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Impact of HLA-B Alleles, Epitope Binding Affinity, Functional Avidity, and Viral Coinfection on the Immunodominance of Virus-Specific CTL Responses[1]

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Immunodominance is variably used to describe either the most frequently detectable response among tested individuals or the strongest response within a single individual, yet factors determining either inter- or intraindividual immunodominance are still poorly understood. More than 90 individuals were tested against 184 HIV- and 92 EBV-derived, previously defined CTL epitopes. The data show that HLA-B-restricted epitopes were significantly more frequently recognized than HLA-A- or HLA-C-restricted epitopes. HLA-B-restricted epitopes also induced responses of higher magnitude than did either HLA-A- or HLA-C-restricted epitopes, although this comparison only reached statistical significance for EBV epitopes. For both viruses, the magnitude and frequency of recognition were correlated with each other, but not with the epitope binding affinity to the restricting HLA allele. The presence or absence of HIV coinfection did not impact EBV epitope immunodominance patterns significantly. Peptide titration studies showed that the magnitude of responses was associated with high functional avidity, requiring low concentration of cognate peptide to respond in in vitro assays. The data support the important role of HLA-B alleles in antiviral immunity and afford a better understanding of the factors contributing to inter- and intraindividual immunodominance. ***The Journal of Immunology,*** **2006, 176: 4094–4101.**

The term "immunodominance" is as widely used as it is loosely defined. Most commonly, immunodominant B or T cell responses or Ags are referred to as those that can be most frequently detected in a group of individuals (frequency of recognition) or that induce the immune response of greatest magnitude (strength of response) within a single individual (1, 2). For all practical purposes, it would likely be advantageous to discriminate interindividual from intraindividual immunodominance, because the first one assesses the frequency of Ag recognition among a group of individuals expressing a certain HLA allele, whereas the second determines the relative magnitude among different responses in a single subject. However, even less clear than the definition of immunodominance are the factors that contribute to inter- or intraindividual dominance.

A number of parameters have been implicated in affecting one or the other form of immunodominance, including the nature of the restricting MHC allele (3, 4), efficiency of epitope processing and translocation into the endoplasmatic reticulum (5), the degree of sequence variability in epitopes derived from highly variable pathogens such as HIV (6, 7), and Ag availability by either cross-presentation of exogenous Ag or processing of intracellular (viral) proteins (8, 9). Besides these factors, interactions among different T cell populations and cross-reactivity between "self" and/or "other" pathogen Ags, as well as the presence of antagonistic epitopes, may further impact immunodominance patterns (10).

Of note, a previous study by Sette et al. (11) established a binding affinity threshold that was associated with the vast majority of known CTL epitopes. Studies in HLA transgenic mice confirmed the relevance of this threshold, while also indicating some correlation between affinity and the propensity to be immunogenic (12, 13). Furthermore, previous studies in the human system proposed a relationship between binding affinity and the magnitude and breadth of responses for variants of a single epitope but did not examine those relationships over a heterogeneous set of epitopes (7, 14). Finally, studies in the context of the more complex system of malaria infection indicate that, provided that a peptide can bind to a specific HLA molecule, subsequent antigenicity and immunogenicity may not directly correlate with the affinity of epitope binding per se. However, none of these preceding studies have directly addressed relationships between affinity and immunodominance systematically, and on a large scale. Thus, although considerable work has been invested in elucidating the most important factors determining immunodominance, many studies have either used a selected range of previously defined epitopes or have

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been limited to single MHC allele and epitope combinations (2, 7). Yet, a clear understanding of factors responsible for pronounced immunodominance patterns would greatly benefit vaccine design and provide deeper insight into mechanisms responsible for shaping pathogen-specific immune responses, as well as help to better understand as to how the immune system copes with the multitude of infections and exposures to potential pathogens over the lifetime of an individual (1, 10).

The present study was conducted to shed some light on the relevance of multiple factors in determining immunodominant CTL responses against two human pathogens, EBV and HIV. For both pathogens, single-epitope or HLA allele-specific immunodominance assessments have been performed in the past; however, such studies have often been limited to traditionally well-studied, HLA class I alleles such as HLA-A02 (2, 7, 15). Relatively little is thus known about immunodominant epitopes presented on alleles that are infrequent in traditionally well-studied, Caucasian-dominated cohorts. Similarly, the effects of viral coinfections and sequence variability, especially for highly variable pathogens such as HIV, are often not considered when determining immunodominance patterns, and potential differences among HLA-A-, HLA-B-, and HLA-C-restricted responses have not been addressed, despite some recent reports that point toward an important role of HLA-B alleles in mediating the most effective antiviral immunity (16). In this study, 276 previously defined, HIV- or EBV-derived CTL epitopes were used to stimulate PBMC from 135 HIV- and/or EBV-infected, fully HLA-typed individuals (17). Response patterns were recorded according to the described restricting HLA allele and compared with the HLA binding affinity of these epitopes as well as the magnitude and functional avidity of the responses. Although epitope binding affinity was not by itself associated with the dominance of responses, the functional avidity of responses was found to correlate with the magnitude of epitope-specific ELISPOT reactivity. The data also show a direct association between the magnitude and the frequency of epitope-specific responses and a dominant role of HLA-B alleles in restricting responses to these two viral pathogens.

Materials and Methods

Study subjects

Ninety-eight HIV-infected individuals were recruited from a previously described cohort in the Boston area (18). Fifty-four of these individuals, as well as 37 HIV-negative subjects, were tested for responses against a set of EBV-derived CTL epitopes (17, 19). The HIV-infected individuals were mostly (80%) treated with highly active antiretroviral therapy and presented with an overall median viral load of 330 copies/ml. There was no difference in the EBV response rates between the treated and untreated individuals (data not shown). For all 135 subjects, HLA typing was performed as described previously (18). The study was approved by the respective institutional review boards of all participating hospitals, and all subjects provided written informed consent before recruitment.

Assessment of CTL responses

PBMC were separated from whole blood and used in direct ex vivo ELISPOT assays as described (17). The peptide sets used consisted of 184 optimally defined HIV-derived, CTL epitopes included in the 2001 edition of the Los Alamos National Laboratory HIV Immunology Database CTL epitope list (20). The set of EBV-derived, CTL epitopes has largely been described (21) and completed with more recently identified epitopes and consisted of 92 reportedly HLA class I-restricted CTL epitopes (17). Cells were incubated overnight with single peptides, and ELISPOT plates were developed using Mabtech reagents detecting IFN-γ production by epitope-specific cells. The resulting number of spots was counted using the AID ELISPOT Reader Unit (Autoimmun Diagnostika), and results were expressed as spot-forming cells (SFC)[3] per million input cells. Thresholds for positive responses were determined as at least 5 spots (50 SFC/10^6) per well and responses exceeding "three times mean of negative wells" and "mean of negative wells plus three SDs."

Peptide titration assays to determine functional avidity

The functional avidity of responses was assessed by performing limiting peptide dilutions and determining the peptide concentration required to induce half-maximal responses in in vitro assays (22–24). Peptides were added in serial 10-fold dilutions ranging from 100 μg/ml to 10 pg/ml to ELISPOT plates and incubated with freshly isolated PBMC for 16 h. Half-maximal stimulatory Ag doses (SD_{50}) were determined as the peptide concentration needed to achieve a half-maximal number of spots in the ELISPOT.

Epitope binding to alleles in the HLA-A02, HLA-A03, HLA-B07, and HLA-B58 supertypes

A previously described HLA binding assay was used to determine binding affinities of all 276 peptides to a total of 16 alleles in the HLA-A02, HLA-A03, HLA-B07, and HLA-B58 supertypes (25, 26). The assay is based on the inhibition of binding of a radiolabeled standard probe peptide to detergent solubilized HLA class I molecules by the test peptide(s). Briefly, 1–10 nM radiolabeled probe peptide was coincubated for 2 days at room temperature with varying amounts of test peptide and fixed amount of class I molecules, in the presence of 1 mM β_2-microglobulin and protease inhibitors (25). The concentration of each peptide resulting in 50% inhibition of the binding of the radiolabeled index peptide was calculated and is reported as IC_{50} (nM).

Statistical analyses

Statistical analysis was done using GraphPad Prism version 3.0 for Macintosh, Excel (Microsoft), and custom C++ code. Results are generally presented as median values. Statistical analyses included Spearman test for correlations and Mann-Whitney *U*, Fisher's exact, Wilcoxon matched-pairs signed-ranks test, and χ^2 for comparisons among HLA-A, HLA-B, and HLA-C allele-restricted responses. The correlation analyses were performed using a corrected allele frequency, weighted to reflect the alleles' frequency in the tested cohort. In particular, the corrected frequency was taken to be the posterior mean of a β distribution given a prior with empirical mean equal to the uncorrected mean allele frequency in the tested cohort and a sample size equal to two (results were not sensitive to sample size).

Results

HLA-B restricts frequently targeted CTL epitopes in HIV and EBV

To identify dominant CTL epitopes in HIV and EBV and to investigate whether HLA-A, HLA-B, and HLA-C alleles restrict equally strong and frequent responses, a set of 276 previously described HIV- and EBV-restricted CTL epitopes was tested in 135 subjects (17). Of the 135 subjects, 98 individuals were HIV infected and were tested against 184 HIV-derived, optimally defined CTL epitopes listed in the Los Alamos National Laboratory HIV Immunology Database (20). Ninety-one individuals were EBV infected and tested against a panel of 92 CTL epitopes (17, 19). Of these 91 EBV-infected subjects, 54 individuals were coinfected with HIV and tested against both sets of peptides. For all epitopes, the fraction of epitope responders, among the individuals who expressed the described restricting HLA class I allele was recorded and compared for epitopes derived from HIV or EBV and epitopes restricted by either HLA-A, HLA-B, or HLA-C alleles.

For both peptide sets, complete lists of cohort-wide, interindividual immunodominance patterns and numbers of tested subjects expressing the specific HLA class I allele are included in supplementary Table I[4], providing an unprecedented assessment of interindividual epitope dominance for the known CTL epitopes derived from these two pathogens. Among all 184 HIV epitopes, 35

[3] Abbreviation used in this paper: SFC, spot-forming cell.

[4] The online version of this article contains supplemental material.

(19%) were recognized at frequencies ≥50%, with 5 epitopes targeted by 100% of the subjects expressing the appropriate restricting allele, including epitopes presented by HLA-A25, HLA-B07, HLA-B*1501, and HLA-B57. Similarly, 19 EBV epitopes (21%) were targeted by at least half of the individuals who expressed the appropriate HLA class I allele. Although none of these epitopes reached 100% frequency of recognition, two HLA-B08-restricted epitopes were each targeted by 90% of the individuals expressing HLA-B08 (see supplementary Table I*b*). Interestingly, among the 54 HIV and EBV epitopes with recognition frequencies ≥50%, significantly more peptides were HLA-B restricted than HLA-A restricted (34 vs 18, $p = 0.016$), despite the fact that overall, an essentially identical number of HLA-A- and HLA-B-restricted epitopes were tested (130 HLA-A restricted, 132 HLA-B restricted).

In contrast to EBV, HIV is characterized by a highly variable genome, which may affect the response rates to HIV CTL epitopes due to sequence differences between the autologous infecting virus and the test peptide sequence (2). To assess whether this could alter the present analyses, the frequency of recognition for all HIV-derived CTL epitopes was compared with the average entropy, a measure of viral diversity among HIV clade B sequences in the region of the CTL epitope (27). There was no difference among the median entropies for HLA-A-, HLA-B-, or HLA-C-restricted HIV CTL epitopes, indicating that restricting elements of all three loci present conserved as well as more variable epitopes (data not shown). However, there was an overall negative correlation ($p = 0.03$) between the entropy and the frequency of recognition, indicating that epitopes located in more variable parts of the viral genome are either intrinsically less immunogenic in vivo or that response rates against more variable epitopes are potentially underestimated due to differences between peptide test set and autologous virus sequences (7, 18, 28).

Overall, the data showed a wide range of frequency of recognition for both HIV- and EBV-derived CTL epitopes presented by HLA-A, HLA-B, and HLA-C. Interestingly, for both viruses, HLA-B-restricted epitopes were more frequently targeted than HLA-A- or HLA-C-restricted epitopes, although data included in Fig. 1 do not provide strong visual support for this conclusion. However, it is important to note that, because the various HLA alleles were present at different frequencies in the tested cohort, observed frequencies of recognition had, for statistical analyses, to be adjusted for allele frequencies. When adjusting the data in Fig. 1 for allele frequencies, a significantly stronger dominance pattern for HLA-B-restricted epitopes vs non-B-restricted epitopes was evident ($p = 0.00012$). In turn, HLA-A-restricted epitopes scored significantly less frequently than did the non-A-restricted epitopes ($p = 0.00005$), whereas HLA-C-restricted epitopes did not differ significantly from non-C-restricted epitopes ($p = 0.64$). A breakdown by virus showed significance of these comparisons for EBV-derived epitopes (A vs non-A, $p = 0.00013$; B vs non-B, $p = 0.00024$), whereas the association did not reach statistical significance for HIV epitopes (A vs non-A, $p = 0.048$; and B vs non-B, $p = 0.066$). Responses to HIV- and EBV-derived, HLA-A-, HLA-B-, or HLA-C-restricted epitopes also were analyzed on an individual subject basis. To this end, for each individual, the number of "expected" responses (i.e., the number of epitopes with known restrictions by the alleles expressed by the individual tested (19)) was separately compared with the number of detected responses against HLA-A-, HLA-B-, and HLA-C-restricted epitopes. The ratios of expected to detected responses was then compared for all individuals among HLA-A-, HLA-B-, and HLA-C-restricted epitopes. These analyses showed again that, overall, subjects recognized a significantly higher proportion of the HLA-B-restricted epitopes than HLA-A- ($p = 0.000014$) or HLA-C-restricted epitopes ($p = 0.00197$, Wilcoxon Matched-Pairs Signed-Ranks Test; data not shown). These findings are in line with reports that show HLA-B restriction for especially frequent responses such as HLA-B27- and HLA-B57-restricted responses to HIV epitopes and HLA-B08 for EBV epitopes (23, 29–31) and confirm some of our recent findings in larger HIV cohorts where HLA-B-restricted responses were found to dominate the antiviral immune responses (16, 22, 32, 33).

HLA-B alleles restrict stronger responses than HLA-A or HLA-C alleles

To address whether epitopes restricted by HLA-A, HLA-B, or HLA-C alleles differed not only in their interindividual dominance (frequency of recognition), but also in their intraindividual dominance patterns, the magnitude of all responses, expressed as Ag-specific cells per million PBMC, were compared among HLA-A-, HLA-B-, and HLA-C-restricted epitopes. Similar to the frequency analyses, HLA-B-restricted epitopes showed stronger responses than did non-B-restricted epitopes ($p = 0.0053$), whereas HLA-A

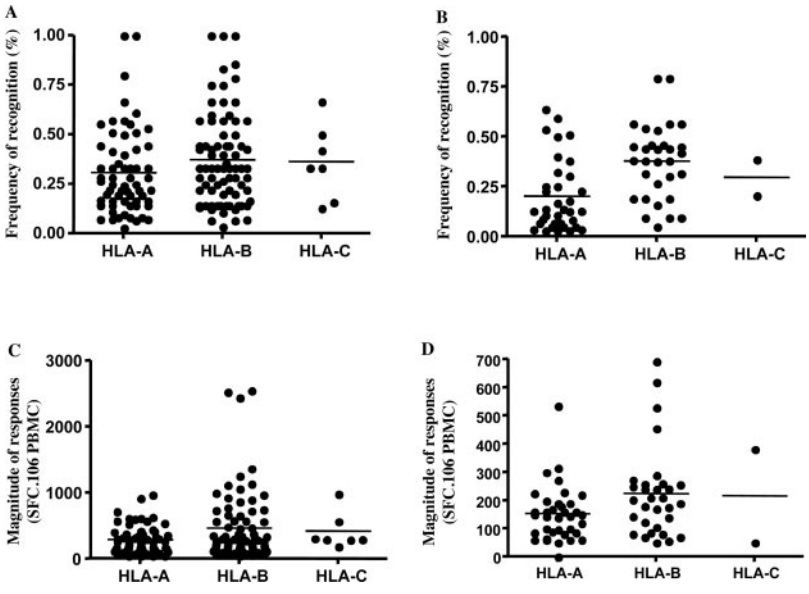

FIGURE 1. HLA-A-, HLA-B-, and HLA-C-restricted CTL epitopes differ in the frequency of recognition and magnitude of responses: Previously defined epitopes in HIV and EBV were tested in 98 and 91 subjects, respectively, and epitope-specific frequency of recognition among individuals expressing the described HLA allele was determined for HLA-A-, HLA-B-, and HLA-C-restricted epitopes derived from HIV (*A*) and EBV (*B*). The median magnitude of responses was calculated for all epitopes targeted at least once in the cohort and compared among HLA-A-, HLA-B-, and HLA-C-restricted epitopes and between HIV (*C*) and EBV (*D*) epitopes. Mann-Whitney *U* analysis was performed to compare epitopes restricted by the different loci.

restricted responses were weaker than non-HLA-A restricted responses ($p = 0.028$; Fig. 1, *C* and *D*). Because these data suggest that the frequency of recognition was associated with the magnitude of responses, allele-adjusted frequencies and median epitope-specific magnitudes were compared directly with each other. Although data in Fig. 2 show the direct, unadjusted values for frequency of recognition and magnitude of responses, the statistical analyses used a rank-order statistical approach using weight-adjusted frequency and magnitude values to accommodate differences in the HLA allele frequencies in the tested cohort. The analyses showed a significant direct association between intra- and interindividual immunodominance when HIV- and EBV-derived epitopes were analyzed separately (HIV, $p = 0.0031$; EBV, $p = 0.001$) or together ($p = 2.5 \times 10^{-6}$). Given the small number of epitopes for some alleles and differences in allele representation in the cohort, data are presented in a total analyses rather than in single allele-specific analyses, which, in some cases, showed statistical significance that withstood correction for multiple comparison (data not shown and Fig. 2). Thus, and although the scattering of data points in Fig. 2 is considerable, the present data demonstrate a statistically significant association between the magnitude of responses and their frequency of recognition. To rule out that this association was due to weaker responses falling more frequently under the detection limit than stronger responses, the analyses were repeated by using a higher ELISPOT cutoff and by limiting the analysis to the top 20% of epitopes (ranked by magnitude). Regardless of this correction, the analysis still yielded statistically significant associations even considering a much smaller data set (data not shown).

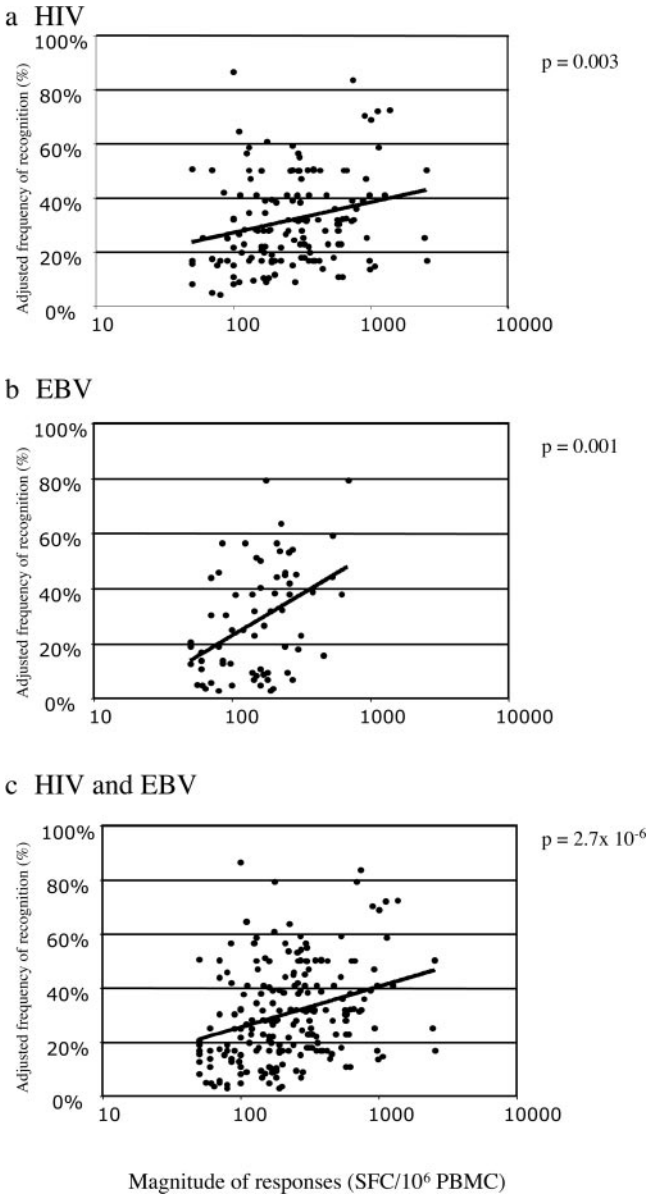

FIGURE 2. Correlation between magnitude of responses and frequency of recognition: The frequency of epitope recognition among individuals expressing the described, restricting HLA allele and the median magnitude of responses among the epitope responders were compared for HIV epitopes (*a*), EBV (*b*) epitopes and for both viruses together (*c*). Spearman correlations were performed using adjusted allele frequencies as described in *Materials and Methods*.

HLA binding affinity is not associated with immunodominance

To address whether intra- and interindividual immunodominance were associated with epitope binding to the restricting HLA class I molecule, binding affinities for 87 HIV- and 48 EBV-derived epitopes restricted by a total of 16 different alleles were compared with magnitude and the frequency of epitope-specific responses. Binding data were generated for HLA-A and HLA-B alleles as described in *Materials and Methods* and did not show significant differences between HIV and EBV epitopes (data not shown), indicating that both viruses yield CTL epitopes of comparable binding affinities. Analyzing the frequency of recognition and the median magnitude of all 135 epitopes for which binding data were available, no significant association was observed between binding affinity and magnitude or the frequency of recognition (Fig. 3, *A* and *B*). Among the 135 epitopes tested for binding, 41 did not show strong binding ($IC_{50} > 500$ nM) to their described, restricting allele. Although this could potentially indicate wrongly assigned HLA restriction alleles (34), there are numerous examples of well-defined and frequently targeted epitopes with binding affinities >500 nM (2, 7, 14). In fact, when looking at epitopes with $IC_{50} < 500$ or > 500 nM, there was no significant enrichment of rarely (<5%) or never-targeted epitopes in the group of epitopes with $IC_{50} > 500$ nM. In addition, focusing on the 94 good binders only, the results showed again that there was no significant association between binding and the frequency of recognition or the magnitude of responses (all $p > 0.3$).

Furthermore, to assess whether potential peptide binding competition to the same presenting HLA class I allele could affect immunodominance patterns (2), the HLA-A02 and HLA-B07 epitopes were analyzed separately, because these were the two HLA-A and HLA-B alleles with the most described epitopes available. The comparison of the binding affinities of the 43 HLA-A02-restricted epitopes did not reveal any association between binding and either the frequency of recognition, or the median magnitude of epitope-specific responses ($p = 0.38$ and $p = 0.33$, respectively). Similarly, no associations between these parameters and epitope binding affinity were found for the 20 HLA-B07-restricted epitopes ($p = 0.34$ and $p = 0.47$, respectively). As before, the analyses were repeated by including only those epitopes that showed good HLA binding ($IC_{50} < 500$ nM) to HLA-A02 or HLA-B07, respectively; again, the results did not change (all $p > 0.3$). These data are in line with the absence of an overall association between epitope binding and dominance and indicate that immunodominance patterns among epitopes presented by the same allele are not dictated by epitope binding alone.

The relationship between binding affinities and immunodominance was further analyzed for EBV-derived epitopes encoded by lytic or latent Ags. The rationale for this analysis was that high

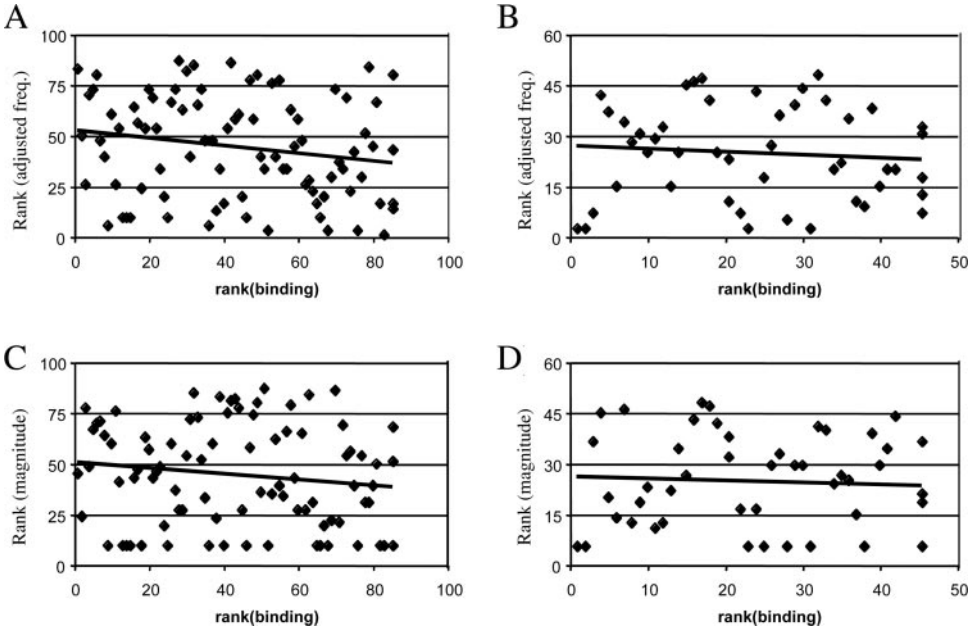

FIGURE 3. Epitope binding does not correlate with magnitude of response or frequency of recognition: Epitope binding affinities for 87 HIV epitopes and 48 EBV-derived epitopes were compared with frequency of recognition (*A* and *B*) or the median magnitude of response (*C* and *D*). Spearman correlations were performed using adjusted allele frequencies as described in *Materials and Methods*.

viral loads in acute EBV infection may theoretically drive responses of low avidity against highly expressed lytic Ags, whereas limited Ag availability in chronic infection may preferentially maintain high avidity responses against latent Ags expressed during the later stages of infection (19). No difference in the median binding affinities between lytic cycle ($n = 18$) and latent cycle ($n = 30$) epitopes was observed, indicating that lytic and latent EBV proteins contain epitopes of comparable binding affinities. Furthermore, there was no association between the magnitude of EBV lytic or latent Ag-specific responses and epitope binding affinity (data not shown). A comparison between lytic and latent epitope binding affinity and the frequency of recognition was not performed as previous work has shown a gradual shift from lytic to latent Ag-specific responses between acute and chronic stages of EBV infection would have biased such an analysis (19, 31).

EBV response patterns are not significantly altered by HIV coinfection

Because a fraction of the individuals tested for EBV-specific responses were HIV coinfected, the data allowed to investigate whether HIV coinfection could cause shifts in the EBV response patterns (35). All HIV-infected subjects also were EBV infected and likely acquired HIV after EBV, given that EBV infection most frequently occurs before adolescence. Thus, all HIV-positive individuals were considered chronically EBV infected; which also was the case for all HIV-negative individuals included here (19). The magnitude of EBV epitope-specific responses did not differ between the HIV-positive and the HIV-negative subjects ($p = 0.1$; data not shown), indicating that HIV infection did not drive more robust EBV responses or, alternatively, that the HIV-infected subjects were not significantly immune compromised in their EBV-specific immunity. When the overall frequencies of recognition of EBV epitopes were compared between the 54 HIV-positive and the 37 HIV-negative subjects, no significant direct correlation was observed, suggesting that some epitope response rates could be different between the two groups. Subsequent detailed analysis indeed identified two HLA-A02-restricted epitopes that were less frequently targeted in the HIV-positive, compared with the HIV-negative group (epitope FLYALALL in LMP2, $p = 0.015$; and epitope YVLDHLIVV in BRLF1, $p = 0.017$). However, the statistical significance was lost after correction for multiple comparisons. These data indicate that, although some fluctuations of response rates between HIV-infected and HIV-negative individuals may be observed, the number of individuals tested would likely need to be considerably increased to document potential statistically significant shifts in the response patterns of single epitopes upon HIV infection. Nevertheless, the data are in line with murine studies that show reduced memory responses to an initial viral infection upon infection with a heterologous second virus and provide some candidate epitopes on which to test this observation in the human setting (36).

Functional avidity is associated with magnitude of response but not with epitope binding affinity

Because HLA binding affinity alone does likely not reflect the affinity at which the TCR on the epitope-specific T cell interacts with the HLA/peptide complex (3), the functional avidity of HIV-specific responses was compared with peptide binding as well as magnitude and frequency of recognition. Functional avidity was determined by using serial peptide concentrations and defining the SD_{50} as the peptide concentration yielding half-maximal counts in ELISPOT assays (22, 24). Thus, these assays provide a measure of the overall avidity by which the epitope-specific T cells interact with cells presenting the cognate epitope. Given that peptide stability, epitope binding to HLA molecules, CD8 dependence, TCR density, and likely a number of other factors contribute to variable degrees to the overall functional avidity, this does not directly reflect the affinity by which the TCR on epitope-specific CTL interacts with the HLA/peptide complex (24, 37, 38). In order not to bias the analysis toward frequently targeted epitopes, and thus generally toward epitopes of higher magnitude (see above, Fig. 2), epitopes presented on less frequent alleles and emerging as subdominant responses were included as well. Thus, 21 HLA-A-, 19 HLA-B-, and five HLA-C-restricted epitopes were tested in the context of 22 different HLA alleles, with a total of 70 SD_{50} determined (Fig. 4). Overall, no differences in the SD_{50} for HLA-A-, HLA-B-, and HLA-C-restricted epitopes were observed (Fig. 4*A*). Similarly, when SD_{50} were compared with the epitope binding affinity, no associations became evident (Fig. 4*B*). However, a statistically significant association between SD_{50} and the magnitude

HLA restriction	Sequence	# indiv. tested	binding[1]	SD50% [2]	magnitude[2]
A01	YFPDWQNYT	2	ND[3]	5	685
A02	SLYNTVATL	4	9.1	0.05	335
A02	LVGPTPVNI	1	101	0.005	480
A02	KLTPLCVTL	1	ND	0.1	530
A02	ILKEPVHGV	1	ND	0.01	110
A02	LTFGWCFKLV	1	ND	0.05	60
A02	FLGKIWPSYK	1	40	5	200
A03	RLRDLLLIVTR	1	9.6	0.5	450
A03	AVDLSHFLK	1	0.65	0.5	620
A03	KTKPPLPSVKK	1	1.4	0.05	580
A11	AVDLSHFLK	1	5.9	0.5	50
A24	RYPLTFGW	2	ND	0.001	252
A24	LFCASDAKAY	1	ND	5	240
A24	RYLKDQQLL	1	ND	5	80
A24	YLKDQQLL	1	ND	0.005	170
A25	ETINEEAAEW	1	ND	0.005	380
A29	SFNCGGEFFY	2	ND	0.0001	855
A29	FNCGGEFFY	1	ND	0.1	80
A31	RLRDLLLIVTR	1	3.8	0.05	450
A32	PIQKETWETW	1	ND	1	88
A68	DTVLEEMNL	2	> 40000	0.5	565
B07	TPQDLNTML	2	4597	0.75	659
B07	RPNNNTRKSI	1	23	1	1075
B07	IPRRIRQGL	1	8.3	1	163
B07	TPGPGVRYPL	1	5.1	0.5	210
B1501	GLNKIVRMY	2	ND	0.25	510
B1516	SFNCGGEFF	1	ND	0.5	765
B2705	IRLRPGGKK	2	ND	0.5	520
B2705	KRWIILGLNK	2	ND	0.001	1510
B2705	GRRGWEALKY	2	ND	5	215
B35	WASRELERF	1	17432	0.01	220
B35	NPDIVIYQY	1	9.0	0.0005	180
B35	VPVWKEATTTL	1	10730	0.1	310
B44	AENLWVTVY	1	ND	5	160
B44	AEQASQDVKNW	2	ND	0.5	525
B51	EKEGKISKI	1	14059	0.1	270
B53	QASQEVKNW	5	9.7	0.1	595
B53	EPVDPRLEPW	4	54	0.075	405
B57	KAFSPEVIPMF	3	21	0.005	740
B57	QASQEVKNW	1	157	10	350
Cw04	QASQEVKNW	5	ND	0.05	755
Cw04	SFNCGGEFF	1	ND	0.5	640
Cw07	RRQDILDLWIY	1	ND	0.0005	460
Cw08	KAAVDLSHFL	2	ND	0.01	540
Cw08	RAEQASQEV	1	ND	0.01	220

1) Epitope binding is indicated as 50% inhibitopry concentration (nM)
2) Median values are indicated where multiple individuals were tested
3) ND indicates "not done"

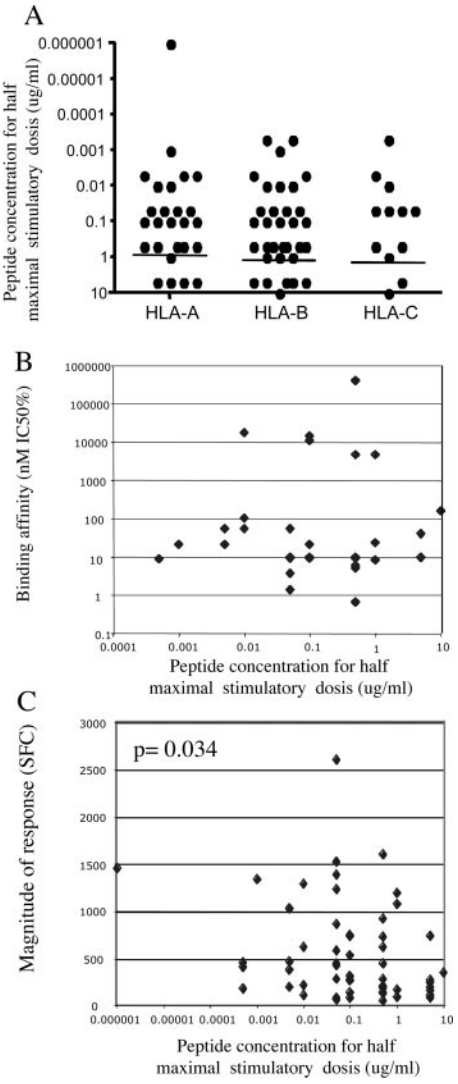

FIGURE 4. Functional avidity, but not HLA binding, is associated with magnitude of recognition: The functional avidity, defined as the peptide concentration required to achieve half-maximal reactivity (SFC/10^6 PBMC) in ELISPOT assays was determined for 46 different HIV epitopes, partially tested multiple times as indicated in the table (*left*). Functional avidities in HLA-A-, HLA-B-, and HLA-C-restricted epitopes were compared among each other (*A*), to epitope binding affinities (*B*), or to magnitude of responses (*C*). *A–C* contain all 70 data points, whereas the table reflects median magnitudes and SD_{50} in cases where multiple individuals were tested for the same epitope.

of response was observed ($p = 0.028$; Fig. 4*C*). The association with the magnitude of response was not observed anymore when the epitope HLA-binding data was factored in and compared as the product of avidity and peptide binding affinities (data not shown, $p = 0.23$). Together, the data indicate that binding affinity alone does not determine the functional avidity of epitope recognition, and that the overall avidity with which CTL and APC interact may play an important role in defining the magnitude of responses (38).

Discussion

The present study compared the impact of HLA-allele usage, functional avidity, HLA binding affinity, and viral coinfection on the interand intraindividual immunodominance of CTL responses against HIV- and EBV-derived, HLA class I-restricted epitopes. These data show that magnitude and frequency of recognition, the two major aspects of immunodominance, are related to each other, and that functional avidity, reflecting TCR avidity to HLA/peptide complexes, is a more important determinant for the magnitude of responses than the peptide binding affinity to HLA molecules. The studies also reveal that, overall, HLA-B alleles are more frequently inducing detectable responses than either HLA-A or HLA-C alleles, and that these responses are generally of greater magnitude than responses restricted by molecules of the HLA-A or HLA-C loci. These findings are in line with a recent report indicating that HLA-B-restricted CTL responses carry the bulk of the immune response against HIV (16). The data presented here strongly suggest that this phenomenon may be expanded to other viral infections, because EBV-derived epitopes were also found to induce more frequent and stronger responses when presented on HLA-B than on HLA-A or HLA-C alleles. The importance of HLA-B alleles has also been documented recently in influenza infection, where HLA-B08-restricted responses dominated the antiviral immune response (39). Moreover, this is in agreement with the observation that HLA-B alleles, more so than HLA-A and HLA-C alleles, have been associated with slower HIV disease progression, and further supports the notion that the HLA-B locus evolved under strong selective pressure (16, 33). However, as shown in a recent study from our laboratory on HLA-B*1503-restricted CTL responses, dominant responses are not necessarily the ones mediating immune control, and a better understanding of immunodominance patterns may allow for the further discrimination of beneficial from less favorable responses for inclusion in vaccine design (32, 33).

An improved understanding of immunodominance patterns also may be helpful to identify more or less immunogenic variants of the same epitope, especially when studying viral pathogens with a high sequence diversity. For instance, a recent study on HLA-A*0201-restricted responses in individuals followed from acute HIV infection showed that among two common epitope sequence variants in HIV Vpr, only one was able to induce responses in vivo

(7). In this case, epitope binding to the restricting HLA allele was diminished for the less immunogenic variant, suggesting that epitope binding may contribute to its reduced immunogenicity. Although this is in line with earlier reports, the present study did not find a direct association between HLA binding and either frequency of recognition or magnitude of responses (2, 29). Rather, the magnitude of responses was directly associated with the functional avidity, indicating that the affinity with which the TCR of the epitope-specific T cell interact with the HLA/peptide complex has a more pronounced impact on the magnitude of responses than epitope binding alone or the relative surface expression of proteins encoded by the HLA-A, HLA-B, or HLA-C loci, which also might impact the magnitude of responses (3, 40). However, this observation does not diminish the potential important role of epitope binding, as illustrated in the above HIV Vpr example, where reduced binding corresponded to a lack of in vivo immunogenicity. It is thus conceivable that epitope binding may be a crucial factor for the de novo induction of the response, whereas subsequent selection of high-avidity TCR populations are then determining its magnitude (3). This also would be in agreement with the finding that epitope binding affinity is a useful parameter for epitope prediction approaches, which has allowed for the identification of novel CTL epitopes in essentially all closely studied viruses (41–44). In the present study, initial comparisons between epitope binding and magnitude and frequency of responses were repeated by limiting the analysis to only those epitopes which showed strong binding to their described, restricting HLA class I allele. These control analyses were performed to account for the possibility that some of the epitopes tested here can be presented on more than one allele leading to epitope-specific responses to occur on additional HLA restriction elements expressed by the same subject and that some of the described HLA restrictions may be erroneous (34, 45, 46). However, these analyses showed no association between epitope binding affinity and the frequency or magnitude of responses; again suggesting that epitope binding is not predictive of the strength and frequency of the detected responses. This is of special relevance for epitopes that have been derived by epitope prediction approaches and which may have yielded Ag sequences with limited relevance for antiviral defense. However, rare recognition and poor binding were not restricted to those alleles that have frequently been used in epitope prediction approaches and included HLA-B-restricted epitopes that were never targeted in the tested cohort.

The direct correlation between the magnitude of responses and the frequency of recognition initially raised concerns that less frequent responses scored less often because they would more frequently fall under the detection limit of the ELISPOT assays that were used. However, limiting this analysis to the 20% of epitopes with the highest magnitude of response did not change the outcome, reflecting the fact that 90% of all median magnitudes of responses were >100 SFC/10^6 PBMC and thus well above cutoff. Furthermore, it is important to note that the screening ELISPOT assays were performed with likely saturating peptide concentrations, so that weaker responses were not missed due to their reduced functional avidity. Together, although these considerations cannot conclusively rule out that some weaker responses did indeed get lost in our screenings, they strongly support the notion that the magnitude of responses is directly associated with the frequency of recognition.

Overall, the present data provide an extensive immunodominance analysis of previously described, HIV- and EBV-derived CTL epitopes, demonstrating that inter- and intraindividual dominance are closely linked and that HLA-B-restricted CTL epitopes are targeted more frequently and with higher magnitudes than non-B-restricted CTL targets. These associations were statistically highly significant for EBV but failed to reach statistical significance for the HIV epitopes. Although previously published data from our lab (16) strongly support an important role of HLA-B in the response to HIV as well, the weaker associations seen in this study may have resulted from viral adaptation to epitopes restricted by some of the most frequent alleles in the cohort (32). Nevertheless, the data also show that the magnitude of responses was more closely linked to the functional avidity of the response than to the affinity with which the epitope binds its restricting HLA allele, suggesting that TCR interactions with the epitope/MHC complex have a profound effect on the strength of responses. Finally, the observation that HLA-B alleles restrict a significant portion of the antiviral CTL response to EBV, HIV (this study and Ref. 16), as well as influenza virus (39), highlights the importance of this most diverse HLA class I locus in host defense and provides valuable guidance for future vaccine design, where immunodominance patterns will need to be considered (47, 48).

Disclosures

The authors have no financial conflict of interest.

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