Human Endogenous Retrovirus K(HML-2) Gag- and Env-Specific T-Cell Responses Are Infrequently Detected in HIV-1-Infected Subjects Using Standard Peptide Matrix-Based Screening

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T-cell responses to human endogenous retrovirus (HERV) K(HML-2) Gag and Env were mapped in HIV-1-infected subjects using 15mer peptides. Small peptide pools and high concentrations were used to maximize sensitivity. In the 23 subjects studied, only three bona fide HERV-K(HML-2)-specific responses were detected. At these high peptide concentrations, we detected false-positive responses, three of which were mapped to an HIV-1 Gag peptide contaminant. Thus, HERV-K(HML-2) Gag- and Env-specific T-cell responses are infrequently detected by 15mer peptide mapping.

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uman endogenous retroviruses (HERVs) are the DNA remnants of ancient retroviruses that infected the germ line of our evolutionary ancestors (2). The complement of HERVs in the human genome is diverse, representing all three major branches of the retroviral tree: gamma-epsilon, spuma, and delta-lenti-alpha beta retroviruses (see reference 14 for a review). The potential for HERVs to serve as a source of antigens was highlighted by a recent study which mapped the epitope specificity of a renal cell carcinoma (RCC)-reactive CD8+ T cell to a HERV-E-derived peptide (26).

Previously, we presented the hypothesis that the manipulation of an infected cell by HIV-1 to favor retroviral expression may result in the expression of HERV antigens. Supporting this, we have reported the detection of T-cell responses to peptides derived from diverse HERV families, selected for their predicted binding to common major histocompatibility complex class I (MHC-I) molecules, in HIV-1-infected subjects but not in uninfected controls (12, 27). We have since reported that the presence of strong HERV-specific T-cell responses is associated with control of HIV-1 in chronic infection (25). This has led us to speculate that HERV antigen expression in HIV-1-infected cells may serve as a surrogate marker that could be targeted in novel T-cell-based HIV-1 vaccines. In testing this model, we have decided to focus on the HML-2 lineage of the HERV-K class II superfamily. Recent proliferation of HERV-K(HML-2) is evidenced by the presence of human-specific and polymorphic insertions. These recently integrated HERV-K(HML-2) proviruses are comparatively intact, and many contain complete open reading frames for viral proteins (3, 5, 10, 13, 19–21, 23, 28–30). We have recently demonstrated that the expression of HERV-K(HML-2) Gag and Env proteins is induced upon HIV-1 infection of primary CD4+ T cells and that a HERV-K(HML-2) Env-specific CD8+ T-cell clone specifically eliminates cells infected with diverse isolates of HIV-1 (Jones et al., submitted for publication).

Here, we took a comprehensive approach to evaluating a potential role for HERV-K(HML-2) Gag- and Env-specific T-cell responses in natural control of HIV-1 infection. T-cell responses to 15mer peptides (manufactured at >70% purity), overlapping by 11 amino acids and spanning HERV-K(HML-2) Gag and Env, were measured by a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay. In contrast to our previous reports, which used peptides predicted to be optimal epitopes for common MHC-I alleles, this method allowed for the screening of subjects irrespective of their HLA type. Critically, we are also focusing on the Gag and Env antigens of the HML-2 lineage of HERVs, based on our evidence for HIV-1-induced expression of these proteins (Jones et al. submitted), whereas previous reports considered responses to diverse HERV families. ELISPOT assays were performed using standard procedures with the Mabtech IFN-γ ELISPOT assay. Briefly, cryopreserved cells were thawed and rested overnight in AIM-V medium (Invitrogen) supplemented with 60 U/ml of Benzonase (Sigma). Cells were then plated at 2 × 105 cells/well in AIM-V medium and cultured for 16 h with peptides (final concentration, 0.5% dimethyl sulfoxide [DMSO]), 0.5% DMSO alone, or 2 μg/ml staphylococcal enterotoxin B (SEB) (Sigma). Plates were then washed, probed with antibodies (Abs) following the manufacturer’s instructions, and developed with the AP color development reagent (Bio-Rad). Plates were dried overnight, and spots were counted using a CTL ImmunoSpot system. Criteria for positive responses are given in the legend of Fig. 1. Initially, we screened a number of HIV-1-infected and uninfected subjects for HERV-K(HML-2) Gag- and Env-specific CD8+ T-cell responses using master pools containing 172 peptides (Gag) or 164 peptides (Env) at 0.1 μg/ml/peptide, but we consistently observed a lack of responses. We rea-

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soned that HERV-K(HML-2)-specific CD8+ T-cell responses may be of lower avidity than those specific for exogenous viruses, such as HIV-1, as higher-avidity clones specific for HERV-K(HML-2) may have been deleted by thymic selection. We therefore moved to a higher-sensitivity approach that has been successfully applied for other antigens and arranged peptides into matrix pools of 10 to 18 peptides each (1, 4, 6, 11, 17, 22, 24). In addition to allowing us to test higher per-peptide concentrations, this approach allows for mapping of responses to an individual 15mer peptide. Peripheral blood mononuclear cells (PBMC) from HIV-1-infected subjects, comprising 11 chronic progressors, 9 vi-ral controllers, and 3 subjects in acute/early infection (see reference 15 for definitions), as well as 6 uninfected controls, were screened using these matrix pools (Table 1). We observed a lack of responses to HERV-K(HML-2) Gag and Env while detecting clear responses to HIV-1 Gag and cytomegalovirus (CMV) pp65 peptide pools. To further increase our ability to detect low-avidity responses, we rescreened these subjects with matrix pools at 10 μg/ml/peptide. Under these conditions, we observed 22 responses to HERV-K(HML-2) in HIV-1-infected subjects and a lack of responses in HIV-1-uninfected controls. When we attempted to confirm these responses, however, we observed that the majority of these proved irreproducible with newly synthesized batches of 15mer peptide (while responding to the original batch of individual 15mer peptides in parallel). Only three responses, all detected in viral controllers, were reproduced across multiple batches of peptides. One subject, number 77, made weak responses to one epitope each in Gag and Env: HERV-K(HML-2) Gag QSAIKPLK-GKVPAGS (mean, 60 spot-forming units [SFU]/million PBMC) and HERV-K(HML-2) Env HKMNKMVTSEEQMKL (mean, 65 SFU/million PBMC). This response has been subsequently fine-mapped to CIDSTFNWQHR (Jones et al., submitted).

Intriguingly, the 19 responses which proved irreproducible with new batches of peptides were detected exclusively in HIV-1-infected subjects. This led us to consider that perhaps they may be true HERV-K(HML-2)-specific CD8+ T-cell responses that are specific for some modified version of the relevant HERV-K(HML-2) peptide which was present in only the first batch of

FIG 1 T-cell responses can be mapped using matrix pools of HERV-K(HML-2) Gag- and Env-derived peptides. (A and B) Peripheral blood mononuclear cells (PBMC) from HIV-1-infected subject 125 were screened by IFN-γ ELISPOT using peptide matrix pools. Results are shown in mean spot forming units (SFU)/million PBMC. Tests were performed in duplicate, and error bars are standard deviations. Background levels were established by measuring responsiveness to 0.5% DMSO, and a pool of CMV pp65 peptides was included as a positive control. The horizontal line depicts the cutoff, with a positive response based on both (i) >3× background and (ii) >50 SFU/million PBMC after background subtraction. (A) The response to HERV-K(HML-2) Env peptide matrix pools 7 and 26 maps to HERV-K(HML-2) Env pep124. (B) The response to HERV-K(HML-2) Gag peptide matrix pools 9 and 21 maps to pep100, while the response to HERV-K(HML-2) Gag peptide matrix pools 4 and 23 maps to pep121. (C) IFN-γ ELISPOT results with 200,000 PBMC from subject 125/well are shown, confirming the response to the HERV-K(HML-2) Env pep124 15mer peptide common to matrix pools 7 and 23. The responses to these matrix pools are also shown in parallel. HK, HERV-K(HML-2).
peptides. To study this possibility, we generated CD8⁺ T-cell clones specific for HERV-K Env SVSVQSVNFVNDWQKN (peptide 124 [pep124]), HERV-K Gag GIGQNWSTISQQALM (pep100), and HERV-K Gag YENANPECQSAIPL (pep121) using a previously described method of IFN-γ capture followed by limiting dilution cloning (16). These responses had been detected from subject 125, an HIV-1-infected chronic progressor, by mapping, based on these preassembled matrix pools, which led us to test corresponding individual peptides, at which level these responses were confirmed. Second, the original peptides were shipped directly from the manufacturer in two separate aliquots to our labs in Toronto and San Francisco. The contaminant-specific T-cell clones responded to the corresponding HERV-K(HML-2) peptides at both sites. We propose that the contamination issues encountered in this study may be ones for which synthetic peptides are frequently produced at manufacturing facilities. Thus, in addition to iterating the need for caution, highlighted by others (7–9), in considering the possibility of foreign contaminants in peptide preparations, we emphasize a specific need to consider the possibility of HIV-1 peptide contaminants when screening HIV-1-infected subjects for responses to HERV-K(HML-2) and other non-HIV-1 antigens. This can be guarded against by testing at least a subset of identified responses against newly synthesized batches of peptide provided by a different supplier.

Our original presentation of HERV-specific T-cell responses in HIV-1-infected subjects (12) used different batches of peptides (from a different manufacturer) than those used for our follow-up studies (25). The fact that we observed comparable magnitudes and frequencies of responses between these studies demonstrates the reproducibility of these earlier results. The discrepancy between the infrequent detection of T-cell responses in the present study and the frequent HERV-specific T-cell responses that we have previously reported likely results from a number of factors.

#### Clinical data of subjects screened for HERV-K(HML-2)-specific T-cell responses

| Subject ID | Classification       | Viral load (copies/ml) | Absolute CD4 count (cells/mm³) | Estimated duration of infection (mos) |
|------------|----------------------|------------------------|-------------------------------|--------------------------------------|
| 1          | HIV negative         | NA                     | ND                            | NA                                   |
| 71         | HIV negative         | NA                     | ND                            | NA                                   |
| 176        | HIV negative         | NA                     | ND                            | NA                                   |
| 484        | HIV negative         | NA                     | ND                            | NA                                   |
| 651        | HIV negative         | NA                     | ND                            | NA                                   |
| 652        | HIV negative         | NA                     | ND                            | NA                                   |
| 2          | Acute/early infection| 332                    | 360                           | 2                                    |
| 314        | Acute/early infection| 10,560                 | 780                           | 2                                    |
| 348        | Acute/early infection| 4.00 × 10⁵             | 1,137                         | 1                                    |
| 7          | Chronic progressor   | 1.00 × 10⁶             | 240                           | 11                                   |
| 10         | Chronic progressor   | 22,415                 | 132                           | ND                                   |
| 12         | Chronic progressor   | 1.00 × 10⁶             | 510                           | ND                                   |
| 17         | Chronic progressor   | 4.00 × 10⁶             | 660                           | ND                                   |
| 19         | Chronic progressor   | 2.00 × 10⁶             | 600                           | ND                                   |
| 125        | Chronic progressor   | 1.00 × 10⁶             | 580                           | 90                                   |
| 378        | Chronic progressor   | 5.36 × 10⁶             | 460                           | 97                                   |
| 380        | Chronic progressor   | 1.34 × 10⁶             | 490                           | 66                                   |
| 411        | Chronic progressor   | 3.36 × 10⁶             | 430                           | ND                                   |
| 412        | Chronic progressor   | 1.45 × 10⁶             | 710                           | 86                                   |
| 415        | Chronic progressor   | 2.20 × 10⁶             | 530                           | ND                                   |
| 4          | Viral controller     | 499                    | 803                           | 120                                  |
| 9          | Viral controller     | <50                    | 760                           | ND                                   |
| 77         | Viral controller     | 79                     | 670                           | ND                                   |
| 148        | Viral controller     | <50                    | 1,070                         | ND                                   |
| 194        | Viral controller     | <50                    | 1,440                         | 97                                   |
| 374        | Viral controller     | <50                    | 990                           | 239                                  |
| 410        | Viral controller     | 876                    | 740                           | ND                                   |
| 419        | Viral controller     | 648                    | 390                           | ND                                   |
| 481        | Viral controller     | <50                    | 670                           | 35                                   |

a NA, not available; ND, not determined.

b ID, identification number.

c >1 year.

We tested the possibility that this peptide may be derived from HIV-1 by screening these clones with HIV-1 peptide matrix pools (NIH AIDS Research and Reference Reagent Program catalog numbers R117-Gag and 6208-Pol) and observed clear recognition of HIV-1 Gag peptide pools 5, 20, and 21 (Fig. 2B). The two 15mer peptides mapped by these responses, AAEWDRLHPVHAGPI and DRLHPVHAGPIAPGQ, share the HLA-B*35-restricted T-cell epitope HPVHAGPI, corresponding with the B35⁵ status of the subject. The possibility that these “clones” may be oligoclonal, with distinct subsets specific for the HERV-K(HML-2)-derived peptides and HPVHAGPI, was ruled out by flow cytometry experiments indicating responsiveness of the entire population to the original HERV-K(HML-2)-peptide preparations (Fig. 2C). Thus, the CD8⁺ T-cell responses identified using our original HERV-K(HML-2) peptides in subject 125 are specific for an HIV-1-derived peptide contaminant. We have since confirmed the presence of the HPVHAGPI peptide in these peptide preparations by nano-liquid chromatography (LC) Orbitrap mass spectrometry. While we did not identify a specific contaminant for the other 16 of the 19 irreproducible responses [observed against other HERV-K(HML-2) peptides], we extrapolate upon this result to propose that these may each be due to low-level HIV-1 peptide contaminants, explaining their exclusive presence in HIV-1-infected subjects.

We can rule out that the contamination occurred in our hands based on two lines of evidence. First, peptides were pooled by the manufacturer. It was mapping, based on these preassembled matrix pools, which led us to test corresponding individual peptides, at which level these responses were confirmed. Second, the original peptides were shipped directly from the manufacturer in two separate aliquots to our labs in Toronto and San Francisco. The contaminant-specific T-cell clones responded to the corresponding HERV-K(HML-2) peptides at both sites. We propose that the contamination issues encountered in this study may be ones for which synthetic peptides are frequently produced at manufacturing facilities. Thus, in addition to iterating the need for caution, highlighted by others (7–9), in considering the possibility of foreign contaminants in peptide preparations, we emphasize a specific need to consider the possibility of HIV-1 peptide contaminants when screening HIV-1-infected subjects for responses to HERV-K(HML-2) and other non-HIV-1 antigens. This can be guarded against by testing at least a subset of identified responses against newly synthesized batches of peptide provided by a different supplier.
Critically, in our initial study, we considered T-cell responses to diverse families of HERV, with only six peptides representing HERV-K(HML-2). Of these six, four were from Pol—an antigen which was not included in the present study. Additionally, in previous studies, we tested individual peptides predicted to be optimal epitopes in an HLA-matched manner. This is a more sensitive method than using pools of 15mers for detecting CD8\(^+\) T-cell responses (4, 6, 18). In the current study, we attempted to maximize the sensitivity of screening with 15mer peptides by testing up to 10 \(\mu g/ml\) peptide. Although this did yield some bona fide responses, these were in the minority. The bona fide and false HERV-K(HML-2)-specific T-cell responses detected in this study had titers that were determined to decrease similarly, losing responsiveness between 1 to 10 \(\mu g/ml\), confirming that we are unable to further boost sensitivity of 15mer screening without compromising specificity. As the use of predicted optimal epitopes is not amenable to comprehensive screening of subjects with diverse HLA types, alternative approaches, such as expansion of T cells with HERV-K(HML-2) gag and env mRNA-transfected dendritic cells, will be explored in future studies. At present, it is unclear whether the low frequency of HERV-K(HML-2) Gag- and Env-specific T-cell responses observed in the current study reflects the ineffectiveness of screening with 15mer peptides or whether it represents a true dearth of T-cell responses against the Env and Gag antigens of this particular lineage of HERV in natural HIV-1 infection. Given our recent demonstration of anti-HIV-1 activity by a HERV-K(HML-2)-specific T-cell clone (Jones et al. submitted), the finding that such responses are not commonly induced in natural infection would highlight the potential for intervention by HERV-K(HML-2)-targeted therapeutic vaccination strategies.

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