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**Robust, Vaccine-Induced CD8+ T Lymphocyte Response against an Out-of-Frame Epitope**

Nicholas J. Maness,*†,1 Nancy A. Wilson,*†,1 Jason S. Reed,* Shari M. Piaskowski,*
Jonah B. Sacha,* Andrew D. Walsh,* Elizabeth Thoryk,† Gwendolyn J. Heidecker,†
Michael P. Citron,* Xiaoping Liang,† Andrew J. Bett,† Danilo R. Casimiro,† and
David I. Watkins*†

Rational vaccines designed to engender T cell responses require intimate knowledge of how epitopes are generated and presented. Recently, we vaccinated 8 *Mamu-A*02+ rhesus macaques with every SIV protein except Envelope (Env). Surprisingly, one of the strongest T cell responses engendered was against the Env protein, the *Mamu-A*02–restricted epitope, Env788–795RY8. In this paper, we show that translation from an alternate reading frame of both the Rev-encoding DNA plasmid and the rAd5 vector engendered Env788–795RY8–specific CD8+ T cells of greater magnitude than “normal” SIV infection. Our data demonstrate both that the pathway from vaccination to immune response is not well understood and that products of alternate reading frames may be rich and untapped sources of T cell epitopes. *The Journal of Immunology*, 2010, 184: 67–72.

CD8+ T lymphocytes are important for killing both tumor and pathogen-infected cells. Hence, there is immense interest in creating vaccines that elicit these responses. Indeed, because an Ab-based AIDS vaccine is unlikely in the near future, the goal for a vaccine is now to control viral replication and therefore to slow disease progression and prevent transmission. To achieve this, several laboratories are investigating the use of vaccines that solely elicit T cell responses (1, 2). Most of these vaccines employ rDNA and/or viral vectors that encode HIV proteins (or SIV proteins, in the case of these macaque studies). Understanding the pathway from vaccination to T cell response is a critical step toward rational vaccine design.

Despite the importance of T cells in controlling HIV and SIV (3, 4), it is becoming clear that much remains to be learned about these cells and the epitopes they target. For instance, it is now established that the processes of viral gene expression can lead to the translation of viral alternate reading frames (ARFs) and the production of T cell epitopes derived from these “mistranslation” events (5, 6). Similar processes may lead to the translation of portions of functional viral proteins in regions of open reading frame (ORF) overlap and T cell epitopes contained therein. The contribution of these processes to the production of epitopes either during infection or after vaccination is poorly understood.

Recently, we showed that *Mamu-A*02+ rhesus macaques vaccinated with a DNA/rAd5 regimen encoding all of the SIV proteins, except Envelope (Env), made strong CD8+ T cell responses against the *Mamu-A*02–restricted Env-encoded epitope, Env788–795RY8 (RY8) (7). In this paper, we show that the vaccine-induced RY8–specific response was much stronger than that elicited by “normal” viral infection. Further, we demonstrate that the RY8 epitope was produced by translation of a small portion of the Env protein from both the overlapping Rev-encoding DNA plasmid and the rAd5 viral vector encoding Rev. Together, our data indicate that T cells targeting out-of-frame encoded peptides might be more ubiquitous and important than currently appreciated. These data strongly suggest that there is much to discover about how vaccines are translated to induce immune responses and that rational vaccine design could benefit from a greater understanding of how and when these nontraditional translation events might occur.

**Materials and Methods**

*Detection and analysis of immune responses*

We measured cellular immune responses in the vaccinated animals, using IFN-γ ELISpot as previously described (2, 7), 2 wk after an rAd5 boost in vaccinated animals. Data represent the average of two replicate wells minus the average of all negative (no peptide) wells and are reported as spot-forming cells (SFCs) per million PBMCs, with 100,000 cells added per well. Responses were considered positive if the SFC count was greater than 5 spots (50 SFC per million PBMCs) and greater than twice the background plus 2 SD. These responses were compared with archived data (performed and analyzed in identical fashion) from *Mamu-A*02+ animals previously infected with SIVmac239 from other studies. Specifically, these nonvaccinated animals were in the acute (3–10 wk postinfection) or chronic (>6 mo postinfection) phase of SIV infection and expressed a variety of MHC class I (MHC-I) molecules in addition to *Mamu-A*02. Comparisons of immune responses were performed using a two-tailed *t* test.

**Plasmid synthesis, mutagenesis, and transfection of B cells**

The Rev and Gag plasmids were made as described (7). The Env plasmid was made as described elsewhere (8). Mutation of the Rev plasmid to encode an
FIGURE 1. Whole-proteome ELISPOT of the eight vaccinated animals. Total cellular immune response was measured using IFN-γ ELISPOT 2 wk after rAd5 administration. Peptides were 15-mers overlapping by 11, spanning the entire known SIVmac239 proteome. ELISPOT responses were considered positive if the mean number of SFCs exceeded background plus 2 SD and was >50 SFCs per million PBMCs. Because total PBMC was assayed, these responses are composed of both CD4 and CD8 responses.
were used as APCs. In each experiment, 2–5 × 10^6 cells were transfected with 5 μg Rev (wild type or containing RY8 escape mutation), Gag, or Env plasmids using the Nucleofector device (Lonza, Walkersville, MD), with solution C and program G-16. The cells were then cultured in RPMI 1640 with 10% FBS and no antibiotics for 24 h.

Culture and Ad5 infection of monocyte-derived dendritic cells

CD14+ monocytes were isolated from PBMCs using magnetic bead-bound anti-CD14 Abs and LS separation columns (Miltenyi Biotech, Auburn, CA). The cells were cultured according to Ignatius et al. (9), using RPMI supplemented with 1% FBS (R1) and GM-CSF (1000 U/ml) and IL-4 (100 U/ml). After 4 d of culture, 1 × 10^6 cells were resuspended in 200 ml media and rAd5 viruses were added at ratios of 100:1 (viral particles: cells) and 1000:1 for 90 min. The volumes were then brought up to 1 ml with complete R1 media supplemented with GM-CSF and IL-4 and cultured for 24 or 48 h.

Recognition assays and intracellular cytokine staining

For recognition assays, APCs—either DNA-transfected 721.221 B cells or rAd5-infected monocyte-derived dendritic cells (MDDCs)—were mixed with RY8- or Rev44–51RL8 (RL8)-specific T cells at a ratio of 1:1 (100,000 of each); then intracellular cytokine staining (ICS) for the detection of IFN-γ and TNF-α was performed as previously described (7).

Results

**Rhesus macaques vaccinated with all of the SIV sequences, except Env, made robust T cell responses against an Env epitope**

Previously, we vaccinated eight Mamu-A*02+ rhesus macaques with all of the SIV proteins except Env to test the hypothesis that a vaccine that solely elicits cellular immune responses can control AIDS virus replication (7). The vaccine regimen consisted of three injections of DNA (i.m.), with each DNA plasmid synthesized to encode a single viral protein. We then boosted the DNA primed responses with a single injection (i.m.) of rAd5 and GM-CSF and IL-4 and cultured for 24 or 48 h.

The existence of strong T cell responses against the RY8 epitope in all vaccinated animals, and the fact that the DNA encoding RY8 is contained in the Rev-encoding plasmid, led us to hypothesize that RY8 was translated in both the Env and Gag plasmids. However, they did recognize cells transfected with the Gag plasmid. This epitope was likewise presented, indicating that the Rev plasmid as well as wild type, whereas recognition by RY8-specific T cells was largely abrogated (Fig. 4). It is interesting that recognition was far greater with the RY8-specific cells derived from the vaccinated animal, r02089, than with cells from either of the other animals. However, in our experience, variation is often substantial between cell lines recognizing either infected or transfected cells. Despite this, the recognition depicted in Fig. 3 clearly shows that the Rev plasmid is translated in both the rev and env genes.
the Env reading frames and that T cell epitopes can be derived from both. The above data were collected using a plasmid that was recloned from the original vaccine stock. We repeated the transfection assays using the original vaccine stock and achieved the same results (data not shown). In addition, we attempted to PCR amplify and sequence the plasmid using a series of Env-specific primers. Sequence was obtained only when using a primer that aligned within the region of Env/rev exon 2 overlap. These controls, along with the data presented above, leave little doubt that the Rev-encoding plasmid was the source of the strong RY8-specific response in vaccinated animals.

The Rev-encoding rAd5 vector produces RY8

Finally, we tested whether cells infected with the rAd5 vector could present RY8. We first cultured MDDCs from animals that expressed Mamu-A*02, both Mamu-A*02 and Mamu-B*08, or neither. We chose to use dendritic cells because they are excellent APCs and because rAd5 vectors are known to infect them (12). Next, we infected the MDDCs with the rAd5 vectors encoding Rev or Gag and tested whether they could present the RY8 or RL8 epitopes to Ag-specific T cell lines. As with the DNA plasmid data, we found robust recognition of Ad5-infected MDDC after 24 h (data not shown), which became even greater after 48 h (Fig. 5). There was no recognition of MDDC that did not express the correct MHC (data not shown). Together with the DNA data, we have clearly demonstrated that an immunodominant CD8+ T lymphocyte response can be directed against an out-of-frame epitope with no clear mechanism of translation.

Discussion

Data presented in this study and other recent data from our laboratory (8) paint an emerging picture of the sources of T cell responses to out-of-frame epitopes.

FIGURE 4. Recognition of cells transfected with the vaccine plasmid encoding Rev. We transfected 721.221 cells that stably express either Mamu-A*02 or Mamu-B*08 with the plasmid encoding Gag from the vaccine, the Rev plasmid, the Rev plasmid engineered to express an escaped version of RY8, or an Env-encoding plasmid. At 24 h after transfection, we used ICS to see if T cell lines specific for Env 788–795RY8 or Rev 44–51RL8 could recognize the cells, as measured by TNF-α and IFN-γ production. We tested for recognition by RY8-specific cells grown from an animal vaccinated in this study (RY8 Vacc.), RY8-specific cells grown from an SIV-infected animal from a previous study (RY8 non Vacc.), and RL8-specific cells grown from an SIV-infected animal from a previous study (RL8).
The protein source of a given epitope is clearly important, and likely plays a primary role both in the timing of epitope generation (13, 14), with important exceptions (15), and in the ability of that epitope to escape T cell responses (16–18). However, at the very least, caution should be exercised when drawing whole-protein conclusions based on epitope-specific data. In some cases, epitopes can be derived from novel translation events of portions of viral proteins. This model of the sources of T cell epitopes shares fundamental similarities with that of the defective ribosomal product hypothesis, which states that T cell epitopes are primarily derived from defective protein products that do not achieve stable conformation, and are rapidly degraded by the proteasome into T cell epitopes (19). Indeed, the products of these unique translation events may be prime examples of defective ribosomal products.

The important mechanism by which the RY8 epitope is translated remains unknown. We sequenced the Rev-encoding vector and found that three stop codons are present in the \textit{env} reading frame in the region overlapping \textit{rev} exon 1, indicating that translation of the \textit{env} reading frame must occur farther downstream or via an unknown splicing mechanism. In addition, there are no AUG initiation codons in the \textit{env} reading frame. However, it is possible that translation of the RY8 epitope was due to translation initiation at a CUG codon, as has been described (20–23). Several CUG codons (encoding leucines) are found in the \textit{env} reading frame upstream of the epitope, including two that contain conserved features of a Kozak consensus, either a position $-2$ cysteine or a position $-3$ purine (24). It is also possible that translation occurs by way of a $+1$ ribosomal frameshift. This phenomenon is difficult to predict but is well documented in yeast (25) and may facilitate translation of a portion of the Env protein containing the RY8 epitope.

An important observation of this study is that the relative dominance of the RY8-directed response was greater than that elicited during either acute or chronic SIVmac239 infection. It is unknown if this is due to its peculiar source of translation or to some other factor associated with this vaccine regimen. Indeed, the
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Disclosures
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