A Shared Epitope of the Interphotoreceptor Retinoid-Binding Protein Recognized by the CD4+ and CD8+ Autoreactive T Cells

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A Shared Epitope of the Interphotoreceptor Retinoid-Binding Protein Recognized by the CD4⁺ and CD8⁺ Autoreactive T Cells

Hui Shao, Yong Peng, Tianjiang Liao, Meiqin Wang, Ming Song, Henry J. Kaplan, and Deming Sun

We previously demonstrated that cultures of rat uveitogenic T cells rapidly become dominated by CD4⁺ cells, but activation of CD8⁺ autoreactive T cells also occurred during the in vitro culture of in vivo-primed T cells. In the present study, we show that the commonly used uveitogenic peptide, interphotoreceptor retinoid-binding protein (IRBP) 1–20, generated both CD4⁺ and CD8⁺ autoreactive T cells in the C57BL/6 (B6) mouse and that this 20-mer contains at least two distinct antigenic epitopes. To determine whether the CD8 response was Ag-specific and whether CD4⁺ and CD8⁺ IRBP1–20-specific T cells recognize distinct antigenic epitopes, we prepared highly purified CD4⁺ and CD8⁺ T cells from IRBP1–20-primed mice and tested their proliferative response to a large panel of truncated peptides derived from IRBP1–20. The results showed that both CD4⁺ and CD8⁺ T cells recognized the same spectrum of peptides. In addition, peptides P10–18 were found to bind effectively to CD8⁺ IRBP1–20-specific T cells when complexed with recombinant H-2Kb and also stimulate the proliferation and cytokine production of CD4⁺ IRBP1–20-specific T cells. Our results document for the first time that CD8⁺ and CD4⁺ autoreactive T cells display characteristic epitope recognition and they both recognize the same core epitope. The Journal of Immunology, 2005, 175: 1851–1857.

Uveitis is a common cause of human visual disability and blindness. Recurrent uveitis is associated with severe clinical complications, such as cataract formation, and glaucoma. Experimental models have been generated in rodents by immunization with several different ocular Ags, one of which, interphotoreceptor retinoid-binding protein (IRBP), has been widely studied in mice (1–3).

Early studies found that uveitogenic T cells in either the mouse or rat were predominantly CD4⁺ (4–6); however, we recently demonstrated that the uveitogenic T cells induced in the Lewis rat by immunization with a synthetic IRBP-derived peptide, IRBP1177–1191, consisted of both CD4⁺ and CD8⁺ T cells (7). Therefore, we examined the situation in a mouse model of uveitis. We first used the CSFE staining technique (7–10) to examine the Ag-specific proliferation and/or expansion of in vivo-primed CD8⁺ IRBP1–20-specific T cells. Because typical antigenic epitopes for CD8⁺ T cells consist of 9–10 aa (11, 12), we also determined the minimal antigenic epitope required for the activation of CD4 and CD8 T cell subsets by measuring the stimulatory activity of truncated peptides covering the whole length of IRBP1–20 on CD4⁺ and CD8⁺ IRBP1–20-specific T cells and the binding to CD8⁺ IRBP1–20-specific T cells of these peptides in the form of a complex with recombinant MHC class I (H-2Kb) molecules. The results showed that both the CD4 and CD8⁺ autoreactive T cells recognized the same core epitope.

Materials and Methods

Animals and reagents

Pathogen-free female C57BL/6 mice (8–10 wk old) were purchased from The Jackson Laboratory and were housed and maintained in the animal facilities of the University of Louisville. Institutional approval was obtained and institutional guidelines regarding animal experimentation followed.

The sequences of IRBP 1–20 and the truncated peptides are listed in Table I. All were synthesized by Pepscan Systems and were >85% pure. Abs against mouse H-2D⁰ (KH95), mouse H-2Kb (AF6-88), and mouse H-2Kb/H-2D⁰ mAbs were obtained from BioLegend.

Animal model of experimental autoimmune uveitis (EAU)

Briefly, to prepare T cells, donor mice were immunized s.c. with 200 μl of an emulsion containing 200 μg of IRBP1–20 and 500 μg of Mycobacterium tuberculosis H37Ra (Difco) in IFA (Sigma-Aldrich), distributed over six spots on the tail base and flank. Uveitis was induced in naive B6 mice by adoptive transfer of 5 × 10⁶ IRBP1–20-specific T cells as described previously (13–15). The animals were examined three times a week for clinical signs of uveitis by fundoscopy, starting at the second week post-transfer. Fundoscopic evaluation for longitudinal follow-up of disease was performed using a binocular microscope after pupil dilation using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. The incidence and severity of EAU were graded on a scale of 0–4 in half-point increments using the criteria described previously (16), which are based on the type, number, and size of lesions present.

Pathological examination

Inflammation in the eye was confirmed by histopathology. Whole eyes were collected, immersed for 1 h in 4% phosphate-buffered glutaraldehyde, and transferred to 10% phosphate-buffered formaldehyde until processed. The fixed and dehydrated tissue was embedded in methacrylate, and 5-μm
The presence or absence of disease was evaluated blind by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously (15). Sections were cut through the papillary-optic nerve plane and stained with H&E. The presence or absence of disease was evaluated by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously (15).

### IRBP1–20-specific T cells

T cells from IRBP1–20-immunized B6 mice were isolated 13 days postimmunization (p.i.) from lymph node cells or spleen cells by passage through a nylon wool column, then 1 × 10^7 cells in 2 ml of RPMI 1640 medium in a 6-well plate (Costar) were stimulated with 20 μg/ml IRBP1–20 in the presence of 1 × 10^5 irradiated syngeneic spleen cells as APCs. After 2 days, the activated lymphoblasts were isolated by gradient centrifugation on Lymphoprep (Robbins Scientific) and cultured in RPMI 1640 medium supplemented with 15% IL-2-containing medium (supernatant form Con A-stimulated rat spleen cells).

#### Proliferation assay

T cells from IRBP1–20-immunized B6 mice were prepared and seeded at 4 × 10^7 cells per well in 96-well plates, then cultured at 37°C for 48 h in a total volume of 200 μl of medium with or without IRBP-derived peptides in the presence of irradiated (2000 rad) syngeneic spleen APCs (2 × 10^5), and [3H]thymidine incorporation during the last 8 h was assessed using a microplate scintillation counter (Packard). The proliferative response was expressed as the mean cpm ± SD of triplicate determinations.

### Purification of CD4^+ and CD8^+ T cells using StemSep columns

CD4^+ and CD8^+ -enriched T cell populations were isolated from freshly prepared draining lymph node and spleen cells using negative selection StemSep kits (Stem Cell Technology). The mouse lymph node and spleen cells were incubated with mixtures of bispecific Ab complexes, each of which binds both to dextran beads and to CD4 or CD8 and to a cell surface Ag on mouse hemopoietic cells (CD11b; Mac-1), NK cells (DX5), B cells (CD45R; B220), erythroid cells (TER119), or polymorphonuclear leukocytes (Ly-6G; Gr-1). The cells were then incubated for 15 min at 4°C with StemSep Magnetic Colloid, loaded into a magnetic column, and washed with 15 ml of medium according to the manufacturer’s protocol. The flow-through fraction containing either CD4^+ or CD8^+ -enriched cells was collected, and the purity of the isolated cell fraction was determined by flow cytometry using FITC-conjugated anti-TCR Abs and PE-conjugated anti-CD4 or anti-CD8 Abs.

### CFSE staining

T cells from the draining lymph nodes and spleen from immunized mice were prepared by passage through a nylon wool column and stained with the vital dye CFSE (Molecular Probes) as described previously (8). Briefly, the cells were washed and resuspended at 50 × 10^6 cells/ml in medium with or without 15 ml of medium according to the manufacturer’s protocol. The cells were then incubated for 15 min at 4°C with StemSep Magnetic Colloid, loaded into a magnetic column, and washed twice with 15 ml of medium according to the manufacturer’s protocol. The flow-through fraction containing either CD4^+ or CD8^+ -enriched cells was collected, and the purity of the isolated cell fraction was determined by flow cytometry using FITC-conjugated anti-TCR Abs and PE-conjugated anti-CD4 or anti-CD8 Abs.

### Table 1. Truncated peptides derived from IRBP1–20

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRBP1–20</td>
<td>GPTHLFQPSLVLDMAKVLLD</td>
</tr>
<tr>
<td>P2–20</td>
<td>PTHLFQPSLVLDMAKVLLD</td>
</tr>
<tr>
<td>P3–20</td>
<td>THLFQPSLVLDMAKVLLD</td>
</tr>
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<td>P4–20</td>
<td>HLFQPSLVLDMAKVLLD</td>
</tr>
<tr>
<td>P5–20</td>
<td>LFQPSLVLDMAKVLLD</td>
</tr>
<tr>
<td>P6–20</td>
<td>FQPSLVLDMAKVLLD</td>
</tr>
<tr>
<td>P6–19</td>
<td>FQPSLVLDMAKVL</td>
</tr>
<tr>
<td>P6–16</td>
<td>FQPSLVLDMAK</td>
</tr>
<tr>
<td>P6–14</td>
<td>FQPSLVLD</td>
</tr>
<tr>
<td>P7–18</td>
<td>QPSLVLDM</td>
</tr>
<tr>
<td>P8–18</td>
<td>PSVLDM</td>
</tr>
<tr>
<td>P9–18</td>
<td>SLVDM</td>
</tr>
<tr>
<td>P10–20</td>
<td>LVDM</td>
</tr>
<tr>
<td>P10–19</td>
<td>LVDM</td>
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<tr>
<td>P10–18</td>
<td>LVDM</td>
</tr>
<tr>
<td>P2–18</td>
<td>PTHLFQPSLVLDMAKVL</td>
</tr>
<tr>
<td>P2–16</td>
<td>PTHLFQPSLVLDMAK</td>
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<tr>
<td>P2–15</td>
<td>PTHLFQPSLVLDM</td>
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<td>P3–19</td>
<td>THLFQPSLVLDMAKVLLD</td>
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<tr>
<td>P3–18</td>
<td>THLFQPSLVLDMAK</td>
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<td>P3–16</td>
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<tr>
<td>P3–14</td>
<td>THLFQPSLVLDM</td>
</tr>
<tr>
<td>P3–13</td>
<td>THLFQPSLVLD</td>
</tr>
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</table>

For further information, please refer to the original source [1852 SHARED EPITOPES OF IRBP-SPECIFIC CD4^+ AND CD8^+ T CELLS](http://www.jimmunol.org/).
Flow cytometric detection of T cells binding IRBP peptides complexed with recombinant MHC class I (H-2Kb) dimers

The MHC class I (H-2Kb) molecule used was a fusion protein containing mouse H-2Kb and mouse IgG1 obtained from BD Pharmingen (17). To produce the dimeric form, it was incubated at 4°C for 12–24 h with human β2-microglobulin (both at a final concentration of 0.15 mg/ml) and an excess of the test peptide (1 mg/ml). Double staining was performed by incubating 5 × 10⁵ cells with 0.5 μg of peptide-dimer complexes at 4°C for 30 min in a volume of 0.5 ml. The cells were washed twice in PBS containing 1% BSA and 0.1% sodium azide and stained with a PE-labeled anti-mouse IgG1 Ab, followed by a FITC-conjugated Ab against either CD4 or CD8. The results are presented as PE staining vs FITC staining.

Immunofluorescence flow cytometry

Aliquots of 2 × 10⁵ cells were double-stained with combinations of FITC- or PE-conjugated mAbs against mouse αβ TCR (H57), CD4, or CD8. All Abs were purchased from BD Biosciences. Data collection and analysis were performed on a FACS Calibur flow cytometer using CellQuest software.

Statistical analysis

The data are expressed as the mean ± SD. Each experiment was repeated at least three times.

Results

Detection of CD8+ IRBP1–20-specific T cells using CFSE staining

In a previous study, we demonstrated that, although cultures of rat uveitogenic T cells are predominantly CD4+ cells, culture of the in vivo-primed lymphocytes also contains significant numbers of CD8+ autoreactive T cells (7). To further characterize these CD8+ autoreactive T cells and determine their role in the pathogenesis of mouse EAU, we immunized B6 mice with IRBP1–20, a known uveitogenic peptide derived from IRBP (3, 18), then prepared T cells from these mice at day 13 p.i. and labeled them with CFSE before stimulation with IRBP1–20 in the presence of irradiated syngeneic APCs (spleen cells). The activated T cell blasts were then separated by ficoll gradient centrifugation, cultured in IL-2-containing medium, stained with PE-labeled anti-CD4 or anti-CD8 Abs, and subjected to FACS analysis. As shown in Fig. 1A, at 96–120 h after in vitro activation, ~40% of the IRBP1–20-specific T cells expressed CD8. This T cell response was Ag-specific, as a specificity test (Fig. 1B) showed that a strong response was only seen using the immunizing peptide.
Response of in vivo IRBP1–20-primed T cells to truncated IRBP peptides

Because IRBP1–20 used for immunization contains 20 aa residues, we examined whether this 20-mer peptide contained separate antigenic epitope(s) stimulating either CD4⁺ or CD8⁺ T cells using a panel of 24 truncated peptides derived from IRBP1–20 (Table I) by truncation of the N and/or C terminus, including the peptide FQPSLVLDM (P6–14) which was predicted to contain the best “CD8-stimulating motif” by a computer program (Peptide Binding Predictions programs http://bimas.dccc.nih.gov/molbio/hla_bind). To assess the response of CD8⁺ and CD4⁺ T cells separately, we isolated CD8⁺ and CD4⁺ T cells from the spleen of IRBP1–20-immunized mice using StemSep columns.

The purity of the CD4⁺ and CD8⁺ T cells was verified by staining with FITC-labeled Abs followed by FACS analysis (Fig. 2A), then their proliferative responses to the truncated peptides were determined. A representative proliferation result (Fig. 2B) shows that the peptide IRBP1–20 and many of the truncated peptides had a strong stimulatory effect on both CD8⁺ and CD4⁺ T cells. Although the purified CD8⁺ T cells had a generally lower response to the test peptides than the CD4⁺ T cells, it is apparent that the response of CD8⁺ T cells is not always CD4-dependent. A further group of peptides was tested (Fig. 3), and the results showed that truncated peptides P2–20, P3–20, and P4–20 were as effective as P1–20 in stimulating both CD4 and CD8 T cells (Fig. 3A), indicating that the first three N-terminal residues were not essential. Similarly, the C-terminal residues 15–20 were also functionally redundant in terms of stimulating both sets of T cells (Fig. 3B). Removal of residue 14[M] (P3–13) resulted in no loss of ability to stimulate CD4 cells, but in a 50% decrease in the CD8 cell response, indicating that residue 15 is more important for the CD8 response. In summary, none of the truncated peptides stimulated only CD4 or CD8 T cells.

The short peptide P10–18 was also found to stimulate both a CD4 and a CD8 T cell response (Fig. 3C). Interestingly, addition of 1–3 residues to the N terminus (residues 9, 8, and 7) almost totally abolished this effect (Fig. 3C). Because P3–13 and P10–18 overlap each other by only four amino acids (residues 10–13) and the sequence of the peptides are significantly different, we hypothesize that IRBP1–20 contains at least two separate T cell activation epitopes. Interestingly, the 9-mer peptide, P6–14 (FQPSLVLDM), which was predicted by a computer program to have the highest potential for binding to MHC class I (H-2Db and H-2Kb) molecules, had no appreciable stimulatory effect on CD8 cells (Fig. 2A) and did not bind to the recombinant H-2Kb molecule (see Fig. 5).

FIGURE 3. Residues that are crucial for the activation of IRBP1–20-primed T cells in vitro. The proliferative response of purified CD4⁺ and CD8⁺ IRBP-specific T cells from IRBP1–20-immunized mice to the indicated IRBP peptides was tested. The results shown are the mean cpm values and are representative of those for three separate experiments, each involving pooled T cells from six to eight IRBP1–20-immunized B6 mice; the SD was always <15%.

The response of CD8⁺ IRBP1–20-specific T cells to P10–18 is H-2D/Kb-restricted

From the shortest peptides that still activated T cells, we choose P10–18 for more intensive study. To determine whether the response to this peptide was restricted by Kb or Db of the MHC class I molecules, we performed an Ab-blocking test in which CD8⁺ T
The response of CD8+ IRBP1–20-specific T cells to P10–18 is H-2Dβ/Kb restricted. In thymidine incorporation assays, the proliferative response of $4 \times 10^5$ in vivo-primed CD8+ IRBP-specific T cells to a suboptimal dose (5 μg/ml) of P10–18 was tested in the absence or presence of Abs against H-2Dβ, Kβ, or both (final concentration 10 μg/ml). The results shown are representative of those for four separate tests.

**FIGURE 4.** The response of CD8+ IRBP1–20-specific T cells to P10–18 is H-2Dβ/Kb restricted. In thymidine incorporation assays, the proliferative response of $4 \times 10^5$ in vivo-primed CD8+ IRBP-specific T cells to a suboptimal dose (5 μg/ml) of P10–18 was tested in the absence or presence of Abs against H-2Dβ, Kβ, or both (final concentration 10 μg/ml). The results shown are representative of those for four separate tests.

The cells were then incubated with complexes containing H-2Kβ and IRBP- peptides (see Materials and Methods) and FITC-labeled anti-mouse CD8 Ab detecting bound complex (see Materials and Methods). Fig. 5 shows that the percentage of CD8 IRBP-expanded T cells bound to complexed H-2Kβ molecules depended on the peptide in the complex. The greatest binding of H-2Kβ molecules was seen using peptide P10–18, which bound to 29% of the cells, whereas the 20-mer peptide P1–20 showed lower, but significant, binding (9.5% of the cells). The binding assay was absolutely peptide-dependent; for example, peptide P1–20 maximally stimulated the proliferative response of both CD4 and CD8+ IRBP1–20-specific T cells, but bound only to a low percentage of CD8+ T cells. It is noted that staining of P1–20-specific T cells with P10–18 and Kβ-fusion protein reveals that a small portion of the binding cells are CD8-negative. This might be due to a part of the newly activated CD8 cells that has down-regulated CD8 expression, even though they retain the expression of TCR after a new stimulation.

**FIGURE 5.** Binding to complexes containing H-2Kβ and various IRBP-derived peptides to IRBP1–20-specific T cells. IRBP1–20-specific T cells, prepared from immunized B6 mice 13 days p.i., were stimulated in vitro with 20 μg/ml IRBP1–20 and APCs, then the T cell blasts were separated by Ficoll gradient centrifugation and cultured in IL-2-containing medium for a week. The cells were then incubated with complexes containing H-2Kβ and IRBP- peptides (see Materials and Methods) and FITC-labeled anti-mouse CD8 Ab (y-axis) and FITC-labeled anti-mouse CD8 Ab (x-axis).

**Adoptive transfer of disease by P10–18- and P3–13-activated IRBP1–20-specific T cells**

To determine whether P10–18- and P3–13-activated IRBP1–20-specific T cells had uveitis-inducing capability, T cells from IRBP1–20-immunized B6 mice were separately stimulated in vitro with IRBP1–20, P10–18, or P3–13, and the activated T cell blasts were transferred to naive B6 recipients. As shown in Fig. 6, comparable disease severity, examined by either fundoscopy or histology, was comparable all three groups of mice.

**Discussion**

Autoimmune diseases, such as experimental autoimmune encephalitis (EAE) and EAU, have been believed to be mainly caused by CD4+ autoreactive T cells. This conclusion was supported by the observation that established encephalitogenic T cell lines, such as MBP-specific (19) or proteolipid protein-specific (20, 21) encephalitogenic lines and IRBP-specific (4) or retinal-soluble Ag-specific (5, 6) uveitogenic T cells, are exclusively CD4+ autoreactive T cell lines. However, a pathogenic role of autoreactive CD8+ T cells has been demonstrated in a number of autoimmune diseases, such as diabetes (22, 23), arthritis (24), and proteolipid protein-induced EAE (25) in the mouse. In the study of EAE, other laboratories (26–28) and our own (17, 29) have demonstrated that CD8+ encephalitogenic T cells play a major role in MOG-induced EAE in the B6 mouse. Our recent studies have also shown that coactivation of CD8+ uveitogenic T cells can be readily demonstrated in IRBP-induced uveitis in the rat (7). We have also found, using the
FIGURE 6. Uveitogenic activity of P10–18- and P3–13-activated IRBP1–20-specific T cells. Nylon wool-enriched splenic T cells, prepared from IRBP1–20-immunized B6 mice 13 days p.i., were subjected to in vitro stimulation with 20 µg/ml IRBP1–20, P10–18, or P3–13 and APCs, then 5 x 10⁶ T cell blasts, separated by Ficoll gradient centrifugation, were transferred to each recipient mouse. The clinical score was then monitored by fundoscopy, and the eyes of the recipient mice were subjected to pathological examination 15 days later. A, Mean clinical score of uveitis after adoptive transfer (n = 5). B, Eye histology shows that P10–18- and P3–13-stimulated IRBP-specific T cells are potently pathogenic.

CSFE staining technique, that uveitogenic IRBP-specific T cells in the mouse also contain CD8⁺ T cells (Fig. 1).

To determine whether the activation of CD4 and CD8 autoreactive T cells is driven by separate antigenic epitopes within IRBP1–20, we synthesized a panel of truncated peptides covering the length of IRBP1–20 and tested their stimulatory activity on CD4 and CD8 T cells. The results showed that highly purified CD8⁺ T cells from B6 mice primed in vivo with IRBP1–20 responded significantly to IRBP1–20 and its truncated peptides in the absence of CD4 help (Fig. 2); however, the response of the purified CD8 cells was much weaker than that of their CD4 counterparts, but could be greatly enhanced by adding nanogram per milliliter concentrations of cytokines, such as IL-2 and IL-7 (data not shown).

Our initial experimental goal was to determine whether the 20-mer IRBP1–20 contained separate CD4 and CD8 T cell epitopes. However, repeated assays on a large panel of truncated peptides failed to identify a peptide that stimulated only CD8⁺ or CD4⁺ T cells. Based on the observations that two of the shortest peptides, P3–13 and P10–18, retained the ability to stimulate CD4⁺ and CD8⁺ IRBP1–20-specific T cells and that the response of purified CD8 cells was more effectively blocked by anti-H-2Kb Abs than anti-H-2Db Abs, we assessed their binding to recombinant H-2Kb molecules (our unpublished results).

A typical CD8⁺ T cell antigenic epitope is composed of 9–10 aa (11, 12); however, in our study, many IRBP peptides longer than 9-mers (up to 13-mers) had CD8 stimulatory activity. It has also been recently demonstrated that peptides can fit into the MHC class I groove by inserting both ends into the groove, leaving the rest of the peptide as a “bulge” outside the groove (30, 31). It is also worth mentioning that peptide P6–14, a candidate peptide predicted to bind H-2Db and H-2Kb MHC I molecules by computer software (Peptide Binding Predictions programs (http://bimas.dcr.nih.gov/molbio/hla_bind)), totally failed to stimulate CD8⁺ T cells and did not bind to recombinant H-2Kb.

To determine whether a single epitope was able to stimulate both CD4⁺ and CD8⁺ autoreactive T cells in other systems, we recently re-examined a panel of truncated peptides derived from MOG35–55, an encephalitogenic epitope in the B6 mouse (17, 29), and found that highly purified CD4⁺ and CD8⁺ T cells from MOG35–55-primed B6 mice also respond to the same epitope within this peptide (our unpublished results).

We found that there was not always a correlation between the stimulatory and binding activity of an IRBP peptide. For example, IRBP1–20 and most of the truncated peptides larger that 14-mers had a stimulatory activity on CD8 activation; however, strong binding to peptide-complexed MHC I molecules was only seen with peptides P10–18 and P3–13. One possible explanation is that, in the proliferation assay or in vivo, serum or cellular proteases might further cleave and process the longer peptides to fit the MHC binding groove, whereas, in the absence of APCs, only those peptides that already fit the MHC groove can bind.

In conclusion, our results demonstrate, for the first time, that, unlike the T cell response to viral proteins in which CD8⁺ T cells recognize different antigenic epitopes compared with their CD4⁺ T cell counterparts, CD4⁺ and CD8⁺ IRBP1–20-specific autoreactive T cells recognize the same antigenic epitope(s).

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Disclosures
The authors have no financial conflict of interest.

References


