T Cell Receptor Recognition of MHC Class II-Bound Peptide Flanking Residues Enhances Immunogenicity and Results in Altered TCR V Region Usage

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Summary

Naturally processed MHC class II-bound peptides possess ragged NH2 and COOH termini. It is not known whether these peptide flanking residues (PFRs), which lie outside the MHC anchor residues, are recognized by the TCR or influence immunogenicity. Here we analyzed T cell responses to the COOH-terminal PFR of the H-2Ak immunodominant epitope of hen egg lysozyme (HEL) 52–61. Surprisingly, the majority of T cells were completely dependent on, and specific for, the COOH-terminal PFR of the immunogen. In addition, there were striking correlations between TCR Vβ usage and PFR dependence. We hypothesize that the Vα CDR1 region recognizes NH2-terminal PFRs, while the Vβ CDR1 region recognizes COOH-terminal PFRs. Last, peptides containing PFRs were considerably more immunogenic and mediated a greater recall response to the HEL protein. These results demonstrate that PFRs, which are a unique characteristic of peptides bound to MHC class II molecules, can have a profound effect on TCR recognition and T cell function. These data may have important implications for peptide-based immunotherapy and vaccine development.

Introduction

T cells can recognize antigenic peptides only in the context of major histocompatibility complex (MHC) class I or class II molecules (reviewed by Townsend and Bodmer, 1989; Chien and Davis, 1993). Significant progress has been made in the elucidation of MHC structure and the characterization of MHC-bound peptides (reviewed by Rotzschke and Falk, 1992; Stern and Wiley, 1994). MHC class I molecules require peptides for correct folding, and they form conserved interactions with the NH2 and COOH-terminal ends of the peptide (Townsend et al., 1990; Fremont et al., 1992; Latron et al., 1992; Madden et al., 1992, 1993; Matsumura et al., 1992). This intrinsic requirement is evident from both structural and peptide elution studies and places strict constraints on peptide length (8–10 amino acids for the majority of peptides).

In contrast, MHC class II molecules are not dependent on peptides for folding, and their binding groove is open at both ends (Brown et al., 1993; Stern and Wiley, 1994; Stern et al., 1994; Fremont et al., 1996). These features allow MHC class II molecules to present antigenic epitopes as large nested sets of peptides of variable length (10–35 amino acids) (Rudensky et al., 1991; Chicz et al., 1992, 1993; Hunt et al., 1992). Structural studies of MHC class II:peptide complexes have shown that on either side of the minimal MHC-binding epitope there can be at least two peptide flanking residues (PFRs) that are within the MHC groove and thus potentially accessible to the T cell receptor (TCR) (Stern et al., 1994; Fremont et al., 1996).

The H-2Aα-restricted immunodominant epitope of hen egg white lysozyme (HEL), 52–61, has been used extensively to study TCR recognition (Allen et al., 1984, 1985). The putative MHC anchor residues for this epitope have been assigned by functional and binding studies as D52 and to a lesser extent I58 and R61, thus defining the minimal epitope as 52–61 (Figure 1) (Allen et al., 1987; Nelson et al., 1996). All T cells specific for this peptide are dependent on L56, since this is the only residue in this sequence that differs between HEL and mouse lysozyme (ML) (Allen et al., 1987). This amino acid has therefore been defined as the primary TCR contact residue. Some TCRs require the conserved residues Y53 and/or Q57 for effective recognition. However, T cell dependence on these residues is variable, and so they are referred to as secondary contact residues. Of the four PFRs (S50, T51, W62, and W63), W62 is the only residue that differs between HEL and ML (Figure 1).

Our previous studies have shown that certain murine T cell hybridomas, which lack CD4, require the COOH-terminal PFR W62:W63 for recognition (Vignali and Strominger, 1994a, 1994b). Although peptides lacking these residues failed to stimulate interleukin-2 (IL-2) secretion, they did induce partial CD3 tyrosine phosphorylation, reminiscent of observations made with T cell clones anergized by altered peptide ligands (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Furthermore, the WW PFRs did not affect the affinity of HEL 48–61 for H-2Aα or MHC stability, as determined by floppiness versus compact dimer formation, suggesting that they do not influence MHC structure (Nelson et al., 1993, 1994; Vignali and Strominger, 1994b). The analysis of naturally processed, H-2Aα-eluted peptides from HEL-pulsed B cells has shown that peptides can be generated both with and without these tryptophan residues (Nelson et al., 1992; Vignali et al., 1993).

In the present study, we asked whether the TCR can directly recognize PFRs and examined the consequence of this interaction on peptide immunogenicity, T cell function, and repertoire selection.

Results

PFRs Can Act as Dominant TCR Contact Residues

T cell hybridomas were generated from HEL 48–63-immunized B10.BR mice and were selected according to their ability to respond to both the immunizing peptide...
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Figure 1. The Immunodominant Epitope of HEL for H-2A<sup>k</sup>

A model of the HEL 48±63 peptide used in this study. The homologous murine sequence (ML) is also shown. The numbers refer to the HEL residues; ML residue numbers are one greater than the number shown. The dark shaded box encompasses the putative minimal MHC binding epitope, with arrows indicating residues that have been suggested to bind either the MHC or TCR molecules by functional studies (Allen et al., 1987; Nelson et al., 1996). While there is strong evidence for R52 binding to the NH<sub>2</sub>-terminal, P1 pocket of H-2A<sup>k</sup> molecules, it is unclear whether S60 or R61 binds to the COOH-terminal, P9/P10 pocket. The large arrow above L56 highlights the immunodominant residue for the peptide. The open boxes highlight the PFRs that we predict would be within the MHC groove by extrapolation from current MHC class II:peptide complexes (Stern et al., 1994; Fremont et al., 1996).

and the native HEL protein (data not shown). All 34 hybridomas failed to produce IL-2 in response to the peptide analog HEL 48-L56A-63 (data not shown). This finding is consistent with previous studies demonstrating that L56 is a primary TCR contact residue for this epitope (Allen et al., 1987). Surprisingly, two thirds of this panel (65%; 22 of 34) were also highly dependent on the PFR W62:W63, while the remaining third were completely independent of these residues. For instance, the WW-independent hybridomas WW148 and WW161 responded strongly to HEL 48±61 (Figure 2A). In contrast, the WW-dependent hybridomas WW241 and WW245 failed to produce any IL-2 with the highest concentration of HEL 48±61, even though they responded to low concentrations of HEL 48±63 (Figure 2A). Because the WW PFRs do not alter peptide affinity or MHC stability, they are unlikely to affect MHC structure (Nelson et al., 1993, 1994; Vignali and Strominger, 1994b). It is noteworthy that the hybridomas were either completely dependent or independent of the PFR, with none showing a partial response to HEL 48±61.

Previous studies have shown that a relatively low percentage (~1%) of MHC class II molecules are loaded with peptides derived from either exogenous antigen or synthetic peptide (Vignali et al., 1993). Thus, the inability of WW-dependent hybridomas to respond to HEL 48±61 may be due to low ligand density on the surface of the antigen-presenting cell (APC). To examine this possibility, we produced APCs expressing a single, defined MHC:peptide complex, resulting in a substantially higher percentage of the MHC class II molecules loaded with a defined peptide (Kozono et al., 1994). The A20.J B cell line was transfected with constructs encoding the wild-type H-2A<sup>a</sup> chain and the H-2A<sup>b</sup> chain recombinantly attached to either HEL 48±63, HEL 48±61AA, or HEL 48-L56A-61 (referred to here as CAP [covalently attached peptide] transfectants). As expected, the WW-independent hybridomas WW148 and WW161 responded strongly to both the HEL.48-63.CAP and HEL.48-61AA.CAP transfectants (Figure 2A; righthand bars). Even though these transfectants possessed ligand densities substantially higher than conventional peptide-pulsed APCs, the WW-dependent hybridomas WW241 and WW245 still failed to respond to HEL.48-61AA.CAP while responding strongly to the HEL.48-63.CAP transfectants (Figure 2A). None of the hybridomas responded to the HEL.48-L56A-63.CAP transfectants (data not shown). Thus, the WW-dependent hybridomas were completely unable to respond to HEL 48±61 regardless of ligand density.

Can any amino acid mediate the production of PFR-dependent T cells? This question was initially addressed by generating hybridomas from mice immunized with a HEL 48-63 analog peptide in which the tryptophan residues were replaced with lysine (HEL 48-61KK). Strikingly, the majority of the hybridomas (68%; 32 of 47)
We then determined which of the two PFRs were crucial for TCR recognition. Nine of ten WW-dependent hybridomas responded with equivalent sensitivities to HEL 48–63 and the WA analog peptide, thus having an \( \frac{EC_{50}}{EC_{50}} \) ratio of \( \approx 1 \) (Figure 3A). Their response was comparable to that of the four WW-independent hybridomas tested. However, they failed to recognize the AW peptide, demonstrating that TCR recognition is focused toward W62. The tenth hybridoma, WW242, did not respond to either peptide analog and was thus affected by substitutions at both W62 and W63. Of note, W62 but not W63 differs between HEL and ML (Figure 1). The lack of recognition of W63 may be due either to tolerance induction by the ML peptide or to the inaccessibility of the W63 residue.

In contrast, the KK-dependent hybridomas were affected by substitutions at both PFRs and displayed a more complex pattern of recognition (Figure 3B). One third of the hybridomas (34%; 11 of 32) did not respond to either the AK or the KA analog peptides and thus required both residues for recognition. Another third (34%; 11 of 32) were able to respond fully to only one of the two analog peptides, while the remaining third possessed a phenotype between these two extremes. No preference was observed between the two PFRs, since similar numbers of hybridomas responded to AK only (6 of 11) and KA only (5 of 11). Therefore, the TCR is equally capable of recognizing either of the two COOH-terminal PFRs.

Taken together, these data argue against the possibility that the TCR is merely recognizing a peptide-induced conformational change in the MHC molecule, and instead imply stringent requirements for the recognition of PFRs.

Recognition of PFRs Skews the T Cell Repertoire

There is now substantial structural and functional evidence demonstrating that complementarity-determining region 3 (CDR3) of the TCR, encoded by the V(D)J junction, interacts with the central peptide residues (Jørgensen et al., 1992; Garbozi et al., 1996; Garcia et al., 1996). Furthermore, recent studies have shown that CDR1 and CDR2, which are encoded within the V regions, can also recognize peptide residues in addition to the surrounding MHC molecule (Sant’Angelo et al., 1996). We reasoned that PFRs, which are at the extremity of the MHC groove, would be recognized by the TCR V region and thus result in skewed TCR usage.

To test this notion, we first determined TCR Vα and Vβ gene usage by flow cytometry, polymerase chain reaction (PCR), and DNA sequencing. Analysis of the TCRα Vβ junctional sequence showed that only 3 of 29 WW-dependent and WW-independent hybridomas analyzed were identical (Table 1 and data not shown). Preferential usage of Vα4 and Vα11 was observed with both the WW-dependent and WW-independent hybridomas (Figure 4A). However, no obvious correlation was noted between PFR dependence and Vα gene usage.

In contrast, a dramatic segregation of TCR Vβ gene usage was observed (Figure 4B). A substantial proportion of the WW-dependent hybridomas (80%; 18 of 22) were either Vβ8.3+ (7) or Vβ14+ (11), while two thirds of...
Table 1. TCR Sequence Analysis of HEL 48-63 and 48-63KK-Specific Hybridomas

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TCR usage determined by flow cytometry, reverse transcription-PCR, and DNA sequencing as described in Experimental Procedures. Hybridomas are grouped according to their PFR dependency and specificity. Each group is ordered according to their sensitivity to the immunogen (in descending order). Three WW-dependent and two KK-dependent hybridomas that were found to be identical to those listed are not shown. WW142 and WW133 have identical amino acid sequences but distinct DNA sequences. *Vα11.2 and Vα11.1/6 defined by recognition by RR8.1 (Vα11.1, 11.2), which appears not to recognize Vα11.4. ²WW241 has two in-frame TCRα chains; this hybridoma is KT65 (1) KT50 (2), each antibody recognizes a subset of Vα8 TCR; the expression of Vα16.1 is unknown. ³Subfamily member is either 8.6, 8.8, 8.12, or 8.13. §Unique amino acid and DNA sequence distinct from other Vα4 family members with Y89F, thus designated Vα4.13.
3. Second, TCR Vα may alter the position of TCR Vβ, thus allowing for specific recognition of either the WW or KK PFR. Third, different residues in CDR1β recognize the WW and KK PFRs. In summary, these data show that different PFRs mediate the selection of T cells expressing distinct TCR Vβ elements, supporting the notion that the TCR directly recognizes PFRs.

**PFRs Potentiate T Cell Responses**

Given the high percentage of T cell hybridomas that were dependent on PFRs, we reasoned that peptides possessing such residues might be more immunogenic. To test this possibility, B10.BR mice were immunized with either HEL protein, HEL 48–63, or HEL 48–61, and lymph node T cells were tested for their ability to proliferate in response to all three immunogens (Figure 5A). Three important observations were made. First, HEL 48–63 was as potent an immunogen as the HEL protein. However, T cells from mice immunized with HEL 48–61 responded poorly to their immunogen. Second, mice immunized with HEL or HEL 48–63 generated a much stronger recall response to 48–63 versus 48–61, while the response to the two peptides was identical in HEL 48–61–immunized mice. The former was also observed in C3H.HeJ mice (data not shown). Third, the recall response to HEL protein with T cells from HEL 48–63–immunized mice was almost as good as it was with T cells from HEL-immunized mice. In contrast, T cells from HEL 48–61–immunized mice responded very weakly to HEL. Taken together, these data indicate that PFRs enhance peptide immunogenicity.

We next tested whether PFRs influence the proportion of T cells generated following peptide immunization and expansion in vitro, and/or influence their ability to be reactivated. T cells from peptide immunized mice were expanded in vitro with the immunogen and rested for 11 days. After this period, all of the cells expressed low levels of the activation markers CD69 and CD25 (IL-2Rα). We then determined the percentage of CD25+ T cells among the CD4+ T cell population following restimulation with either the immunogen or HEL 48–61. Substantially higher percentages of T cells from HEL 48–63, 48–61KK−, and 48–61EE−immunized mice (43%, 58%, and 45%, respectively) were reactivated (CD25+) in response to their respective immunogens than T cells from HEL 48–61–immunized mice (29%) (Figure 5B). Furthermore, only 16%–26% of the T cells from HEL 48–63−, 48–61KK−, and 48–61EE−immunized mice could be reactivated with HEL 48–61. Similar results were obtained when CD69 expression was used as a measure of reactivation (data not shown). These data confirm the hybridoma data and suggest that more than half of the T cells generated were dependent on PFRs (dependent on WW, 41%; KK, 54%; and EE, 63%). Formal frequency analysis will need to be performed to establish whether PFRs increase the size of the available precursor population.

The T cell response to HEL 48–61 is focused on L56, since this is the only central peptide residue that differs between ML and HEL and is thus referred to as the immunodominant residue. To test this question, the L56 residue was substituted for an arginine residue, since positively charged peptide residues promoted TCR recognition of PFRs.

The diagonal solid lines indicate where the EC50 values in response to peptides on the x- and y-axes are identical (i.e., the addition of PFRs neither increases nor decreases the response to HEL 48–61). The dashed lines are 1 log10 to either side of the solid line. Hybridomas within the dashed lines are considered PFR independent. See Experimental Procedures for protocol.
peptides, confirming the close association between this unclear whether the TCR can directly recognize these have a significant effect on peptide immunogenicity. Previous studies have shown that NH$_2$-terminal PFRs reactivated 5% fewer cells than HEL 48–63 (37% vs. tide immunogenicity and/or T cell repertoire selection expected, significant expansion of V$_b$9 usage using a panel of monoclonal antibodies (see Experimental Procedures). The percent-ages of T cells reactivated with HEL 48–61. To test this, an experiment similar to that shown in Figure 6A was performed, except that the T cells were reactivated with either the immunogen or HEL 48–61 (Figure 6B). As previously shown, only HEL 48–61KK mediated the preferential expansion of V$_b$14 $^+$ T cells. However, the percentage of T cells reactivated by HEL 48–61 was comparable regardless of the immunogen. Likewise, HEL 48–61 failed to reactivate a substantial proportion of the V$_b$14 $^+$ T cells expanded by either HEL 48–63 or 48–61KK. These data show that most of the T cells generated as a consequence of PFRs are functionally dependent on them.

**Discussion**

Analysis of MHC class II-bound peptides has shown that immunogenic epitopes are presented as large nested sets that vary in length at the NH$_2$ and COOH termini, a characteristic not shared by MHC class I-bound peptides (Urban et al., 1993). This raises two important questions. First, what effect do PFRs have on TCR recognition, and second, do these residues influence peptide immunogenicity and/or T cell repertoire selection and function?

Previous studies have shown that NH$_2$-terminal PFRs can affect MHC class II stability, thus extending the lifespan of the complex and prolonging T cell stimulation (Nelson et al., 1993, 1994). Our previous studies have shown that T cell function, in the absence of CD4, could be significantly affected by the truncation of COOH-terminal PFRs (Vignali and Strominger, 1994b). More recently, residues flanking a measles virus fusion protein were found to influence the responsiveness of some human T cell clones (Muller et al., 1996). However, it was not clear what effect these residues had on MHC class II stability, and there were inconsistencies in the assignment of MHC binding residues when compared with the previously published DR1 motif (Rammensee et al., 1995). While all of these studies highlight the potential influence of PFRs on T cell function, it is still unclear whether the TCR can directly recognize these residues and what the consequence of such an interaction might be.
It has been suggested that MHC molecules loaded with synthetic peptides are antigenically distinct from those loaded with peptides derived from naturally processed protein (Viner et al., 1996). These findings could not account for our data, since the WW-dependent T cell hybridomas responded strongly to native HEL. We also tested the ability of WW- and KK-dependent hybridomas to respond to HEL mutants produced in bacteria, where the W62:W63 residues were replaced with either lysine or alanine. The WW- and KK-dependent hybridomas responded strongly to recombinant wild-type HEL or a HEL.W62K:W63K mutant, respectively, but did not respond to a HEL.W62A:W63A mutant (R. T. C. and D. A. A. V., unpublished data). Taken together, these data demonstrate that the hybridomas used in this study could not distinguish between synthetic peptides and the whole protein.

Could the data presented here be explained by HEL 48–61XX binding to H-2A\(^+\) with a different register than 48–61? There are several reasons why this is unlikely. First, 48–63 and 48–61 both bind with similar affinities, and W62 does not affect affinity or MHC stability (Nelson et al., 1996). Second, PFR-independent T cell hybridomas respond comparably to 48–63 and 48–61 (Figure 2). Third, T cells induced by 48–63 also respond to HEL. Fourth, D52 is critical for binding to H-2A\(^+\), and so any shift in register is highly unlikely.

The data presented here demonstrate that PFRs have a profound effect on T cell function and repertoire selection. The majority of T cells, tested either as hybridomas or as T cell lines, were completely dependent on PFRs despite their ability to respond to low concentrations of peptide. We had previously observed such dependence on PFRs only in the absence of CD4 (Vignali and Strominger, 1994b). However, all of the T cells used in the present study expressed CD4. Dependence on PFRs could be due either to direct recognition by the TCR or to alteration by the PFR of the conformation of the MHC class II:peptide complex. Evidence in favor of a direct interaction between the TCR and PFR comes from studies with peptide analogs and analysis of TCR V\(\beta\) usage. The data show that the majority of hybridomas dependent on PFRs would not tolerate any amino acid substitutions at these positions. In addition, all of the WW-dependent hybridomas were dependent on W62 rather than W63, while the KK-dependent hybridomas displayed a mosaic of preferences for either residue. The

**Figure 6. Distinct Patterns of TCR V\(\beta\) Usage Are Observed with Peptides Containing Different PFRs**

(A) TCR V\(\beta\) expression of the CD25\(^+\) T cells described in Figure 5B was determined by flow cytometry. The peptides used for immunization and in vitro restimulation are indicated in the top left corner of each bar chart. Data are presented as the percentages of reactivated, CD4\(^+\)CD25\(^+\) T cells and the means of two or three experiments ± SEM for each of the V\(\beta\) elements indicated. “Other” represents the percentage of cells for which antibodies are not available (V\(\beta\)1, V\(\beta\)15, V\(\beta\)16, V\(\beta\)18, and V\(\beta\)20) and was determined by subtraction of all of the V\(\beta\)j populations indicated from the total number of reactivated cells. The percentage of CD4\(^+\)V\(\beta\)5\(^+\) and CD4\(^+\)V\(\beta\)11\(^+\) T cells was typically <1% and is not shown. See Experimental Procedures for detailed protocol. The dashed horizontal lines in the 48–61 bar chart indicate the percentage of CD4\(^+\) T cells in normal B10.BR mice expressing the TCR V\(\beta\) elements indicated. This is used as a benchmark for evaluating the effect of HEL 48–61 immunization on V\(\beta\) repertoire selection. The bars filled with a diagonal pattern indicate V\(\beta\)j elements that are >1 SEM above the percentage of cells found in B10.BR mice. The solid horizontal lines in the first four bar charts depict the percentages of CD25\(^+\) T cells for each of the TCR V\(\beta\)j elements observed following immunization and restimulation with HEL 48–61 (bottom bar chart). This is used to determine the effect of adding COOH-terminal PFRs on the V\(\beta\)j repertoire. To highlight enhanced V\(\beta\)j usage, filled bars indicate V\(\beta\)j elements that are >1 SEM above the horizontal line, while the cross-hatched bars indicate V\(\beta\)j elements that are greater than the horizontal line but within 1 SEM.

(B) These experiments were conducted as described above except that the T cells were split into two groups after resting and were reactivated with either the immunogen (filled bars) or HEL 48–61 (hatched bars). Data are presented as the means of two or three experiments ± SEM and are represented as the percentages of reactivated cells as a proportion of the cells reactivated by the immunogen. See Experimental Procedures for protocol.
TCR usage has been observed before, alteration of key NH$_2$-terminal PFRs, while the TCR V-peptides and their PFRs.

Figure 7. A Model for TCR Recognition of MHC Class II-Bound Peptides and Their PFRs

A model of the HEL 50-63: H-2A$^+$ complex. The TCR orientation and CDR3 interactions are based on the two TCR:MHC class I structures (Garbozi et al., 1996; Garcia et al., 1996). Using data presented in this study and elsewhere (Sant’Angelo et al., 1996), a similar model of TCR binding to MHC class II molecules is proposed. The dashed ovals represent the area covered by the V$a$ CDR1 (a1), CDR2 (a2), and CDR3 (a3) loops, while the solid ovals represent the area covered by the V$b$ CDR1 (b1), CDR2 (b2), and CDR3 (b3) loops. We propose that CDR1$a$ interacts with the NH$_2$-terminal PFR, while CDR1$b$ interacts with the COOH-terminal PFR.

is conserved between HEL and ML (Figure 1). It is difficult to argue that such fine specificity could be mediated by PFR-induced alterations in MHC structure. Last, analysis of T cells from peptide-immunized mice showed that different PFRs elicited distinct patterns of TCR V$b$ usage. On the basis of these results, it is difficult to argue that the TCR does not directly recognize these residues.

One of the most striking findings of this study was the influence of PFRs on TCR V$b$ usage. While skewed TCR usage has been observed before, alteration of key TCR contact residues did not result in a substantial change in TCR V usage (Jorgensen et al., 1992). However, in the present study, we showed that peptides differing in PFRs elicited T cells expressing distinct TCR V$b$ usage. This suggests that the TCR V$b$ domain directly interacts with PFRs. Recent studies have solved the structure of two TCR:peptide:MHC class I complexes and have shown that the TCR binds to MHC molecules in a diagonal orientation, such that V$a$ is positioned toward the NH$_2$ terminus of the peptide and V$b$ is positioned toward the COOH terminus (Garbozi et al., 1996; Garcia et al., 1996). This structure confirmed functional studies that had previously proposed a diagonal pattern of TCR:MHC class I interaction (Sun et al., 1995). Our data and studies performed by Sant’Angelo and colleagues (1996) are consistent with these findings and suggest that a similar orientation may also apply to TCR:MHC class II complexes. Further extrapolation from the TCR:peptide:MHC class I structures suggests that the V$a$ CDR1 loop would be positioned over the NH$_2$-terminal PFR, while the V$b$ CDR1 loop would be positioned over the COOH-terminal PFR (Garbozi et al., 1996; Garcia et al., 1996). TCR recognition of NH$_2$-terminal PFR was not analyzed in the present study, since it is unlikely to be significant because of conservation of S50:T51 between ML and HEL (Figure 1). However, we see no theoretical reason why recognition of NH$_2$-terminal PFRs should not occur with other peptides. This notion is supported by the work of Sant’Angelo and colleagues (1996), who have suggested that the V$a$ region of an H-2A$^+$-restricted TCR recognizes an amino acid in the NH$_2$-terminal P2 position of the peptide (analogous to Y53 in the HEL 48-63 peptide; Figure 1). Taking these findings together, we propose a generalized model for TCR recognition of any MHC class II:peptide complex in which the TCR V$a$ CDR1 loop can bind to NH$_2$-terminal PFRs, while the TCR V$b$ CDR1 loop can bind to COOH-terminal residues (Figure 7).

Is there any correlation between PFR dependence and the amino acid composition of the selected V$b$ CDR1 region? Although there does not appear to be any striking correlations comparable to those identified for CDR3:peptide interactions (Jorgensen et al., 1992), there are some intriguing parallels (Table 2). First, the only TCR V$b$ domain consistently selected by tryptophan PFR is V$b$14. This is the only V$b$ that has a serine at position 27, which is normally a relatively conserved histidine. This residue may be preferred because of its small size and/or its ability to form hydrogen bonds with tryptophan. Second, four of five of the V$b$ domains that have an aspartic acid at position 28 are specifically expanded by the lysine PFR (V$b$6, V$b$8.1, V$b$10, and V$b$13). A salt bridge may therefore mediate this interaction. Third, four V$b$ domains have aromatic amino acids at position 29. Two of these, V$b$2 (W29) and V$b$8.1 (Y29), are expanded by phenylalanine PFRs. A third, V$b$16 (Y29), may be responsible for the large expansion of T cells in the group depicted as “other” in Figure 6A (“other” includes the five V$b$s for which we do not have antibodies, i.e., V$b$1, V$b$15, V$b$16, V$b$18, and V$b$20). Despite these notable correlations, exact determination of

<table>
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<th>Table 2. TCR V$b$ CDR1 Regions</th>
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<td>TCR-V$b$ CDR1</td>
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<td>24 24a 25 26 27 28 29</td>
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<tr>
<td>V$b$1 H L G H N A</td>
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<td>V$b$2 K N S Q Y P W</td>
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<td>V$b$3 E K G H P V</td>
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<td>V$b$4 K L G H N A</td>
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<td>V$b$5 I S G H L/S S/N</td>
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<td>V$b$6 N F N H D T</td>
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<td>V$b$7 D M S H E T</td>
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<td>V$b$8.1 T N N H D Y</td>
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<td>V$b$8.2 T N N H N N</td>
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<td>V$b$8.3 T N S H N Y</td>
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<td>V$b$9 T M N H D T</td>
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<td>V$b$10 T L G H D T</td>
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<td>V$b$11 I S G H S A</td>
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<td>V$b$13 I S G H D T</td>
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<td>V$b$14 K G S S P N</td>
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<td>V$b$15 V G F Q A T S</td>
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<td>V$b$16 V S N H L Y</td>
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<td>V$b$18 D S Q V V S</td>
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<td>V$b$20 E K G H T A</td>
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The TCR V$b$ CDR1 amino acid sequences are depicted as described by Arden et al. (1995). Only those V$b$s that are expressed in B10.BR mice are shown.
the structural basis for PFR dependence will require x-ray crystallography.

Can PFR recognition occur with any immunogenic epitope? While our study only analyzed a single epitope, the data show that different PFRs can mediate dependence and skew TCR Vβ usage. Furthermore, changing the immunodominant central peptide residue did not appear to alter significantly the influence of PFRs. Taking these data together, we believe that such recognition can occur with many immunogenic epitopes, unless self-tolerance suppresses such a response.

There is increasing interest in developing peptide-based vaccines to reduce the possibility of inducing autoimmunity against a host protein homologous to the immunogen or of inducing an inappropriate immune response that may exacerbate rather than alleviate a disease (reviewed by Berzovsky, 1995; Nardin et al., 1995). What are the possible implications of TCR recognition of PFRs on the immune response and vaccine development? The data presented in this study suggest that TCR recognition of PFRs may significantly enhance peptide immunogenicity. The most important observation was that T cells from mice immunized with peptides containing PFRs exhibit a far stronger recall response to the native HEL protein. This issue is of particular importance for vaccine development, since infection with a pathogen will always result in exposure to the native form of the antigen. It is tempting to speculate that recognition of both NH2- and COOH-terminal PFRs could further potentiate the immune response. While this hypothesis is currently under investigation, our data imply that peptide-based vaccines are likely to be far more immunogenic with the inclusion of PFRs, assuming that the naturally processed antigen also includes such residues.

Peptide elution studies have shown that PFRs are a unique characteristic of MHC class II-bound peptides (reviewed by Urban et al., 1993). This feature could result in the availability of more residues to the TCR on peptides bound to MHC class II molecules as compared to those bound to MHC class I molecules (Vignali and Strominger, 1994a). Since there appeared to be no difference in peptide sensitivity and IL-2 production between PFR-dependent and -independent hybridomas, it is possible that PFR-recognition may allow an MHC class II-restricted response to develop a more diverse T cell repertoire. Indeed, immunization with peptides containing PFRs gave rise to T cells expressing unique TCR Vβ elements. While this is consistent with the idea that TCR recognition of PFRs increases the size of the precursor T cell population, formal limiting dilution analysis will be required to resolve this issue. Furthermore, this underlying distinction between MHC class I and class II-restricted responses clearly warrants further investigation.

A number of studies have shown that substitution of key TCR contact residues can give rise to peptides that antagonize (inhibit) the response to agonist (stimulatory) peptides (Smiek et al., 1991; De Magistris et al., 1992; Evavold et al., 1993; Jameson et al., 1993). Given the striking dependence on PFRs exhibited by the T cells analyzed in our study, it is intriguing to question whether peptides lacking these residues could act as peptide antagonists. In this regard, we have recently shown that HEL 48–61 can antagonize PFR-dependent hybridomas stimulated with the agonist HEL 48–63 (R. T. C. and D. A. A. V., unpublished data). These observations differ from previous studies in which key residues were substituted rather than deleted. Given that naturally processed peptides can be generated from native HEL with or without the flanking tryptophan residues (Nelson et al., 1992; Vignali et al., 1993), the potential consequence of PFR recognition on immunoregulation and peptide-based immunotherapy will need to be determined. Two observations are relevant to this issue. First, previous studies have shown that differential processing of antigens may occur in different APCs or in different intracellular compartments (Moreno et al., 1991; Parra-Lopez et al., 1997). Second, recent studies have established that endogenous altered peptide ligands can affect peripheral T cell responses (Vidal et al., 1996).

In summary, we believe that the results from this study constitute compelling evidence that the TCR can directly recognize PFRs and that such residues can be as crucial for TCR recognition as the central peptide residues. Furthermore, recognition of PFRs had a profound effect on TCR Vβ usage that was unique for different PFRs. Finally, PFRs appear to significantly enhance peptide immunogenicity, as was particularly evident from their effect on the recall response to the native HEL protein. These findings establish the immunological significance of PFRs and could have significant implications for immunoregulation, vaccine development, and peptide-based immunotherapy.

**Experimental Procedures**

**Generation of Murine T Cell Hybridomas**

T cell hybridomas were produced essentially as described (Woodland et al., 1993). In brief, B10.BR mice (Jackson Laboratories, Bar Harbor, ME) were immunized in the base of the tail with 10 nmol of HEL 48-63 (2 × 50 μl at 100 μM) in complete Freund’s adjuvant (Gibco-BRL, Gaithersburg, MD). After 8 days, inguinal and periaortie lymph node cells were removed into Hank’s balanced salt solution and single-cell suspensions generated by teasing through 70 μm cell strainers (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Lymph node cells were depleted of CD8+ T cells by panning with 53.6.7, 19.178, and 2.43, and stimulated at 3 × 10⁵/ml with HEL (1 μM; Sigma, St. Louis, MO), irradiated splenocytes (10⁵/ml), and rIL-2 (10 U/ml; Genzyme, Cambridge, MA) in complete S-MEM (Gibco-BRL) with 10% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA, or HyClone, Logan, UT) (complete S-MEM: 2 mM glutamine, 1 mM pyruvate, 100 μM nonessential amino acids, 5 mM HEPES, 5.5 × 10⁻³ M 2-mercaptoethanol, 100 μM penicillin, 100 μg/ml streptomycin; all from Gibco-BRL). After 3 days viable cells were separated over Lymphocyte Separation Medium (Organon Teknika, Durham, NC) and cultured overnight (10⁵/ml) with 10 U/ml rIL-2. The following day, cells were fused with the T cell thymoma BW-TCR-μ, using PEG-1500, washed, and cloned by limiting dilution into 96-well microtiter plates. After 24 hr, hypoxanthine–aminopterin–thymidine (HAT) (Gibco-BRL) selection media was added to select for BW-T cell hybridomas. Cells were expanded into 24-well plates under HAT selection and then into flasks with media containing hypoxanthine-thymidine. A second fusion was performed using lymph node T cells from HEL 48-61KK–immunized mice as described above except that HEL 48-61KK and irradiated dendritic cells (3 × 10⁵/ml) were used for the restimulation in vitro. Preliminary screening involved analysis of CD3 and CD4 expression by flow cytometry and of IL-2 production in response to the immunogen and HEL where appropriate. Hybridomas that were less than 90% CD3+ CD4- were cloned.
by fluorescence-activated cell sorting (FACS) as previously described (Vignali and Strominger, 1994b; Vignali et al., 1996) or enriched by pinching with anti-CD4 (OKI.5).

Antigen Presentation Assays

Assays were performed essentially as described elsewhere (Vignali and Strominger, 1994b; Vignali et al., 1996). In brief, T cell hybridomas (5 × 10⁶/well) were stimulated with LK35.2 or CH12.1.16 cells (2.5 × 10⁶/well) as APCs and pulsed with synthetic peptides (Cender for Biotechnology[CTB]core facility at St. J.ude Children’s Research Hospital[5]CRH) or Chiron Technologies[MImotopes][Raleigh, NC] or with HEL (Sigma). Peptides were purified by reverse-phase high-pressure liquid chromatography (Vidac C-18, The Nest Group, Southborough, MA), verified by mass spectrometry, and quantified by amino acid analysis (Chiron Technologies or Harvard Microchemistry Unit, Harvard University, Cambridge, MA). Supernatants (50 μl) were removed after 24 hr for estimation of IL-2 secretion by culturing with the IL-2-dependent T cell line CTLL-2. Two types of assay were performed. In one, absolute IL-2 concentrations were quantified by titrating culture supernatants against a recombinant murine IL-2 standard (Genzyme) in 50 μl of medium and culturing with 10⁵ CTLL-2 cells (50 μl) for 24 hr. Proliferation was determined by adding 20 μl of Alamar Blue (Alamar Biosciences, Sacramento, CA) diluted 1:2 in medium, incubating overnight, and measuring absorbance at 570 nm in a fluorometer. In the other assay, the concentration of peptide (EC₅₀) required to stimulate a 100% maximal CTLL-2 response was determined by culturing 100 μl of supernatant with 10⁵ CTLL-2 cells (100 μl) for 24 hr. Proliferation was determined by pulsing with 3H-thymidine (1 μCi/ml) (Du Pont, Wilmington, DE) for the final 6 hr of culture.

CAP Transfectants

APCs expressing H-2A/K molecules containing a single CAP were produced essentially as described by Koizono and colleagues (1994). Three CAP constructs were produced by attaching the appropriate HEL peptide sequence to the 5′ end of the H-2A/K chain using recombinant PCR. They are referred to as HEL-48-63-CAP, HEL-48-61AA-CAP, and HEL-48-L56A-63-CAP. These were constructed in three stages.

First, three fragments containing overlapping sequences were created: (1) H-2A/K signal peptide and the first three residues of the mature protein, produced by PCR using an H-2A/K chain cDNA as template (kindly provided by R. N. Germain, National Institutes of Health, Bethesda, MD); DV59e forward primer, Asp718-5′CCCTGGGTAGTAGCTCAGCTACCAGC3′; and DV59b reverse primer, 5′CTCTGATGACCTGGAAAGCTGCTGCT3′, and DV59b reverse primer, 5′TCCGTATCTGCCCTACGAGAAGCTT3′; (2) HEL 48-36 plus the linker originally described by Koizono et al., (1994)(GGTGYDYGILQNSRWGGGSLVPRSGGSG), generated by annealing two oligonucleotides: DV53a, 5′GATGCAGGTAGTACCTAGGCATCGAGCC3′; and DV59a forward primer, Asp718-5′CCCTGGGTAGTAGCTCAGCTACCAGC3′, and DV59b reverse primer, 5′TCCGTATCTGCCCTACGAGAAGCTT3′; and DV59b reverse primer, 5′TCCGTATCTGCCCTACGAGAAGCTT3′; and (3) HEL 49-83 plus the linker originally described by Koizono et al., (1994)(GGTGYDYGILQNSRWGGGSLVPRSGGSG), generated by annealing two oligonucleotides: DV53a, 5′GATGCAGGTAGTACCTAGGCATCGAGCC3′; and DV59a forward primer, Asp718-5′CCCTGGGTAGTAGCTCAGCTACCAGC3′, and DV59b reverse primer, 5′TCCGTATCTGCCCTACGAGAAGCTT3′, and DV59b reverse primer, 5′TCCGTATCTGCCCTACGAGAAGCTT3′.

For the construction of HEL-61AA-CAP, HEL-48-L56A-63-CAP, and HEL-48-L56A-63-CAP, the three constructs described above were joined using DV59e and DV59d, and then this product was added to fragment 3 and the construct completed using DV59e and DV59f as primers. The PCR product was then cut with Asp718 and DV59d as primers. The PCR product was then cut with Asp718 and DV59d as primers. The PCR product was then cut with Asp718 and DV59d as primers.

Flow Cytometry

Analysis of T cell hybridomas for CD3 and CD4 expression was performed as described elsewhere (antibodies from Pharmingen, San Diego, CA) (Vignali and Strominger, 1994b; Vignali et al., 1996).

TCR Vβ gene usage was initially determined by flow cytometry using a panel of monoclonal antibodies (details in Tomonari et al., 1989; Deckhut et al., 1993; Cole et al., 1994; Daly et al., 1995). Antibodies used were KTS5 (anti-Vβ8 [subset]), KTS6 (anti-Vβ8 [subset]), RR8.1 (anti-Vβ11 [subset]), B20.6 (anti-Vβ2), KJ 25 (anti-Vβ3), KT-4 (anti-Vβ4), MR9-4 (anti-Vβ1.5,1.2), MR9-8 (anti-Vβ5.1), RR4-7 (anti-Vβ6), TR130 (anti-Vβ7), F23.1 (anti-Vβ8.1,8.2.8.3), F23.2 (anti-Vβ8.2), KJ 16 (anti-Vβ8.1,8.2.2), MR5-2 (anti-Vβ8.2,8.3), Vβ8.3 (anti-Vβ8.3), MR10.2 (anti-Vβ9), KT-106 (anti-Vβ10), RR3-15 (anti-Vβ11), Vβ12 (anti-Vβ12), MR12-5 (anti-Vβ13), and 14-2 (anti-Vβ14) antibodies kindly provided by M. Blackman, Immunology, SJ CRH.

Reverse Transcription-PCR Analysis and Sequence Determination

Total RNA (~10 mg) was prepared from 10⁶ T cells using STAT-60 (TEL-TEST, Friendswood, TX). To produce cDNA, 2 μg of RNA and 0.2 μg of oligo dT (Promega) in 10 μl of diethyl pyrocarbonate (0.1%)–H₂O was heated to 90°C for 2 min, cooled slowly to 50°C, and placed in ice. RNA was reverse transcribed at 42°C for 60 min in the presence of 1.2 μl of SuperScript II RT (Gibco-BRL), 4 μl of Superscript II RT buffer, 2 μl of DTT, 2 μl of dNTP (10 mM), and 0.8 μl of RNasin (Promega). The cDNA was heated to 70°C for 5 min and H₂O added to 100 μl. PCR reactions were performed by using 5 μl of cDNA, 2 μl of dNTP (10 mM), 25 pmol of each primer, Taq buffer, and 4 U of Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). The PCR primers were optimized using VectorNTI (Informax, Gaithersburg, MD), as follows. Forward primers: Vo₁–5′GAGCGAGCAGACAATCTCCT3′; Vo₂–5′GACGAGTAGCTGAGCCAAGACCTCAAC3′; Vo₃–5′GCTAGCTGAGTACCTAGGCATCGAGCC3′; and DV13-2 reverse primer, BamHI-5′ATGCGCTGAGTACCTAGGCATCGAGCC3′. The PCR products were then cut with Asp718 and DV59d as primers.

The PCR products were then cut with Asp718 and DV59d as primers. The PCR product was then cut with Asp718 and DV59d as primers. The PCR product was then cut with Asp718 and DV59d as primers. The PCR product was then cut with Asp718 and DV59d as primers.
the TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing was performed by the CBT at SJ CRH using PE-ABD PRISM dye primer chemistry and a PE-ABD PRISM 377 automated DNA sequencer. Sequence alignment and verification was performed using the GCG Wisconsin Package V8.1 (Genetics Computer Group, Madison WI) running on a DEC Alpha Server 8200.

TCR Vβ usage of hybridomas that were negative by flow cytometry was determined by reverse transcription–PCR. The Vβ1, Vβ3, Vβ16, Vβ18, and Vβ20 primers and protocol are described elsewhere (Cole et al., 1994).

**T Cell Proliferation Assay**

B10.R mice (Jackson Laboratories) were immunized with 10 nmol of either HEL, HEL 48-63, or HEL 48-61 as described above (see Generation of Murine T Cell Hybridomas). After 10 days, lymph node cells (5 x 10^6/well) were removed and cultured in 96-well flat-bottom plates in complete S-MEM with 5% FCS for 3 days. Lymph node cells from individual mice were stimulated with HEL peptides or protein and proliferation measured by pulsing with [³H]thymidine (1 μCi/well; Du Pont, Wilmington, DE) for the final 12 hr of culture.

**Preparation of Dendritic Cells**

Spleens were removed from B10.R mice (Jackson Laboratories), diced into small pieces with scissors, and suspended in S-MEM + 5% FCS, 1500 U/ml Collagenase Type II (Worthington, Freehold, N J) and 300 U/ml DNase I (Sigma), 4 ml per spleen. After 1 hr of gentle shaking at 37°C, the digested spleen cells were teased through a 70 μm sieve and erythrocytes lysed using Gey’s solution (8.3 g/L NH₄Cl, 1 g/L KHCO₃; Sigma). Cells were then plated out on 10 cm tissue culture–treated Petri dishes (7.5 x 10^6 cells in 15 mL S-MEM + 5% FCS per plate) and incubated overnight. Nonadherent cells were collected and resuspended at 5 x 10^6/mL in S-MEM plus 15% FCS, and 8 ml of cells layered over 3 ml of metrizamide (145 mg/mL S-MEM plus 15% FCS; Nycodenz, Gibco-BRL) and centrifuged at 600 × g for 20 min. The interface, containing mainly dendritic and activated B cells, was collected and washed. This crude preparation was either used as APCs or depleted of B cells by panning with RA3.6B1/2 (anti-B220), yielding ~85% pure N418™ (anti-mCD11c) dendritic cells by flow cytometry.

**T Cell Activation and TCR Vβ Usage Assay**

B10.R mice were immunized at the base of the tail with 10 nmol of peptide (Q x 50 μL of 100 μM) in complete Freund’s adjuvant. After 8 days, CD4^+ inguinal and periaortic lymph node T cells were isolated and enriched by panning with the following: 53.6.7, 19.178, and 2.43 (anti-mCD8); RA3.6B1/2 (anti-B220); and anti-MHC class II antibodies P7/7 (anti-H-2A/E), 10.2.16 and H116±32 (anti-H-2A k), and 14.4.4 and 17.3.35 (anti-H-2K b). Cells (5 x 10^6/ml) were stimulated with the immunizing peptide at 3 mM and irradiated dendritic/ B cells (3 x 10^7/ml) in 24-well plates with 1 ml of media. After 24 hr, 20 units of IL-2 in 1 ml of media was added. On day 3, wells were expanded 3-fold and given IL-2 (10 units/ml). After 11 days, viable T cells were separated over Lymphocyte Separation Medium (Organon Teknika) and reactivated with one-tenth the number of irradiated dendritic cells and 1 μg peptide in 96-well round-bottom plates. The following day cells were first stained with a panel of biotinylated TCR Vβ domain–specific antibodies (see Flow Cytometry, above), followed by Neutrulate Avidin-PE (Southern Biotech., Birmingham, AL), anti-mCD25 FITC (IL-2Rα), and anti-mCD4 Cy-Chrome (Pharmingen). Flow cytometric data were collected using a FACScan (Becton Dickinson Immunocytometry Systems, San J ose, CA).

The percentage of reactivated CD4^+ T cells was determined by expression of the CD25 activation marker. The percentage of reactivated cells shown in Figure 5B was determined using the following equation:

\[
\frac{\text{Total CD4}^+ \times \text{CD25}^+ \times \text{reactiv}}{\text{Total CD4}^+ \times \text{reactiv} \times 100}
\]

where reactiv = the number of T cells following reactivation with peptide, and unactiv = the number of T cells in the absence of peptide.

The percentage of reactivated cells expressing specific Vβ elements as shown in Figure 6A was then determined using the following equation:

\[
\frac{\text{Total CD4}^+ \times \text{CD25}^+ \times \text{Vβ}^+ \times \text{reactiv}}{\text{Total CD4}^+ \times \text{CD25}^+ \times \text{Vβ}^+ \times \text{reactiv} \times 100}
\]

Data presented in Figure 6B, in which T cells were reactivated with either the immunogen or HEL-48-61, were determined using the following equation:

\[
\frac{\text{Total CD4}^+ \times \text{CD25}^+ \times \text{Vβ}^+ \times \text{immunogen-reactiv}}{\text{Total CD4}^+ \times \text{CD25}^+ \times \text{Vβ}^+ \times \text{reactiv} \times 100}
\]

where immunogen-reactiv = T cells reactivated with the immunogen, and peptide-reactiv = T cells reactivated with either the immunogen or HEL-48-61.

**Acknowledgments**

We are very grateful to Marcia Blackman for anti-TCR antibodies, R. N. Germain for plasmids, Sherri Surman for performance of the hybridomas fusions and initial TCR Vα analysis, Lolita Harris for initial characterization of the hybridomas, Haiyan Liu and Lisa (Lu Zheng) Liu for assistance with TCR analysis, Duyen Nguyen for technical assistance, and our colleagues in CBT for DNA sequencing and oligonucleotide synthesis. We also thank Roseann Lambert and J im Houston for assistance with flow cytometry and fluorescence-activated cell sorting and Julia Hurwitz and William Walker for their critical review of the manuscript. D. A. A. V. and D. L. W. are supported by the National Institutes of Health (AI-39480 to D. A. A. V. and CA-56570 to D. L. W.), a Cancer Center Support CORE grant (S 5 P 30 CA21765-17), and the American Lebanese Syrian Associated Charities.

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