta} sequence data indicated that PA_{113-732}-specific clones used multiple TCR V{beta} types. Interestingly, although the CDR3 regions within each TCR sequence were 100% diverse, naive DR0101-restricted PA_{113-732}-reactive cells were enriched for V{beta}13.6 and V{beta}15. The PA_{113-732}-reactive T cells in the memory repertoire were also enriched for V{beta}13.6. However, the percentage of PA_{113-732} CD4^{+} T cells with V{beta}13.6 in the naive repertoire was lower than in the naive repertoire. Previous studies have documented that T cells with high-affinity TCR were selectively favored to expand (3, 42, 43). Therefore, given their relative lack of expansion, it could be argued that PA_{113-732} CD4^{+} T cells that use V{beta}13.6 may have lower avidities for DR0101/PA_{113-732} complexes than PA_{113-732}-specific T cells, which use V{beta}V. We did not analyze V{beta}15 usage in memory PA_{113-732}-specific T cells because a V{beta}15 Ab was not available. Thus, it remains unclear whether T cells that use V{beta}15 were preferentially expanded.

In summary, we used direct ex vivo tetramer analysis to estimate the frequency of epitope-specific naive CD4^{+} T cells in human PBMC. The observed frequencies for PA-specific naive T cells ranged from 2 to 10 per million naive CD4^{+} T cells for the more dominant epitopes, but were 0.2 per million or lower for the subdominant epitope. Based on the consistent hierarchy observed, these data also strongly suggest that frequencies of naive T cells play a crucial role in determining the immunodominant hierarchy in the memory repertoire after Ag exposure. PA-specific T cells expand two orders of magnitude following immunization. At the level of TCR usage, at least one preferred V{beta} in the naive repertoire was preserved in the memory repertoire. Given the correspondence between anthrax-specific frequencies and those other models Ags such as influenza, it is likely that these observations will hold true in other settings.

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Disclosures

The authors have no financial conflicts of interest.

References


