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The Alloreactive and Self-Restricted CD4$^+$ T Cell Response Directed Against a Single MHC Class II/Peptide Combination$^1$

Jean-Paul Kovalik,* Nagendra Singh,²* Sanjeev K. Mendiratta,³* W. David Martin,⁴* Leszek Ignatowicz,† and Luc Van Kaer⁵*

The cellular basis for allograft rejection derives from the strong T cell response to cells bearing foreign MHC. While it was originally assumed that alloreactive T cells focus their recognition on the polymorphic residues that differ between syngeneic and allogeneic MHC molecules, studies with MHC class I-restricted CTL have shown that MHC-bound peptides play a critical role in allore cognition. It has been suggested that alloreactive T cells depend more strongly on interactions with the MHC molecule than with the associated peptide, but there is little evidence to support this idea. Here we have studied the alloreactive and self-restricted response directed against the class II H2-Ab molecule bound with a single peptide, Ep, derived from the H2-Ea chain. This MHC class II-peptide combination was a poor target and stimulator of alloreactive CD4$^+$ T cells, indicating that MHC-bound peptides are as important for alloreactive CD4$^+$ T cells as they are for alloreactive CTL. We also generated alloreactive T cells with exquisite specificity for the Aβ/Ep complex, and compared their reactivity with self-restricted T cells specific for the same Aβ/Ep complex. Our results showed that peptide-specific alloreactive T cells, as compared with self-restricted T cells, were more sensitive to peptide stimulation, but equally sensitive to amino acid substitutions in the peptide. These findings indicate that alloreactive and self-restricted T cells interact similarly with their MHC/peptide ligand. The Journal of Immunology, 2000, 165: 1285–1293.

Lymphocytes recognize foreign peptide Ags bound by self-MHC molecules, a property termed self-MHC restriction (1). Self-MHC restriction of T cell responses is acquired during the positive selection of immature T cells in the thymus (2–4). However, 1–10% of all T cells that are positively selected appear to be also capable of alloreactivity, i.e., to react strongly with MHC molecules expressed on allogeneic tissue to which they were not previously exposed (5, 6). This strong reactivity may, in part, be due to the inherent affinity of TCRs for MHC molecules (7, 8).

Precisely how alloreactive TCRs interact with their ligands remains controversial. In one model, it is proposed that T cells interact only with polymorphic residues that differ between the self and foreign MHC molecules and that the interaction with foreign MHC is not affected by the bound peptide (9). The high frequency of alloreactivity would then result from the high density of the allogeneic determinant as compared with the low density of a specific peptide bound with a self-MHC molecule. Some evidence for this model is available. For example, purified HLA-A2 molecules were able to stimulate alloreactive T cells in the absence of any bound peptide (10). In another study alloreactive T cell clones were isolated that recognize determinants on H2-K$^b$ that are independent of peptide (11). In general, however, peptide-independent recognition has been difficult to detect and probably reflects only a small subset of allosequences. In another model, it is proposed that alloreactive T cells recognize a complex between the foreign MHC molecule and an associated peptide (12). This foreign MHC/peptide combination mimics self-MHC plus foreign peptide. Numerous studies with MHC class I-restricted CTL support this hypothesis (5, 6, 13–27). Although some alloreactive T cells had a strict requirement for the type of peptide that associates with the foreign MHC (24–27), most alloreactive CTL displayed substantial degeneracy for peptide recognition (19, 21–23).

A number of similarities and differences regarding the interaction of alloreactive and self-restricted T cells with MHC/peptide complexes have been noted. First, alloreactive T cells with very high affinity for their MHC ligand have been identified (28, 29). Second, it is unclear whether alloreactive and self-restricted T cells interact with MHC/peptide complexes in the same way. Crystallographic studies of TCR/self-MHC/peptide complexes have indicated a diagonal orientation of the TCR with respect to the MHC/peptide (30–35). Despite the overall conservation of the TCR footprint in the different crystals, the actual positions of the TCR domains and the distribution of MHC vs peptide contacts varied widely. It also remains to be determined whether the overall orientation and the exact positions of complementarity determining region (5) loops with respect to MHC and peptide ligands of alloreactive TCRs is similar to that of self-restricted TCRs. Third, it has been argued that alloreactive T cells depend more strongly on interactions with residues located on the α-helices of the allo-MHC and less on interactions with peptide residues (28, 35–38). This idea is supported by studies that compared the ligand requirements for self vs alloantigen recognition of different MHC alleles.
by a single T cell clone (28, 35–38). However, a different conclusion was made in a recent study that compared self vs alloantigen recognition of a single MHC allele, H2-Ld, by a large panel of self-restricted and alloreactive CTL clones (39). Ld-restricted and Ld-alloreactive CTLs were equally sensitive to changes in the sequence of the Ld molecule, indicating that self-restricted and alloreactive T cells are comparably dependent on MHC molecules. Thus, the overall dependence of alloreactive T cells for interaction with MHC vs peptide residues remains controversial.

Here, we have studied the alloreactive and self-restricted T cell response directed against the MHC class II molecule H2-A\textsuperscript{b} bound with a peptide derived from the H2-\textalpha-chain (Ep). We first showed that among a large panel of alloreactive T cell clones generated against H2-A\textsuperscript{b}-molecules bound with the normal wide array of peptides present in wild-type cells, only a small fraction was able to react with the H2-A\textsuperscript{b}/Ep complex. We also demonstrated that cells expressing only the H2-A\textsuperscript{b}/Ep complex are very poor stimulators of naive alloreactive CD4\textsuperscript{+} T cells in MLCs. We then raised a panel of alloreactive T cell hybridomas against the A\textsuperscript{b}/Ep complex. While the majority of these clones cross-reacted with wild-type H2b cells, some clones had exquisite specificity for this complex. While the majority of these clones cross-reacted with a peptide derived from the H2-E\textsuperscript{p} molecule, indicating that self-restricted and alloreactive T cells are comparably dependent on MHC molecules. Thus, the overall dependence of alloreactive T cells for interaction with MHC vs peptide residues remains controversial.

**Materials and Methods**

**Mice**

A\textsuperscript{b}/Ep invariant chain (Ii)-negative mice have been described (7). Ii mutant mice (40) on a C57BL/6 (B6) background were obtained from Dr. Elizabeth K. Bikoff (Harvard University School of Medicine, Boston, MA). H2-DM mutant mice (41) were bred to a B6 background in our laboratory. MHC class II mutant mice (42) on a B6 background were obtained from Drs. Diane Mathis and Christophe Benoist (Harvard University School of Medicine, Boston, MA). All of these mutant strains were maintained and bred under specific pathogen-free conditions in the animal facility at Vanderbilt University School of Medicine (Nashville, TN). B6 (H2\textsuperscript{b}), BALB/c (H2\textsuperscript{b}), SJL (H2\textsuperscript{b}), RII (H2\textsuperscript{b}), and CBA/J (H2\textsuperscript{b}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility.

**Peptides**

The following peptides were used: the E\textsuperscript{a}-derived peptide Ep\textsubscript{52–68}, asFEAQGALANIAVDKA and its single amino acid substituted variants: Ep\textsubscript{55A}, Ep\textsubscript{56G}, Ep\textsubscript{59G}, Ep\textsubscript{61G}, Ep\textsubscript{63A}, Ep\textsubscript{64G}, and Ep\textsubscript{66A}; a set of truncated versions of Ep\textsubscript{52–68} that include Ep\textsubscript{53–68}, Ep\textsubscript{54–68}, Ep\textsubscript{55–68}, Ep\textsubscript{56–68}, Ep\textsubscript{57–68}, Ep\textsubscript{58–68}, Ep\textsubscript{59–68}, Ep\textsubscript{60–68}, and Ep\textsubscript{61–68}. All of the peptides were >90% pure as shown by reverse-phase HPLC and mass spectrometry. Peptides were obtained from the biopolymer facility of the Howard Hughes Medical Institute at the University of Texas Southwestern Medical Center (Dallas, TX) and the Duke University School of Medicine (Durham, NC).

**Mixed lymphocyte cultures**

Primary MLRs were performed with spleen responder cells from naive mice depleted of CD8\textsuperscript{+} T cells. Depletion of CD8\textsuperscript{+} T cells was performed by staining with anti-CD8 Abs (clone 2.43) (obtained from Dr. Barney Graham, Vanderbilt University) followed by panning on plates coated with rabbit anti-rat IgG (Cappel, Organon Teknika, West Chester, PA). Purified responder cells (1 × 10\textsuperscript{5}) were then mixed with varying numbers of irradiated spleen stimulator cells for 3 days at 37\textdegree C. Cells were pulsed with 0.5 uCi [\textsuperscript{3}H]thymidine (NEN Life Science Products, Boston, MA) followed by further culture for 16 h. [\textsuperscript{3}H]Thymidine incorporation was measured by harvesting cells with a cell harvester (Tomtec, Orange, CT) and counting the amount of radioactivity with a betaplate reader (Wallac, Gaithersburg, MD).

**Generation of T cell hybridomas**

Mice were immunized i.p. with 2 × 10\textsuperscript{5} irradiated (1000 rad) spleen cells from B6 or A\textsuperscript{b}/Ep\textsuperscript{+} mice. Seven to 10 days later, 5 × 10\textsuperscript{5} spleen cells from these immunized animals were restimulated in vitro with 4–5 × 10\textsuperscript{5} irradiated B6 or A\textsuperscript{b}/Ep\textsuperscript{+} spleen cells for 3 days. Cells were counted and fused with Bw5147 α β cells (obtained from Dr. Willi Born, National Jewish Center, Denver, CO) using polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN). Hybridomas were obtained by plating fused cells in hypoxanthine/aminopterin/thymidine selection media (Boehringer) and those remaining in hypoxanthine/aminopterin/thymidine selection medium (Boehringer) and those remaining were plated under limiting dilution conditions, hybridomas can be considered clonal.

**T cell hybridoma stimulation assay**

Hybrids were screened by coculturing a fixed number (1 × 10\textsuperscript{5}) of hybridomas with irradiated splenocytes from B6 controls (1 × 10\textsuperscript{5}) in a flat-bottom 96-well plate. After 24 h culture, 50 μl supernatants were harvested and assayed for the presence of IL-2 by using the IL-2-dependent cell line HT-2. The supernatants were mixed with 5 × 10\textsuperscript{5} HT-2 cells and incubated at 37\textdegree C for 20 h. IL-2-dependent proliferation of HT-2 cells was tested by uptake of [\textsuperscript{3}H]thymidine as described above. Reactivity of hybrids was graded as strong, weak, or none when they produced >50, 10–50, or <10%, respectively, of the IL-2 produced in the optimal response (against cells from B6 or A\textsuperscript{b}/Ep\textsuperscript{+} mice). To test sensitivity of hybrids to stimulation with peptide, fixed numbers of hybridoma cells (1 × 10\textsuperscript{5}) were cocultured with irradiated B6 splenic APCs (1–3 × 10\textsuperscript{5}) and with a titrated dose of the peptide Ep or one of its peptide variants. Reactivity against peptide variants was graded as strong, weak, or none when hybrids produced >50, 10–50, or <10%, respectively, of the IL-2 as produced by stimulation with the cognate Ep peptide. To test the sensitivity of hybrids to stimulation with anti-CD3, fixed numbers of hybridoma cells (1 × 10\textsuperscript{5}) were mixed with irradiated splenic APCs (5 × 10\textsuperscript{5}) from MHC class II mutant mice; titrated doses of supernatant containing anti-CD3 Ab (145-2C11) were then added to the cultures, and supernatants were tested for IL-2 content after 24 h of coculture as above.

**Results**

**Few H2-A\textsuperscript{b}-specific alloreactive CD4\textsuperscript{+} T cells recognize APC from A\textsuperscript{b}/Ep\textsuperscript{+} mice**

A\textsuperscript{b}/Ep\textsuperscript{+} mice express a single MHC class II-peptide combination, H2-A\textsubscript{b} plus a peptide (Ep) derived from the H2-\alpha-chain (7). Cell-surface expression levels of the class II A\textsuperscript{b} molecules in these animals reach ~10% of wild-type mice (7). Because few studies have evaluated the role of MHC-bound peptides for allore cognition in the class II system, we first evaluated whether this unique class II-peptide combination is an efficient target for alloreactive CD4\textsuperscript{+} T cells. Therefore, we generated large panels of alloreactive H2-A\textsubscript{b}-specific hybrids. Briefly, mice from a set of allogeneic strains (CBA, H2\textsuperscript{a}; BALB/c, H2\textsuperscript{b}; RII/J, H2\textsuperscript{c}; SJL, H2\textsuperscript{d}) were immunized with irradiated spleen cells from B6 (H2\textsuperscript{b}) mice, and after in vitro restimulation hybrids were generated under limiting dilution conditions. These clonal hybrids (nearly 500) were then tested for reactivity with APC from wild-type B6, H2-DM-deficient, IFN-deficient, A\textsuperscript{b}/Ep\textsuperscript{+}, and MHC class II-deficient mice (all from the H2\textsuperscript{a} haplotype). MHC class II molecules in these mice display varying degrees of class II cell-surface expression levels and peptide diversity; in B6 mice A\textsuperscript{b} class II molecules are bound by a wide gemish of self-peptides; in H2-DM-deficient mice, A\textsuperscript{b} molecules are expressed at normal levels and are mostly bound by a peptide, CLIP, derived from the MHC class II-associated li chain (41, 43, 44), but some contamination by non-CLIP peptides has

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\[^6\] Abbreviations used in the paper: Ep, E\textsuperscript{a}-derived peptide (aa 52–68); B6, C57BL/6; Ii, invariant chain; CLIP, class II-associated li chain peptide.
been noted (45, 46); in Ii-deficient mice, Aβ surface expression is reduced 10-fold, and these molecules are thought to be occupied by low-affinity peptides (40, 47–49); in Aβ EpIi2 mice, Aβ surface expression is reduced 10-fold, and these complexes are bound by Ep (7); finally, MHC class II-deficient mice lack class II surface expression altogether (42, 50). The reactivity of these hybrids was graded strong, weak, or none, when IL-2 produced by the hybrids was 50, 10–50, or 10%, respectively, of the IL-2 produced when the hybrids were stimulated with wild-type B6 cells (Table I). Consistent with our previous findings (51), only a small percentage (9%) of the hybrids responded (strongly or weakly) to stimulator cells from H2-DM-deficient mice. Similarly, only 6% of the hybrids reacted with cells from Aβ EpIi2 mice. While it is possible that some hybrids failed to react with Aβ EpIi APC because of the reduced expression of class II molecules in these animals, this interpretation cannot explain the low frequency of reactivity. Indeed, cells from Ii-deficient mice, with similar class II surface levels, were recognized by most (93.5%) of the hybrids. Because none of the hybrids studied reacted with all MHC class II-expressing cells tested (i.e., cells from wild-type B6, Ii-deficient, H2-DM-deficient, and Aβ EpIi2 mice), none of the hybrids fit the criterion for complete peptide independence of alloantigen recognition.

We conclude from these experiments that very few Aβ-specific alloreactive T cells can react with the Aβ/Ep complex expressed by Aβ EpIi2 mice. These findings illustrate the critical importance of MHC-bound peptides for recognition of MHC class II by most alloreactive CD4+ T cells and indicate that truly peptide-independent alloreactive T cells must be rare.

Cells from Aβ EpIi− mice are poor stimulators of alloreactive CD4+ T cells in primary MLCs

To provide further evidence for the critical role of peptide in allorecognition, we tested whether APC from Aβ EpIi− mice can stimulate allogenic CD4+ T cells in primary MLCs. Fig. 1 shows

**FIGURE 1.** Cells from Aβ EpIi− mice are poor stimulators of primary alloreactive CD4+ T cell responses. Splenic responder cells, isolated from the indicated allogeneic mouse strains and depleted of CD8+ T cells, were cocultured with titrated numbers of irradiated splenic stimulator cells from H2-DM-deficient mice. Similarly, only 6% of the hybrids reacted with cells from Aβ EpIi− mice. While it is possible that some hybrids failed to react with Aβ EpIi− APC because of the reduced expression of class II molecules in these animals, this interpretation cannot explain the low frequency of reactivity. Indeed, cells from Ii-deficient mice, with similar class II surface levels, were recognized by most (93.5%) of the hybrids. Because none of the hybrids studied reacted with all MHC class II-expressing cells tested (i.e., cells from wild-type B6, Ii-deficient, H2-DM-deficient, and Aβ EpIi− mice), none of the hybrids fit the criterion for complete peptide independence of alloantigen recognition.

We conclude from these experiments that very few Aβ-specific alloreactive T cells can react with the Aβ/Ep complex expressed by Aβ EpIi− mice. These findings illustrate the critical importance of MHC-bound peptides for recognition of MHC class II by most alloreactive CD4+ T cells and indicate that truly peptide-independent alloreactive T cells must be rare.

**Cells from Aβ EpIi− mice are poor stimulators of alloreactive CD4+ T cells in primary MLCs**

To provide further evidence for the critical role of peptide in allorecognition, we tested whether APC from Aβ EpIi− mice can stimulate allogenic CD4+ T cells in primary MLCs. Fig. 1 shows

**Table I. Summary of the reactivities of randomly selected alloreactive anti-H2-Aβ hybridomas**

<table>
<thead>
<tr>
<th>Hybrids</th>
<th>Reactivity</th>
<th>Stimulator Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B6</td>
</tr>
<tr>
<td>CBA/J</td>
<td>Strong</td>
<td>154 (100%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Strong</td>
<td>132 (100%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>RIII</td>
<td>Strong</td>
<td>94 (100%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>SJL</td>
<td>Strong</td>
<td>118 (100%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*CBA/J, BALB/c, RIII, and SJL mice were immunized with 2 x 10^7 irradiated spleen cells from B6 mice. Ten days later, the spleens of immunized mice were harvested and restimulated in vitro with irradiated B6 spleen cells, and hybridomas were generated by fusion with Bw5147 cells. Reactivities of hybrids were measured by coculture of 1 x 10^5 hybridoma cells with the indicated irradiated splenic stimulator cells (1 x 10^5). IL-2 in the culture supernatants was measured by proliferation of HT-2 indicator cells. Hybrids with no reactivity against B6 cells and hybrids reactive against class II-deficient cells were excluded from the analysis and are not shown. Reactivities of hybrids were graded as strong, weak, or none when IL-2 production was >50, 10–50, or <10%, respectively, of the IL-2 produced when the hybrids were stimulated with B6 cells. The total number and percentage (in parentheses) of hybridomas that reacted strongly, weakly, or not at all with the indicated stimulator cells are shown.
that none of the four allogeneic strains tested generated a significant allo response against AβEpIi2 cells. Likewise, consistent with our previous findings (51), cells from H2-DM-deficient mice were also poor stimulators of allogeneic CD4+ T cells.

Generation of Aβ/Ep-specific alloreactive and self-restricted T cells

To generate alloreactive T cells with exquisite specificity for the Aβ/Ep complex, mice from a panel of allogeneic strains were immunized with irradiated AβEpIi2 APC, and T cell hybridomas were generated. The majority (83%) of these hybrids responded not only to AβEpIi2 cells, but also to wild-type B6 APC, and many responded to Ii-deficient cells (Table II). Hybrids with this type of reactivity lack strict specificity for the Aβ/Ep complex. Instead, this type of reactivity may be consistent with peptide-independent alloreactivity. However, only one of the 13 hybrids tested within this group responded weakly to H2-DM-deficient stimulator cells, indicating that the recognition pattern of these hybrids is not completely independent of the bound peptide. Therefore, this group of hybrids represents clones with specificity for multiple peptides displayed by H2-Aβ. A smaller percentage (17%) of the hybrids responded to stimulators from AβEpIi2 mice but not to any of the

---

**Table II. Summary of the reactivities of hybridomas obtained after immunization of allogeneic mice with cells from AβEpIi2 mice**

<table>
<thead>
<tr>
<th>Hybrids</th>
<th>Reactivity</th>
<th>B6</th>
<th>H2-DM−/−</th>
<th>Li−/−</th>
<th>AβEpIi2</th>
<th>CIH−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/J</td>
<td>Strong</td>
<td>3</td>
<td>0 (0%)</td>
<td>1 (17%)</td>
<td>6 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>2</td>
<td>0 (0%)</td>
<td>4 (67%)</td>
<td>0 (0%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1</td>
<td>6 (100%)</td>
<td>1 (17%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Strong</td>
<td>1</td>
<td>0 (0%)</td>
<td>2 (17%)</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>2</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>9</td>
<td>11 (92%)</td>
<td>9 (75%)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>RIII</td>
<td>Strong</td>
<td>3</td>
<td>0 (0%)</td>
<td>1 (9%)</td>
<td>11 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>2</td>
<td>0 (0%)</td>
<td>3 (27%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>6</td>
<td>11 (100%)</td>
<td>7 (64%)</td>
<td>0 (0%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>SJL</td>
<td>Strong</td>
<td>123</td>
<td>ND</td>
<td>ND</td>
<td>212 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>63</td>
<td>ND</td>
<td>ND</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>26</td>
<td>ND</td>
<td>ND</td>
<td>0 (0%)</td>
<td>212 (100%)</td>
</tr>
</tbody>
</table>

* CBA/J, BALB/c, RIII, and SJL mice were immunized with 2 × 10^7 irradiated spleen cells from AβEpIi2 mice. Ten days later, the spleens of immunized mice were harvested and restimulated in vitro with irradiated AβEpIi2 spleen cells, and hybridomas were generated by fusion with Bw5147 αβ- cells. Reactivities of hybrids were measured by coculture of 1 × 10^5 hybridoma cells with the indicated irradiated splenic stimulator cells (1 × 10^5). IL-2 in the culture supernatants was measured by proliferation of HT-2 indicator cells. Hybrids with no reactivity against AβEpIi2 cells and hybrids reactive against class II-deficient cells were excluded from the analysis and are not shown. Reactivities of hybrids were graded as strong, weak, or none when IL-2 production was >50, 10–50, or <10%, respectively, of the IL-2 produced when the hybrids were stimulated with AβEpIi2 cells. The total number and percentage (in parentheses) of hybridomas that reacted strongly, weakly, or not at all with the indicated stimulator cells are shown.
other stimulator cells (Table II and Fig. 2). Therefore, these hybrids have exquisite specificity for the Ep peptide bound with H2-A^b.

The same protocol was used to generate A^b/Ep-specific self-restricted T cells. In this case, wild-type B6 mice were immunized with irradiated Ab EpIi^2 APC, and T cell hybridomas with specificity for A^b/EpIi^2 stimulator cells were isolated.

Both A^b/Ep-specific alloreactive and self-restricted hybridomas responded to stimulators from A^b/EpIi^2 mice in a dose-dependent manner and failed to react with APC from B6 mice (Fig. 2). Seventeen allogeneic hybrids from three different allogeneic strains and nine self-restricted hybrids were then compared for their sensitivity to stimulation with the Ep peptide and for their sensitivity to amino acid substitutions in the sequence of Ep.

A^b/Ep-specific alloreactive T cells are more sensitive to peptide stimulation than A^b/Ep-specific self-restricted T cells

Prior studies have suggested that the TCRs of alloreactive T cells are more sensitive to stimulation than the TCRs of self-restricted T cells (28, 29). Therefore, we compared the sensitivity of our alloreactive and self-restricted hybrids to stimulation with the Ep peptide. Hybrids were cultured with irradiated B6 APC that were pulsed with graded doses of the Ep peptide. Fig. 3 shows that, on

![Graph showing sensitivity of hybridomas to peptide stimulation](http://www.jimmunol.org/)

**FIGURE 3.** Alloreactive hybrids are more sensitive to stimulation by peptide than self-restricted hybrids. Hybrids (1 × 10^5 cells) derived from either B6, SJL, RIII, or BALB/c mice were incubated with a titrated dose of the Ep peptide in the presence of irradiated B6 APC (3 × 10^5 cells). Stimulation of hybridoma cells was measured by IL-2 production, as assayed by proliferation of HT-2 indicator cells. Each data point in the graphs represents the mean of duplicate wells.
average, alloreactive hybrids were ~10 times more sensitive to stimulation by Ep than the self-restricted hybrids. In contrast, both types of hybrids were equally sensitive to stimulation with anti-CD3 Abs (data not shown). Thus, these findings are in general agreement with the prior studies (28, 29) and show that alloreactive T cells have lower activation thresholds than self-restricted T cells.

$A^b$/Ep-specific alloreactive and self-restricted T cells are equally sensitive to truncation variants of the Ep peptide

Next, we compared the reactivity of alloreactive and self-restricted T cell hybrids to a set of truncation variants of the Ep peptide. Because the minimal binding sequence of Ep to H2-A$^b$ contains residues 57–65 of the Ea chain (52), no residues within this sequence were deleted. Reactivity of hybrids was graded as strong, weak, or none when IL-2 production from at least two of the three titrated doses was >50, 10–50, or <10%, respectively, of the IL-2 produced when the IL-2 source was stimulated with the full-length peptide, which was used as an internal control. Thus, the reactivity of the indicated hybrids (1×10^5 cells) with irradiated B6 splenic cells (1×10^5 in the presence of a titrated dose of peptide (10, 1, and 0.1 μg/ml). Stimulation of hybridoma cells was measured by IL-2 production, as assayed by proliferation of HT-2 indicator cells. For each hybrid, the reactivity to the full-length peptide at a given dose was arbitrarily set at 100%. The reaction of the hybrids to the truncated peptides was compared with the response to the full-length Ep peptide. Reactivity against a peptide was graded as strong (■), weak (□), or none (▲), when IL-2 production from at least two of the three titrated doses was >50, 10–50, or <10%, respectively, of the IL-2 produced when the hybrid was stimulated with the full-length peptide. Cell cultures were performed in duplicate wells.

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Self-restricted and alloreactive hybrids react similarly to truncated variants of the Ep peptide. The indicated hybrids (1×10^5 cells) were incubated with irradiated B6 splenic cells (1×10^5) in the presence of a titrated dose of peptide (10, 1, and 0.1 μg/ml). Stimulation of hybridoma cells was measured by IL-2 production, as assayed by proliferation of HT-2 indicator cells. For each hybrid, the reactivity to the full-length peptide at a given dose was arbitrarily set at 100%. The response of the hybrids to the truncated peptides was compared with the response to the full-length Ep peptide. Reactivity against a peptide was graded as strong (■), weak (□), or none (▲), when IL-2 production from at least two of the three titrated doses was >50, 10–50, or <10%, respectively, of the IL-2 produced when the hybrid was stimulated with the full-length peptide. Cell cultures were performed in duplicate wells.

$A^b$/Ep-specific alloreactive and self-restricted T cell hybrids to these peptide variants were very similar (Fig. 5). All hybrids reacted with the variant at amino acid position 66. A few hybrids from both allogeneic and syngeneic sources lost reactivity to peptide variants at positions 56, 59, and 64, and approximately half of all hybrids from each panel lost reactivity when changes were made at amino acid positions 55 and 63. The peptide was graded as strong (■), weak, or none. Overall, the reactivities of the alloreactive and self-restricted hybrids to these peptide variants were very similar (Fig. 5). All hybrids reacted with the variant at amino acid position 66. A few hybrids from both allogeneic and syngeneic sources lost reactivity to peptide variants at positions 56, 59, and 64, and approximately half of all hybrids from each panel lost reactivity when changes were made at amino acid positions 55 and 63. The one exception to the pattern of similar fine specificity was noted at amino acid position 61. Substitution at this position resulted in loss of reactivity for 24% of the alloreactive hybrids and 55% of the self-restricted hybrids. Despite this small difference, these findings indicate similar fine specificity for peptide among alloreactive and self-restricted hybrids.

Discussion

**Peptide-dependence of alloreactive T cell responses**

Our findings strongly support the peptide-dependent model of alloreactivity. While many studies have provided evidence for a critical role of peptides in the recognition of alloantigens by MHC class I-restricted CTL (5, 6, 13–27), few studies have investigated this issue for MHC class II molecules. This is mostly due to the lack of good experimental systems for the study of class II alloreactivity. Some early studies showed that the recognition of MHC class II by alloreactive CD4$^+$ T cells could be partially inhibited by addition of exogenous Ags to the APCs (53, 54), and some alloreactive CD4$^+$ T cells were shown to be able to distinguish between allogeneic MHC products expressed in different tissues (55, 56). A few examples of alloreactive MHC class II-restricted T cell clones with peptide-specificity are also available (37, 51, 57). The best evidence for a role of peptide in the class II system comes from studies with Ag-processing defective cells (51, 58). In one study, the alloresponse was measured against HLA-DM-deficient T2 cells transfected with mouse class II molecules (58). However, there was a significant amount of uncertainty regarding the nature and complexity of the peptides bound by class II molecules in these transfected cells. In another study, the alloresponse was measured against cells from H2-DM-deficient mice that mostly express CLIP peptides on their class II molecules (51), but again,
not all class II molecules in these mice are bound by CLIP (45, 46). The experimental system used here does not have this limitation, because it is well-established that AβEpIi mice express only a single peptide species (Ep) on their class II molecules (7, 45).

Of nearly 500 alloreactive T cell hybridomas that were generated against wild-type H2b-expressing cells in four different allo- genetic strains, only a small fraction (6%) recognized the Aβ/Ep complex expressed by these animals (Table I). This lack of reactivity was not just caused by the reduced expression of class II molecules in AβEpIi mice, because most hybrids were able to react with cells from Ii-deficient mice that express similar class II surface levels. Therefore, these findings indicate that the majority of alloreactive T cells are peptide dependent. Among the hybrids that reacted with cells from AβEpIi mice (6%), none recognized cells from H2-DM-deficient mice, indicating that they were not peptide independent. Conversely, among the hybrids that recognized H2-DM-deficient cells (9%), none reacted with AβEpIi cells. Thus, among our large panel of alloreactive hybrids, no evidence was found for complete peptide independence.

The critical role of MHC-bound peptides for allorecognition was further demonstrated by using cells from AβEpIi mice as stimulators of alloreactive CD4+ T cell responses. In MLRs, these cells were very poor stimulators of naive alloreactive CD4+ T cells (Fig. 1). Further, among the alloreactive hybrids generated after immunization with cells from AβEpIi mice, only 17% were exquisitely specific for the Aβ/Ep complex (Table II). Thus, peptide specificity of alloreactive T cell responses appears to be the exception rather than the rule. Most of the hybrids (83%) generated after immunization with cells from AβEpIi mice not only reacted with AβEpIi cells but also with wild-type B6 cells and/or Ii-deficient cells. Therefore, these hybrids cross-react with other peptide species (Ep) on their class II molecules (7, 45).

The experimental system used here does not have this limitation, because it is well-established that AβEpIi mice express only a single peptide species (Ep) on their class II molecules (7, 45).

Sensitivities of the hybrids to the peptide variants were graded as strong (■), weak (■), or none (■) as described in the legend to Fig. 4. Cell cultures were performed in duplicate wells.

Strength of alloreactive T cell responses

Our findings may help to provide insight into the cellular and molecular basis for the overall strength of alloreactive responses, as compared with the weak responses typically seen for self-restricted T cells. One factor may by the degeneracy of alloreactive T cells for peptide recognition. As discussed above, most alloreactive T cells described here, except for those with exquisite peptide specificity, were highly degenerate for recognition of peptide. Another factor that may contribute to the overall strength of allo responses is the low activation threshold of these cells. In some prior studies, alloreactive T cell clones with very high avidity for foreign MHC have been identified (28, 29). Our studies support the idea that high reactivity is a general property of alloreactivity. Fig. 3 shows that alloreactive hybrids, on average, were 10 times more sensitive to peptide stimulation than self-restricted hybrids. Thus, peptide degeneracy and high ligand sensitivity are two properties of alloreactive T cells that contribute to the strength of allosresponses.
Ligand requirements for alloreactive vs self-restricted T cells specific for the same MHC/peptide complex

Comparison of the fine specificity of Aα/Ep-specific alloreactive and self-restricted T cells indicated similar sensitivity to truncations (Fig. 4) and single amino acid substitutions (Fig. 5) of the Ep peptide. Several conclusions can be drawn from these observations. First, the alloreactive and self-restricted T cells must recognize Ep when it binds to Aα class II molecules in the same binding register. Second, the overall pattern of interactions of these TCRs with peptide residues is likely to be very similar, suggesting similar TCR contact points. Third, the overall orientation of these TCRs with respect to the MHC-peptide complex is likely to be identical or at least very similar and agrees with the idea that the diagonal footprint of TCRs with respect to MHC/peptide is likely to be conserved for all TCRs, including alloreactive ones (30–35). Fourth, our findings indicate that alloreactive TCRs can be as specific to peptide as self-restricted TCRs. Some prior studies have suggested that alloreactive T cells, including peptide-specific alloreactive T cells, depend more strongly on interactions with residues of the α-helices of the MHC than with peptide residues (28, 35–38). Two of these studies were performed by comparing the crystal structures of a TCR/self-MHC/peptide with a model for interaction of the same TCR with an allo-MHC/peptide ligand (35, 38). These models suggested increased interaction of the TCRs with residues of the allo-MHC. In some other studies, the specificity of a single TCR for recognition of allo- vs self-restricted ligand was compared (28, 36, 37). These studies suggested greater flexibility in the recognition of peptide bound to the allo-MHC as compared with peptide bound to the self-MHC. In contrast, our findings indicate similar peptide ligand requirements for peptide-specific alloreactive and self-restricted TCRs. This conclusion agrees with a recent study that compared the requirements for interaction with α-helical residues of the MHC among a panel of Lα/alloreactive and Lα/self-restricted T cells, including some peptide-specific alloreactive clones (39). These CTL were equally sensitive to changes in the sequence of the Lα molecule, indicating that alloreactive and self-restricted T cells are comparably dependent on MHC molecules. Our findings indicate that this conclusion can be also extended to the peptide requirements of alloreactive and self-restricted T cells.

Implications for T cell repertoire selection

The finding that alloreactive and self-restricted TCRs can react with a particular MHC/peptide ligand in a similar way has important implications for the role of positive selection in shaping the T cell repertoire. Positive selection induces the maturation of thymocytes with intermediate affinity for self-MHC and with a broad range of specificities. Previous studies that examined the alloreactive and self-restricted CTL responses against peptide libraries have suggested that the allo repertoire has a very wide diversity of specificities, perhaps as wide as that of the self-restricted T cell repertoire (68). Similarly, we demonstrated that peptide-specific alloreactive T cells can be readily obtained, indicating that there is no fundamental difference between the self-restricted and allo repertoire. The main difference between the self-restricted repertoire and the allo repertoire is that the former is more effective because cells with some specificity for self-MHC have been strongly enriched.

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