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Requirement for CD4 T Cell Help in Maintenance of Memory CD8 T Cell Responses Is Epitope Dependent

Elizabeth A. Ramsburg,* Jean M. Publicover,*† Dagan Coppock,* and John K. Rose2*

CD4 Th cells play critical roles in stimulating Ab production and in generating primary or maintaining memory CTL. The requirement for CD4 help in generating and maintaining CTL responses has been reported to vary depending on the vector or method used for immunization. In this study, we examined the requirement for CD4 T cell help in generating and maintaining CTL responses to an experimental AIDS vaccine vector based on live recombinant vesicular stomatitis virus (VSV) expressing HIV Env protein. We found that primary CD8 T cell responses and short-term memory to HIV Env and VSV nucleocapsid (VSV N) proteins were largely intact in CD4 T cell-deficient mice. These responses were efficiently recalled at 30 days postinfection by boosting with vaccinia recombinants expressing HIV Env or VSV N. However, by 60 days postinfection, the memory/recall response to VSV N was lost in CD4-deficient mice, while the recall response HIV Env was partially maintained in the same animals for at least 90 days. This result indicates that there are epitope-specific requirements for CD4 help in the maintenance of memory CD8 T cell responses. Our results also suggest that choice of epitopes might be critical in an AIDS vaccine designed to protect against disease in the context of reduced or declining CD4 T cell help. The Journal of Immunology, 2007, 178: 6350–6358.
FIGURE 1. Primary responses to HIV Env are reduced in CD4-deficient mice. Female BALB/c mice were immunized i.m. with $5 \times 10^6$ PFU of rVSV expressing HIV Env (VSV EnvG). Primary anti-HIV Env CD8 T cell responses were assayed in the spleen (A, B, and E) and lung (C, D, and F) of CD4 T cell-intact (A, C, E, and F) and CD4 T cell-deficient (B, D, E, and F) mice 7 days after primary immunization. Ag-specific CD8 T cells were defined as those expressing low levels of CD62L and binding HIV Env tetramer (Tet$^{CD62L^{low}}$, upper left quadrant). Graphs (E and F) represent average percent of CD8 T cells that are CD62L$^{low}$ and bind HIV Env tetramer ± SEM. Data are representative of three separate experiments in each of which there was a minimum four animals per group. Cytotoxicity against the HIV Env peptide was tested in vivo (G) at 8 days postimmunization. Graph represents average specific killing ± SEM where percent-specific killing was determined as a ratio between CFSE$^{high}$ (target) and CFSE$^{low}$ (nontarget) cells recovered from recipient mice. Data are representative of two separate experiments in which a total of 15 CD4$^{+/+}$ and 7 CD4$^{-/-}$ mice were analyzed.
involved as well. Our results also indicate that there could be a rational basis for choosing less CD4-dependent epitopes in design of an effective AIDS vaccine.

Materials and Methods

Inoculation of mice

Mice were either obtained from The Jackson Laboratory or bred and maintained in our laboratory. To obtain CD4<sup>−/−</sup> mice expressing the MHC class I allele H-2d (BALB/c allele), C57BL/6 CD4<sup>+/+</sup> mice were crossed onto the BALB/c background. Mice were housed in microisolator cages in a biosafety level 2-equipped animal facility. Viral stocks were diluted to appropriate titers in serum-free DMEM. For i.m. vaccination, mice were injected with 5 × 10<sup>5</sup> PFU of virus in 50 μl. For i.p. vaccination, mice were injected with 1 × 10<sup>5</sup> PFU of virus in a 100-μl total volume. The Institutional Animal Care and Use Committee of Yale University approved all animal experiments.

Tetramer assay

Splenocytes were obtained by disrupting spleens between the frosted ends of two microscope slides. RBC were removed using RBC-lysing buffer (Sigma-Aldrich). To obtain lymphocytes from lungs, mice were perfused with sterile PBS until lungs were cleared of blood. The lungs were removed, chopped into fine pieces, and digested for 2 h at 37°C in DMEM containing 5% FCS, 150 U/ml collagenase, and 20 μg/ml DNase. After digestion, cells were washed through a metal sieve, filtered, and layered onto a Ficoll gradient. Gradients were centrifuged (2000 rpm for 30 min at room temperature (RT)) in a Sorvall Legend RT centrifuge and lymphocytes
were collected from the interface. Cells were washed and resuspended in DMEM containing 5% FCS. Staining was performed on freshly isolated lymphocytes. Briefly, \(5 \times 10^6\) cells were added to the wells of a 96-well V-bottom plate and were blocked with unconjugated streptavidin (Molecular Probes) and Fc block (BD Pharmingen) for 15 min at RT. Following a 5-min centrifugation at 500 \(g\), splenocytes were labeled with a FITC-conjugated anti-CD62L Ab (BD Pharmingen), an allophycocyanin-conjugated anti-CD8 Ab (BD Pharmingen), and tetramer for 30 min at RT. The N1 tetramer was a PE-conjugated MHC class I Dd tetramer (provided by Dr. L. Lefrancois, Farmington, CT) containing the VSV N1 peptide (N-RGYVYQGL-C). The VSV N2 pentamer was provided by D. Cooper (Wyeth Pharmaceuticals, Pearl River, NY) and the HIV EnvP18 tetramer was provided by the National Institutes of Health AIDS Reagent facility. CD8 T cells which were tetramer positive and activated CD62Llow were identified by flow cytometry. Animals vaccinated with rVSV were used to determine background levels of tetramer binding. Background was routinely \(<0.1\%\) and was subtracted from all reported percentages. No difference in binding or staining was observed in head-to-head comparisons of the tetramer and pentamer reagents (when a N2 tetramer and pentamer were compared).

**CTL assay in vivo**

This assay was performed as described previously (26) using Env peptide p18-I10 (N-RGPGRAFVTI-C; Invitrogen Life Technologies) or VSV N1 peptide (N-RGYVYQGL-C; Invitrogen Life Technologies). On day 7 postinoculation, splenocytes were obtained as described above from an uninfected mouse and resuspended in 1 ml of 5% FBS-DMEM. The donor (target) cells were split into two populations. Env p18-I10 or VSV N1 peptide was added to one population (+ peptide) to a final concentration of \(10^{-6}\) M, and to the other population no peptide was added (=peptide). Cells were incubated at 37°C in 5% CO2 for 45 min with occasional mixing. Cells were washed and resuspended in 1 ml of PBS. One milliliter of 10 \(\mu\)M CFSE (Molecular Probes) was added to peptide cells (final concentration, 5 \(\mu\)M) to generate a CFSE\(^\text{high}\) group and 1 ml of 1 \(\mu\)M CFSE...
was added to peptide cells (final concentration, 0.5 M) to generate the CFSE low group. Cells were vortexed as the CFSE was added and then incubated for 5 min at RT. Cells were then washed three times in PBS and resuspended in PBS at a concentration of 10^8 cells/ml. Ten million cells were incubated for 5 min at RT. Cells were then washed three times in PBS and incubated for 5 min at RT.

### Results

**Primary CD8 T cell responses to HIV Env expressed by VSV are reduced 2- to 3-fold in the absence of CD4 T cells**

Our goal was to determine whether responses to a vaccine Ag (HIV Env protein) expressed in a VSV-based vaccine vector as well as responses to a vector Ag (VSV N protein) could be generated and maintained in the absence of CD4 T cells. Although the immunodominant epitopes of HIV Env have not been identified for C57Bl/6 mice, the response to the HIV Env epitope p18 (N-RGPGRAFVTI-C) that binds MHC class I H-2D^d in BALB/c mice has been extensively characterized (27), and an immunodominant epitope for VSV N (designated N2) was recently defined for BALB/c mice (N-MPYLIDFGC-C; D. Cooper, Wyeth Pharmaceuticals, unpublished observation). This N epitope binds the MHC class I H-2L^d molecule in BALB/c mice. Because CD4 T cell-deficient mice are not available on the BALB/c background, we backcrossed C57Bl/6 CD4^-/- mice onto the BALB/c background to generate BALB/c CD4^-/-.

To analyze the primary responses to HIV and VSV N simultaneously, we immunized CD4^+/+ and CD4^-/- BALB/c mice with a live VSV recombinant (VSV EnvG) expressing an HIV Env protein in which the Env cytoplasmic domain was replaced with the VSV G cytoplasmic domain (28). We then assayed CD8 T cell responses to the VSV N2 epitope bound by the BALB/c allele H-2L^d in the samples from the same mice used to assay responses to Env above. Responses to the VSV N2 epitope were lower (in terms of the percentage of Ag-specific CD8 T cells generated) than those to the HIV Env p18 epitope, but were still substantial (Fig. 2). Interestingly, CD4-intact and CD4-deficient mice produced equivalent numbers of VSV N2-specific CD8 T cells in both the spleen and the lung after the primary immunization (Fig. 2) showing that this primary response is completely independent of CD4 T cell help.

**Memory and recall responses to HIV Env are partially CD4 independent**

Several recent studies have shown that while CD4 T cells are often dispensable in the priming of CD8 T cell responses to live pathogens, they are normally required for long-term maintenance of these responses (13, 14). The signals provided by CD4 T cells for long-term maintenance of CD8 cells could be Ag specific or non-specific and have not been defined.

To examine the requirement for CD4 T cells in the long-term CD8 T cell responses to Env, we assayed memory and recall responses at 30, 60, or 90 days after primary immunization of BALB/c CD4^+/+ or CD4^-/- mice with VSV EnvG. Resting (memory) T cell levels to HIV Env p18 and VSV N2 were assayed simultaneously at each time point by MHC class I tetramer staining and were both able to kill HIV Env-loaded target cells (84.3 ± 1.2% and 84.9 ± 3.1% specific killing, respectively) and there was no significant difference between the two groups (p = 0.83). These results demonstrate clearly that a functional CTL response to HIV Env is generated independent of CD4 T cell help.

**Primary responses to VSV N2 are intact in CD4-deficient mice**

To determine whether CD8 T cell responses to a different Ag might have a different requirement for CD4 T cell help, we assayed responses to the VSV N2 epitope bound by the BALB/c allele H-2L^d in the samples from the same mice used to assay responses to Env above. Responses to the VSV N2 epitope were lower (in terms of the percentage of Ag-specific CD8 T cells generated) than those to the HIV Env p18 epitope, but were still substantial (Fig. 2). Interestingly, CD4-intact and CD4-deficient mice produced equivalent numbers of VSV N2-specific CD8 T cells in both the spleen and the lung after the primary immunization (Fig. 2) showing that this primary response is completely independent of CD4 T cell help.

### Table I. Primary, memory, and recall T cell responses to epitopes

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Primary</th>
<th>Memory and Recall</th>
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<tbody>
<tr>
<td></td>
<td>Day 8</td>
<td>Day 30</td>
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<tr>
<td>Spleen</td>
<td></td>
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<tr>
<td>HIV Env CD4^+/+</td>
<td>12.5 ± 1.1</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>CD4^-/-</td>
<td>4.6 ± 0.6</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>VSV N2</td>
<td>CD4^+/+</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>CD4^-/-</td>
<td>1.3 ± 0.4</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV Env CD4^+/+</td>
<td>47.5 ± 4.8</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>CD4^-/-</td>
<td>22.7 ± 3.2</td>
<td>0.9 ± 0.3</td>
</tr>
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<td>VSV N2</td>
<td>CD4^+/+</td>
<td>7.0 ± 2.1</td>
</tr>
<tr>
<td>CD4^-/-</td>
<td>8.8 ± 1.3</td>
<td>&lt;0.01</td>
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**Note:** Percent-specific lysis was calculated by using the following formula: percent-specific lysis = (1/(ratio for vaccinated mice/ratio for control mice)) × 100, where “ratio” = (percent CFSE low/percent CFSE high).

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in the CD4+/+ mice. In the spleens of CD4−/− mice, the boost generated responses that were 2- to 3-fold greater than those generated during the primary response (compare days 6–8 to days 37, 67, and 97 in Fig. 3A and Table I), while in the CD4−/− mice the responses after boosting were generally no greater than the primary response to the VSV vector (Fig. 3B). In the lung, the memory cell levels were below background in both the CD4+/+ and CD4−/− mice at the 60 and 90 day time points before boosting. However, after boosting, a vigorous recall response was observed 7 days later in both the spleen and the lung of CD4+/+ and CD4−/− animals. The difference between the lung recall response in the CD4+/+ and CD4−/− mice was <2-fold. This result shows that even though the memory cells may have declined to undetectable levels before boosting, they were still present and able to expand to high levels upon restimulation. In all cases, postboost responses (days 37, 67, and 97) were much greater than a primary response to the boosting vector and were therefore true memory recall responses (Tables I and II). For example, control naive CD4+/+ or CD4−/− mice primed with the vaccinia-Env virus generated 3.8 ± 0.4% or 0.7 ± 0.3% tetramer-positive cells, respectively (for HIV EnvP18), 7 days after infection while on day 67 (7 days postboost), CD4+/+ and CD4−/− mice generated 35.4 ± 1.7% and 5.3 ± 1.5% tetramer-positive cells, respectively.

As in the primary response, when CTL function against HIV Env p18 was tested in vivo at 56, 67, or 97 days postpriming (7 days postboost in each case) both CD4+/+ and CD4−/− mice retained the ability to eliminate p18 peptide loaded cells in vivo (Fig. 3E). CD4−/− mice had significantly lower levels of cytotoxicity in vivo (p < 0.04, p < 0.04, p < 0.03 at days 56, 67, and 97) but it is unknown, because the level of killing still approached 100% in the 4-h assay, whether this difference would translate into a functional immunodeficiency in vivo. Together, these data demonstrate that the maintenance and function of significant numbers of memory CD8 T cells specific for HIV Env p18 does not require the presence of CD4 T cells for at least 3 mo after priming.

Memory and recall responses to VSV N2 are almost completely dependent on CD4 T cells

To determine whether the long-term memory and recall CD8 T cell responses to VSV N2 were CD4 dependent or independent, we used a vaccinia virus expressing VSV N (v38; Ref. 30) to boost an additional cohort of mice primed with VSV EnvG. As above, we then assayed resting and recall CD8 T cell responses at 30, 60, and 90 days postprime.

N2-specific memory cells were present in low but detectable numbers in the spleens of CD4+/+ mice at 30, 60, and 90 days, and also in CD4−/− mice at 30 and 60 days (Fig. 4, A and B, and Table I). VSV N2-specific cells were not detectable in the lungs of any unboosted mice at 30, 60, or 90 days postprime (Fig. 4, C and D, and Table I).

When CD4+/+ mice were boosted at 30, 60, or 90 days, vigorous recall responses occurred in the spleen and lung. In contrast to the primary anti-VSV N2 response, in which CD4+/+ and CD4−/− responses were nearly equivalent, the recall response to N2 was absent in the spleens of CD4−/− mice by day 60. For example, at day 67 after a boost at day 60, the recall response was 50-fold below that seen in CD4+/+ mice (Fig. 4, Table I), and was not a true recall as it was equivalent to that seen in naive CD4−/− mice primed with the boosting vector (Tables I and II). Similarly, the postboost expansion of N2-specific cells in the lungs of CD4−/−
Primary CD8 T cell responses to VSV N1 are largely independent of CD4 help in CD4^−/− mice

The above results indicate that the capacity for re-expansion after secondary exposure to Ag is maintained or lost in an Ag- or epitope-specific manner. Although the recall response to VSV N2 epitope has not been directly assayed previously, the primary response to the VSV N epitope (N-RGYVYQGL-C; here designated VSV N1) (31, 32) has been characterized in C57BL/6 mice. An early study using different methods showed a strong requirement that primary CD8 T cell responses to the VSV N1 epitope are largely independent of CD4 T cell help.

Because our results with the VSV N2 epitope contrasted with this previous work, we also analyzed the response to VSV N1 epitope in our system. We immunized mice with rVSV and quantified the CD8 T cell response to the VSV N1 epitope using an MHC class I tetramer (Fig. 5, A and B). When CD8 T cells were assayed at 8 days after primary immunization, there were 2- to 3-fold fewer VSV N1-specific T cells in the spleen of CD4^−/− mice than in spleen of CD4-intact animals (Fig. 5A). A smaller difference was seen in the lung (Fig. 5B). Our results are thus consistent with those of Marzo et al. (25) who used CD4 cell depletion by Ab rather than CD4^−/− mice, but also measured responses to the N1 epitope.

To determine whether the reduction in the number of VSV N1-specific CD8 T cells caused a functional immunodeficiency in the CD4^−/− mice, we performed a cytotoxic T cell assay in vivo. In this assay, killing of target cells loaded with VSV N1 peptide was very efficient and not significantly different in immunized CD4^−/− and CD4^+/+ mice (Fig. 5C). This experiment demonstrates that the VSV N1-specific CD8 T cells generated in the absence of CD4 T cell help are functional in vivo. Our results support the conclusion that primary CD8 T cell responses to the VSV N1 epitope are largely independent of CD4 T cell help.

MHC class II^−/− mice also show no significant defect in primary response to the VSV N1 epitope

Recent studies have demonstrated that T cell development and positive selection can be abnormal in CD4^−/− mice (33). These mice exhibit an abnormal population of cells that express the CD8 coreceptor molecule but are MHC class II restricted. These aberrant CD8 T cells can negatively affect the generation of normal CD8 T cell responses to a variety of stimuli (33). Because such cells might influence our results, we repeated the analysis of CD8 T cell responses in MHC class II^−/− mice (26, 34), in which CD4 T cells do not develop because they cannot be positively selected. There were slightly more anti-VSV N1 T cells generated in the spleen of MHC class II^−/− mice than in CD4^−/− mice (10.2 ±
than CD4

At 30 days after primary immunization with 10^5 PFU vaccinia virus expressing VSV N protein (v38) (n = 10/group). Recall CD8 T cell responses were assayed at 7 days postboost. Graphs represent average percent of CD8 T cells binding VSV N1 tetramer ± SEM in the spleen (A) or lung (B).

**FIGURE 6.** Resting and memory T cell responses to VSV N1 are intact in CD4-deficient mice. Resting CD8 T cell responses were assayed at 30 days after primary immunization and before boosting. Mice were boosted at 30 days after primary immunization with 10^6 PFU vaccinia virus expressing VSV N protein (v38) (n = 10/group). Recall CD8 T cell responses were assayed at 7 days postboost. Graphs represent average percent of CD8 T cells binding VSV N1 tetramer ± SEM in the spleen (A) or lung (B).

2.0% vs 6.6 ± 0.9%, Fig. 5D) at 8 days after primary immunization but the difference was not statistically significant (p = 0.12). In the lung, MHC class II^-/- mice had slightly lower responses than CD4^-/- mice (26.9 ± 6.6% vs 34.6 ± 3.6%, Fig. 5D). This difference was also not statistically significant (p = 0.63). These data confirm that CD4^-/- mice are suitable for analyzing the requirement for CD4 cells in generation of CD8 T cells. Apparently, the class II-restricted CD8 T cells generated in these animals do not negatively affect the generation of Ag-specific CD8 T cells.

**Memory and recall responses to VSV N1 are independent of CD4 help**

To determine the role of CD4 T cell help in maintaining short-term recall CD8 T cell responses to VSV N1 in our system, we assayed anti-VSV N1 responses at 30 days after primary immunization. We also boosted animals 30 days after primary immunization with a vaccinia virus expressing the VSV N protein (v38) and assayed recall responses to VSV N1 at 7 days after the boost. At 30 days after primary immunization a clear population of resting CD8 T cells was present in the spleen of both CD4-intact (1.3 ± 0.1%) and CD4-deficient (0.33 ± 0.03%) mice (Fig. 6A). Tetramer-positive cells were also present in the lungs of both CD4^+/- (8.5 ± 1.9%) and CD4^-/- (2.3 ± 0.3%) mice at this time (Fig. 6B). After boosting, the VSV N1-specific T cells expanded up to 30-fold (Fig. 6A) in both CD4^+/- and CD4^-/- mice. There were no significant differences in the numbers of VSV N1-specific cells in either the spleens or the lungs of CD4-intact vs CD4-deficient animals. Similar results were obtained in MHC class II^-/- mice immunized and boosted on the same schedule (data not shown). Although we have not followed these responses for as long a time as the responses to the N2 or HIV Env epitopes, these data demonstrate that CD8 T cell responses to VSV N1 can be maintained and recalled in the absence of CD4 T cells.

**Discussion**

We undertook this study to determine whether the CD8 T cell response to immunization with rVSV vectors is dependent upon CD4 T cell help. This issue is of particular importance if rVSV vaccines are to be administered to immunocompromised individuals. We observed that some, but not all, CD8 T cell responses to Ags expressed by rVSV are CD4 dependent. Primary responses to rVSV itself, and to foreign Ags expressed by rVSV, were independent of CD4 help. This is consistent with the results of Marzo et al. (25). In contrast, recall responses to Ags expressed by rVSV are CD4 dependent to different degrees, depending on the Ag. Specifically, when CD4-deficient mice were immunized with a rVSV expressing HIV Env, CD8 T cells specific for HIV Env P18 could be recalled and re-expanded for up to 3 mo postimmunization, while those specific for the VSV N2 epitope could not.

There are several possible explanations to account for the difference in recall potential between HIV Env- and VSV N2-specific T cells. Because HIV Env P18 is a dominant epitope, it is possible that “immunodomination” skewed the immune response toward Env and away from N. Related factors, such as the affinity of an epitope for the MHC class I molecule on which it is presented, and competition for class I molecules could also have influenced the outcome. However, because HIV Env P18 and VSV N2 are presented on different MHC class I molecules (Dd and Ld, respectively), it seems unlikely that either of these factors account for the difference. Also, the primary VSV N2 response is the same in animals infected with VSV lacking the Env gene. Another potential explanation is that relative abundance of the two proteins influenced the primary expansion, and subsequent maintenance, of CD8 T cells. However, expression from the VSV genome is polar, with the protein encoded by the first gene (VSV N) expressed at much higher levels than the protein encoded by the fifth gene (HIV Env).

Finally, it would be expected that the lower frequency of anti-VSV N2 cells generated after the primary response would result in retention of a smaller number of memory cells for VSV N2 vs Env p18. This phenomenon was recently described in detail by La Gruta et al. (35). Although memory precursor frequency may be a contributing factor, it would not explain the complete failure of N2-specific cells to re-expand in CD4-deficient animals. At days 8, 30, and 60, the frequency of VSV N2-specific cells in the CD4^+/- and CD4^-/- mice was similar in the spleen. However, on days 60 and 90 cells in the CD4^+/- but not the CD4^-/- mice re-expanded after boosting. Furthermore, HIV Env-specific cells reached undetectable levels in the spleen of CD4^-/- mice at 60 and 90 days postprime, yet still re-expanded to high levels upon boosting. We propose that the requirement for CD4 help in the maintenance of CD8 T cells may be Ag specific due to some ongoing requirement for cognate interaction (either direct or via an APC intermediate) between CD8 T cells and CD4 T cells. Our data are consistent with the following model. After priming with a rVSV vaccine, the number of CD8 T cells generated varies according to the immunodominance of the epitope (Env P18 > N2). These primary responses are effectively independent of CD4 T cell help for both epitopes. After contraction of the primary response, cells enter the CD8 memory pool in numbers proportional to those generated during priming (Env P18 > N2). Thereafter (2–3 mo), a cognate interaction maintains the capacity of the memory CD8 T cells to re-expand in the CD4^+/- mice. This interaction is not maintained in the CD4^-/-
mice and is differentially lost depending on the epitope (N2 quicker than Env P18).

Although the mechanism is not yet clear, our results indicate that there are epitope-specific requirements for CD4 help in the maintenance of memory CD8 T cell responses. Further investigation of this phenomenon could be valuable in the ongoing effort to design Ags for an AIDS vaccine that could protect against disease in the context of reduced or declining CD4 T cell help.

Acknowledgments

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Disclosures

The authors have no financial interest of interest.

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