Identification of MHC Class I H-2 K^b/D^b-Restricted Immunogenic Peptides Derived from Retinal Proteins

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Purpose. To identify H-2 K^b/D^b-binding immunogenic peptides derived from retinal proteins.

Methods. Computer-based prediction was used to identify potentially H-2 K^b/D^b-binding peptides derived from the interphotoreceptor retinol-binding protein (IRBP), soluble retinal antigen (S-antigen), recoverin, phosducin, and pigment epithelium-derived factor (PEDF). The affinity of the peptides was analyzed by their abilities to upregulate the expression of major histocompatibility complex (MHC) class I on TAP-deficient cells (RMA-S cells) with flow cytometry. C57BL/6 mice were immunized subcutaneously, with individual peptides in incomplete Freund's adjuvant (IFA). Eight days after immunization, splenocytes were isolated for cytotoxic T-lymphocyte (CTL) analysis. A ^{51}chromium-release assay was used to detect specific CTL reactivity generated in the cultures. Eyes were enucleated for histopathological analysis on day 21 after immunization with IRBP or IRBP and the immunogenic peptides.

Results. All the 21 predicted peptides were found to upregulate expression of H-2 K^b/D^b on RMA-S cells. Five peptides, the two IRBP-derived peptides $IRBP_{89\text{-}96}$ and $IRBP_{101\text{-}108}$, and the three PEDF-derived peptides, $PEDF_{389\text{-}397}$, $PEDF_{139\text{-}147}$, and $PEDF_{272\text{-}279}$, induced specific CTL responses in vivo, whereas the remaining 16 peptides, including 5 IRBP-derived peptides, 5 S-antigen-derived peptides, 1 recoverin-derived peptide, 1 phosducin-derived peptide, and 4 PEDF-derived peptides, did not induce specific CTL reactivity. The immunogenic peptides alone did not induce inflammation in the eyes, but they could enhance severity of uveitis induced by IRBP.

Conclusions. Five of 21 H-2 K^b/D^b-binding retinal protein-derived peptides were found to be immunogenic, suggesting that these peptides could function as autoantigenic epitopes in the development of inflammatory eye diseases, such as uveitis. (*Invest Ophthalmol Vis Sci.* 2006;47:3939–3945) DOI: 10.1167/iovs.06-0133

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Experimental autoimmune uveitis (EAU) in mice has become an important model for studying uveitis of presumed autoimmune etiology. The histopathological characteristics observed in mice closely resemble those found in human uveitis.[1–5] EAU seems to have common immunologic mechanisms with uveitis in humans,[6] and so dissection of the immunopathogenic mechanisms involved in EAU may contribute to our understanding of the mechanisms involved in human uveitis, both on a basic and a clinical level.

EAU, which is thought to be a CD_4^+ Th-cell–mediated organ-specific autoimmune disease, can be induced by immunization with a group of retinal proteins, such as interphotoreceptor retinol-binding protein (IRBP),[7,8] soluble retinal antigen (S-Ag),[2] recoverin,[9,10] and phosducin.[11–13] Although it is known that CD_4^+ T cells or major histocompatibility complex (MHC) class II haplotypes are involved in the pathogenesis of EAU[14–18] and sympathetic ophthalmia,[19,20] several diseases in humans are known to correlate with MHC class I haplotypes, such as birdshot retinochoroidopathy (HLA-A29, in particular HLA-A29.2),[21–23] Behçet's disease (HLA-B51),[24–26] and acute iritis-ankylosing spondylitis (HLA-B27).[27,28] This indicates that MHC class I–restricted peptides derived from the retinal antigens may also be involved in the pathogenesis of uveitis. In support of this, Shao et al.[29] reported that rat $CD8^+$-specific T cells were uveitogenic in Lewis rats, and McPherson et al.[30] have clearly shown that $CD8^+$ T-cells can recognize β-galactosidase expressed in retina, causing inflammation and uveitis. However, the putative role for MHC class I–restricted peptides in the development of uveitis is still unclear. The purpose of this study was therefore to determine whether H-2 K^b/D^b-binding peptides derives from retinal proteins, including IRBP, S-Ag, recoverin, phosducin, and pigment epithelium-derived factor (PEDF),[31–34] can cause cytotoxic T-lymphocyte (CTL) reactivity in C57BL/6 mice, in an attempt to find out whether MHC class I–restricted peptides play a role in inflammatory eye diseases, such as uveitis.

Materials and Methods

C57BL/6 Mice

Female 6- to 8-week-old C57BL/6 (H-2^b background) mice were purchased from Bomholtgård (Ry, Denmark). Animals were housed in a specific pathogen-free biohazard level-2 facility maintained by the Department of Experimental Medicine at the Panum Institute (Copenhagen, Denmark). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Peptides

Protein sequences used for prediction of peptides derived from murine retinal antigens (IRBP, S-Ag, recoverin, phosducin, and PEDF) were obtained from the FASTA databases (http://www.ensembl.org/ provided in the public domain by the European Bioinformatics Institute, Hinxton, UK). The peptides were predicted to have a binding affinity for H-2 K^b/D^b, using a computer-based prediction program described previously.[35,36] $SV_{324\text{-}332}$ (MOCK), an H-2 K^b/D^b-binding peptide, representing amino acids FARGNYPAL of the Sendai virus protein, was

used as the control peptide in the peptide-binding assay and the cytotoxicity readout assays (described later). $HepBcore_{128-140}$, a T-helper peptide, representing amino acids TPPAYRPPNAPIL of the hepatitis B virus core protein, was used in the peptide immunization protocol[37] (described later). All peptides were synthesized by Schafer-N (Copenhagen, Denmark). Peptides were dissolved in phosphate-buffered saline (PBS). For immunization and for pulsing of target cells, the peptides were further diluted in PBS.

Cells and Reagents

RAM-S cells were transporter-associated with antigen processing (TAP)-deficient and derived from the T-lymphoma cell line RMA.[38] RMA-S cells were cultured in RPMI-1640 medium (Invitrogen-Gibco, Rockville, MD) supplemented with 10% FCS, 2 mM L-glutamine and antibiotics at 37°C and 5% CO_2 in humidified air. Affinity-purified IRBP was isolated from bovine retinas by concanavalin-A-Sepharose chromatography.[39] Human β_2-microglobulin (β_2m) was prepared and purified from the urine of uremic patients, as described previously.[40] The anti-H-2^b-specific antibody 8F12 was obtained from our own laboratory at the Panum Institute[41] and used as the primary antibody to MHC-peptide complexes. FITC-conjugated rabbit anti-mouse IgG (FITC-RAM) for flow cytometry was purchased from Dako (Copenhagen, Denmark). Incomplete Freund's adjuvant (IFA) and complete Freund's adjuvant (CFA) were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant IL-2 was purchased from Chiron (Emeryville, CA). $Na_2{}^{51}CrO_4$ was purchased from DuPont NEN (Boston, MA). L-glutamine, PBS, and supplements (fetal calf serum; penicillin-streptomycin) were obtained from the culture medium facility of the Department of Medical Microbiology and Immunology at the Panum Institute (Copenhagen, Denmark).

Peptide-Binding Assay with Flow Cytometry

A peptide-binding assay with flow cytometry was performed as described elsewhere.[42] Briefly, RMA-S cells were cultured at 26°C overnight and washed with serum-free medium. Then the cells (1×10^6), in 200 μL of 10% RPMI-1640, were added to tubes containing 30 μg of peptide. The cells were incubated at 37°C in a water bath for 1 hour and washed twice with cold (4°C) PBS. The cells were incubated for 30 minutes on ice with 50 μL of the antibody 8F12. After two washes with cold PBS, the cells were incubated for 30 minutes on ice with 50 μL of FITC-RAM (Dako) at a dilution of 1:40. The cells were then washed three times with cold PBS, after which fluorescence intensity was measured with a flow cytometer (FACScan; BD Biosciences, Mountain View, CA). The upregulation of MHC class I molecules versus background (i.e., no peptide-pulsed cells), was calculated as a fluorescence index (FI: the mean fluorescence of the experimental sample divided by the mean fluorescence of the background).

Immunization and Expansion of CTL In Vitro

Female 6- to 8-week-old C57BL/6 mice were subcutaneously injected with 100 μL of emulsion containing 30 μg of peptide along with 120 μg of $HepBcore_{128-140}$ in 50 μL of IFA. Eight days after immunization, spleens were removed to prepare responder cells, and spleens from nonimmunized mice were also taken to prepare stimulator cells. Stimulator cells were pulsed with 30 μg/mL of the indicated peptide and 10 μg/mL human β_2m in 2% RPMI-1640 for 1 hour at 37°C in a water bath, after which the stimulator cells were irradiated (3000 rads). Responder cells, 5×10^6, were cocultured with 5×10^6 irradiated syngeneic stimulator cells, which had been pulsed with the appropriate peptide, in 1 mL 10% RPMI-1640 in 24-well plates. The next day, 100 U/mL IL-2 was added to the cultures, which were incubated in vitro for 5 days at 37°C, 5% CO_2 in humidified air.

^{51}Chromium-Release Assay

Cytotoxicity of CTL was assessed by a ^{51}chromium-release assay, which was performed as previously described.[43] Briefly, after 5 days of culture, the responder cells were harvested, pooled, counted, and adjusted to 2×10^6/mL in 10% RPMI-1640. RMA-S cells were loaded with peptides as indicated at 37°C for 1 hour, then labeled with 100 μCi of $Na_2{}^{51}CrO_4$ containing 30% FCS at 37°C for 1 hour, after which they were washed three times with 2% RPMI-1640 and adjusted to 2×10^4/mL in 10% RPMI-1640. Responder cells were cocultured with target cells (2×10^3) in 200 μL of 10% RPMI-1640 medium in 96-well round-bottomed plates at titrated effector-to-target (E:T) ratios of 100:1, 50:1, 25:1, and 12.5:1. Target cells were cultured alone, either in 10% RPMI-1640 to measure spontaneous release or in 3% CH_3COOH solution to measure maximum release. After 4 hours, 30 μL of supernatant was collected, and radioactivity was measured using a gamma counter (1470 Wizard; Wallac, Turku, Finland). CTL reactivity was calculated as the specific release of ^{51}Cr from target cells using the following equation:

$$\% \text{ Specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

All cytotoxicity data described shown in the present paper represent the mean of four replicate microcultures or assay cultures. The standard deviations were <10% of the mean.

Induction of EAU

C57BL/6 mice were immunized subcutaneously with 0.2 mL of emulsion containing 100 μg of IRBP in CFA (1:1) containing 5 mg/mL *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) along with additional subcutaneously injection of 0.2 mL emulsion supplemented with or without a mixture of five immunogenic peptides (30 μg of each peptide) plus 120 μg of $HepBcore_{128-140}$ in CFA. Concurrent with immunization, 1 μg PTX was injected intraperitoneally. On day 21 after immunization, eyes were collected and immersed in 4% formaldehyde in phosphate buffer until further processed.

Histologic Analysis

Enucleated eyes were fixated in Lilly's fluid (Bie & Berntsen, Rødovre, Denmark) for 24 hours. Before fixation, the superior calottes of the eyes were dissected to ensure optimal fixation and later to facilitate infiltration with paraffin. After fixation, the central ring of the eyes, containing the anterior chamber, lens, and optic disc, and the surgical lesion, was cut out, embedded in paraffin, and sectioned in series of sections, each of 4 μm thickness. Sections were stained with hematoxylin and eosin, and coverslipped. Histologically, any sign of retinouveitis was graded as follows: grade 0, no pathologic changes; grade 0.5, five or fewer inflammatory cells per eye, no tissue damage; grade 1, mild infiltration with inflammatory cells, retinal edema, focal retinal detachment, 0 to 2 small granulomas in choroid and retina, and perivasculitis; grade 2, moderate infiltration, retinal edema and detachment, focal retinal photoreceptor damage, medium-sized granulomas, perivasculitis and vasculitis; grade 3, moderate inflammation, marked retinal edema and detachment, retinal photoreceptor damage, many medium-sized granulomas, subretinal neovascularization; grade 4, heavy infiltration, complete retinal detachment with subretinal bleeding, extensive photoreceptor damage, large confluent granulomas, subretinal neovascularization. Grading was performed in a blinded fashion by two independent observers (i.e., sections were independently graded twice). In the event of discrepancy, a consensus was reached, producing a validated score for all eyes.

Statistics

Wilcoxon's test was used to analyze the difference between the percentage of specific release for the immunizing peptide and the corresponding control peptide. The Mann-Whitney test (one-tailed probability) was used to analyze the difference between groups with

different immunization for EAU induction. A probability below 0.05 was considered significant.

Results

Predicted Peptides for H-2 K^b/D^b Binding Derived from Retinal Proteins

Twenty-one peptides derived from retinal proteins were predicted for H-2^b binding based on a prediction algorithm developed in our laboratories.[35,36] The results are shown in Table 1. Seven peptides were derived from IRBP, five from S-Ag, seven from PEDF, one from recoverin, and one from phosducin. The affinities (K_D) for H-2 K^b/D^b binding were predicted to be below 100 nM. The table also includes the FI, showing that all the predicted peptides bound to and upregulated H-2 K^b/D^b molecules on RMA-S cells (described later).

Upregulation of H-2 K^b/D^b on RMA-S Cells in the Presence of Predicted Peptides

To investigate whether the predicted peptide can upregulate MHC class I expression on H-2^b TAP-deficient cells we measured H-2 K^b/D^b expression on RMA-S cells in the absence or presence of individual predicted peptides using flow cytometry. We found that all the predicted peptides could upregulate expression of H-2 K^b/D^b on RAM-S cells. The peptides increased the expression of H-2 K^b/D^b molecules on RMA-S cells twofold or more when compared with RMA-S cells cultured in the absence of peptide. For some peptides, the ability to bind to H-2 K^b/D^b was comparable to that of a positive control peptide MOCK (Fig. 1). The results, expressed as FIs for all predicted peptides are shown in Table 1.

Induction of Specific CTL Reactivity in Response to Immunization with Peptides Derived from IRBP and PEDF

To detect whether the predicted peptides could generate specific cytotoxic activity in vivo, C57BL/6 mice were immunized subcutaneously with individual peptides along with $HepBcore_{128\text{-}140}$ mixed in IFA, and a ^{51}Cr-release assay was used to access cytotoxicity. As shown in Table 1, we found that five peptides, the two IRBP-derived peptides $IRBP_{89\text{-}96}$ (ISYEPSTL) and $IRBP_{101\text{-}108}$ (QAPVLTNL), and the three PEDF-derived peptides $PEDF_{139\text{-}147}$ (SRIVFERKL), $PEDF_{272\text{-}279}$ (SIIFFLPL), and $PEDF_{389\text{-}397}$ (LNQPFLFVL), induced specific CTL responses in vivo. Figure 2 shows data from a representative immunization experiment including mice immunized with $IRBP_{89\text{-}96}$, $IRBP_{101\text{-}108}$, $PEDF_{139\text{-}147}$, $PEDF_{272\text{-}279}$, and $PEDF_{389\text{-}397}$, respectively. As shown in Figures 2A and 2B, the two IRBP-derived peptides generated high specific ^{51}Cr release at an E:T ratio from 12.5:1 to 100:1. However, nonspecific ^{51}Cr releases were also detected for these two peptides, especially at an E:T ratio of 50:1. The three PEDF-derived peptides (Figs. 2C–E) induced low levels of specific ^{51}Cr release compared with two immunogenic peptides derived from IRBP. The remaining 16 peptides, including 5 IRBP-derived peptides, 5 S-Ag-derived peptides, one recoverin-derived peptide, one phosducin-derived peptide, and 4 PEDF-derived peptides, did not induce specific CTL reactivity.

Table 1. Predicted Peptides for H-2 K^b/D^b Binding Derived from Retinal Proteins and the CTL Response Induced in C57BL/6 Mice by Immunization with Peptides in IFA

Peptide	MHC Restriction	Sequence	Predicted Affinity K_D (nM)	FI	CTL Response
IRBP-derived					
$IRBP_{23\text{-}31}$	D^b	FQPSLVLDM	14	3.4	0/6
$IRBP_{19\text{-}27}$	K^b	PTHLFQPSL	28	4.3	0/6
$IRBP_{546\text{-}553}$	K^b	AAEEFAFL	14	4.6	0/6
$IRBP_{432\text{-}440}$	D^b	VLPGNVGYL	15	4.6	0/6
$IRBP_{37\text{-}45}$	K^b	DNYCFPENL	11	5.2	0/6
$IRBP_{89\text{-}96}$	K^b	ISYEPSTL	42	5.4	3/6
$IRBP_{101\text{-}108}$	K^b	QAPVLTNL	50	5.9	2/6
S-antigen-derived					
$S\text{-}Ag_{335\text{-}343}$	K^b	TVSGFLGEL	15	4.1	0/6
$S\text{-}Ag_{119\text{-}127}$	K^b	FLLTFPDYL	1	5.6	0/6
$S\text{-}Ag_{76\text{-}84}$	D^b	MGLTFRRDL	12	6.5	0/6
$S\text{-}Ag_{115\text{-}123}$	K^b	NTYPFLLTF	55	6.5	0/6
$S\text{-}Ag_{277\text{-}284}$	K^b	KTLVLVPL	91	6.7	0/6
Recoverin-derived					
$RECO_{174\text{-}182}$	D^b	GTLANKEIL	41	2.3	0/6
Phosducin-derived					
$PHOS_{107\text{-}115}$	K^b	PRYGFVYEL	44	3.2	0/6
PEDF-derived					
$PEDF_{389\text{-}397}$	K^b	LNQPFLFVL	12	3.2	2/6
$PEDF_{386\text{-}394}$	D^b	DYHLNQPFL	38	3.5	0/6
$PEDF_{56\text{-}64}$	K^b	AVSNFGYDL	98	5.3	0/6
$PEDF_{338\text{-}345}$	K^b	ESPDFSKI	82	6	0/6
$PEDF_{139\text{-}147}$	K^b	SRIVFERKL	7	6.1	3/6
$PEDF_{272\text{-}279}$	K^b	SIIFFLPL	1	6.4	1/6
$PEDF_{271\text{-}279}$	K^b	MSIIFFLPL	1	6.7	0/6
$SV_{324\text{-}332}$*	K^b/D^b	FARGNYPAL	—	6.1	—

CTL response data denote the number of mice showing significant CTL responses in the group of six mice immunized with individual peptides.

* $SV_{324\text{-}332}$ (MOCK), an H-2 K^b/D^b-binding peptide, representing amino acids FARGNYPAL of the Sendai virus protein, was used as the positive control in the peptide-binding assay with flow cytometry.

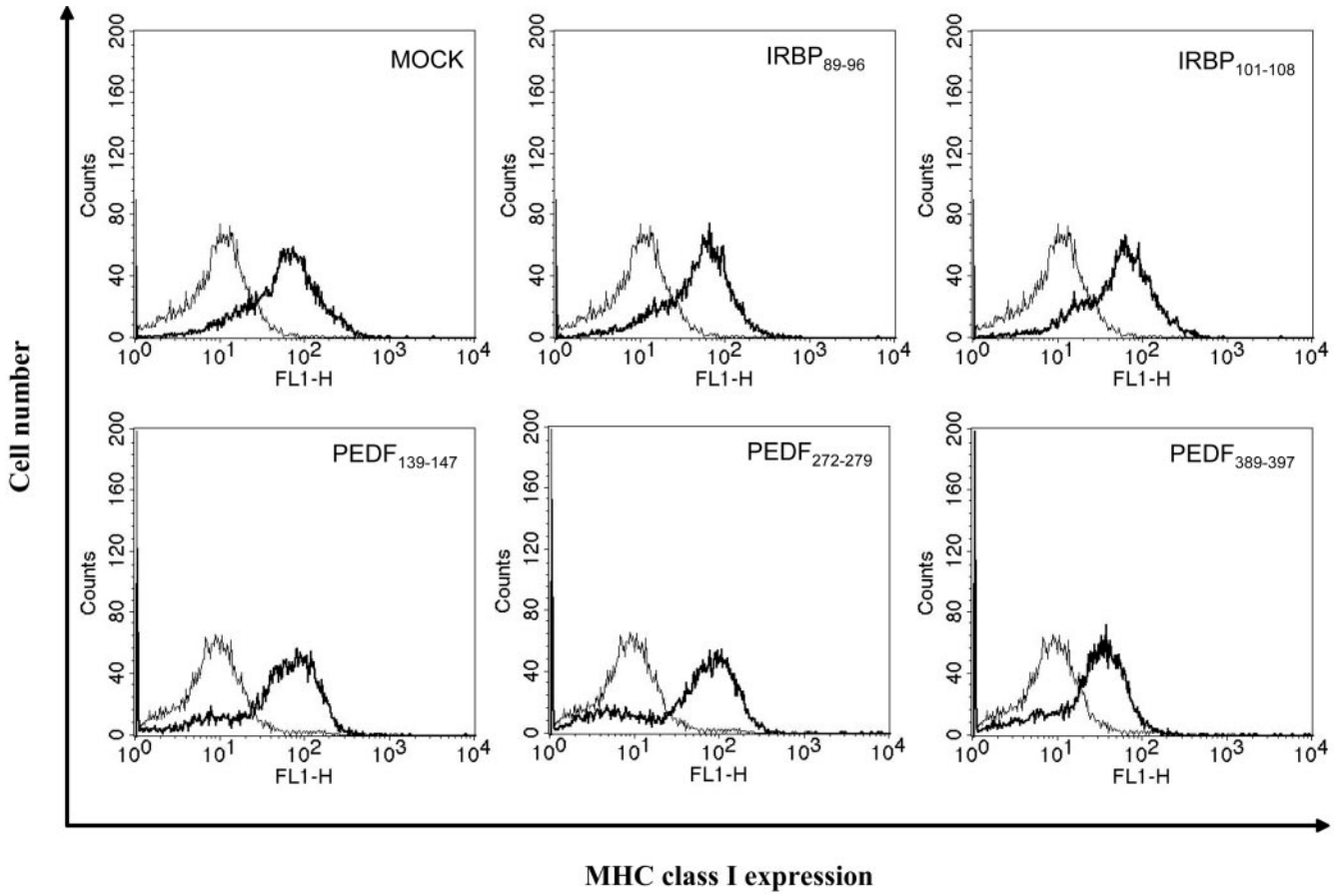

FIGURE 1. A peptide-binding flow cytometry assay for five immunogenic peptides. At 26°C, RMA-S cells displayed empty (for peptide), unstable MHC-I molecules on their cell surfaces that were stabilized after exposure to exogenously added MHC-I-binding peptide. The RMA-S cells were incubated overnight at 26°C, then pulsed with the indicated peptide. The amount of 8F12 bound to RMA-S cells was measured using indirect fluorescence after the addition of FITC-RAM and by subsequent analysis for fluorescence in a flow cytometer. *Thin lines*: RMA-S cells incubated in culture medium alone as background control; *thick lines*: RMA-S cells incubated in the presence of MOCK peptide as positive control or indicated peptides. The peptide-induced stabilization of MHC-I expressed by RMA-S cells was also defined as the FI in Table 1.

Effect of Immunogenic Peptides on the Severity of IRBP-Induced Uveitis

To investigate whether these five immunogenic peptides induce inflammation in murine eyes, C57BL/6 mice were immunized subcutaneously with a mixture of the five peptides (30 μg of each peptide) along with 120 μg of $HepBcore_{128-140}$ in 1:1 CFA. On day 21 after immunization, eyes were removed for histopathological analysis. We did not find any inflammation in the eyes of mice to be induced by the immunogenic peptides alone (data not shown). However, when a mixture of the immunogenic peptides was injected subcutaneously into the mice along with IRBP (see the Materials and Methods section), the peptides enhanced the severity of IRBP-induced uveitis (Fig. 3). Thirty mice were included in each group, and the eyes were scored individually from grades 0 to 4, each point in Figure 3 representing the average score of two eyes in one mouse. The average score of the group immunized with IRBP and peptides was 0.6 versus 0.2 in the group immunized with IRBP alone ($P < 0.05$).

DISCUSSION

Maintenance of the immune privilege of the eye is of fundamental importance in preserving the delicate structures of the retina from destruction by an inflammatory response with subsequent loss of vision. However, in several cases, inflammatory responses were observed within the eye. Thus, it is well known that damage to one eye can evoke an autoimmune reaction in the opposite eye called sympathetic ophthalmia, which is defined as a bilateral granulomatous panuveitis occurring after a penetrating injury (traumatic or surgical) to one of the eyes. The pathogenesis of sympathetic ophthalmia suggests that drainage of ocular antigens, including a soluble fraction from the outer segments of photoreceptor cells, into the lymphatic system, in conjunction with adjuvant activity of molecules entering through the perforating wound, elicits a delayed bilateral cellular autoimmune response.[44]

Despite the immune-privileged status of the eye, uveitis can still occur occasionally, probably due to an autoimmune etiology. Experimental autoimmune uveitis in mice, which can be induced by immunization of retinal antigens or peptides derived from retinal antigens, has therefore become an important model for studying uveitis of presumed autoimmune etiology.

Several investigators have reported that sympathetic ophthalmia[19,20] and EAU[14–18] are MHC class II–restricted diseases. Thus, it is well recognized that MHC class II–restricted peptides derived from IRBP can induce EAU in mice. However, some forms of uveitis are also reported to correlate strongly with MHC class I, including birdshot retinochoroidopathy (HLA-A29, in particular HLA-A29.2),[21–23] Behçet's disease (HLA-B51)[24–26] and acute iritis/ankylosing spondylitis (HLA-B27),[27,28] suggesting that MHC class I–restricted peptides

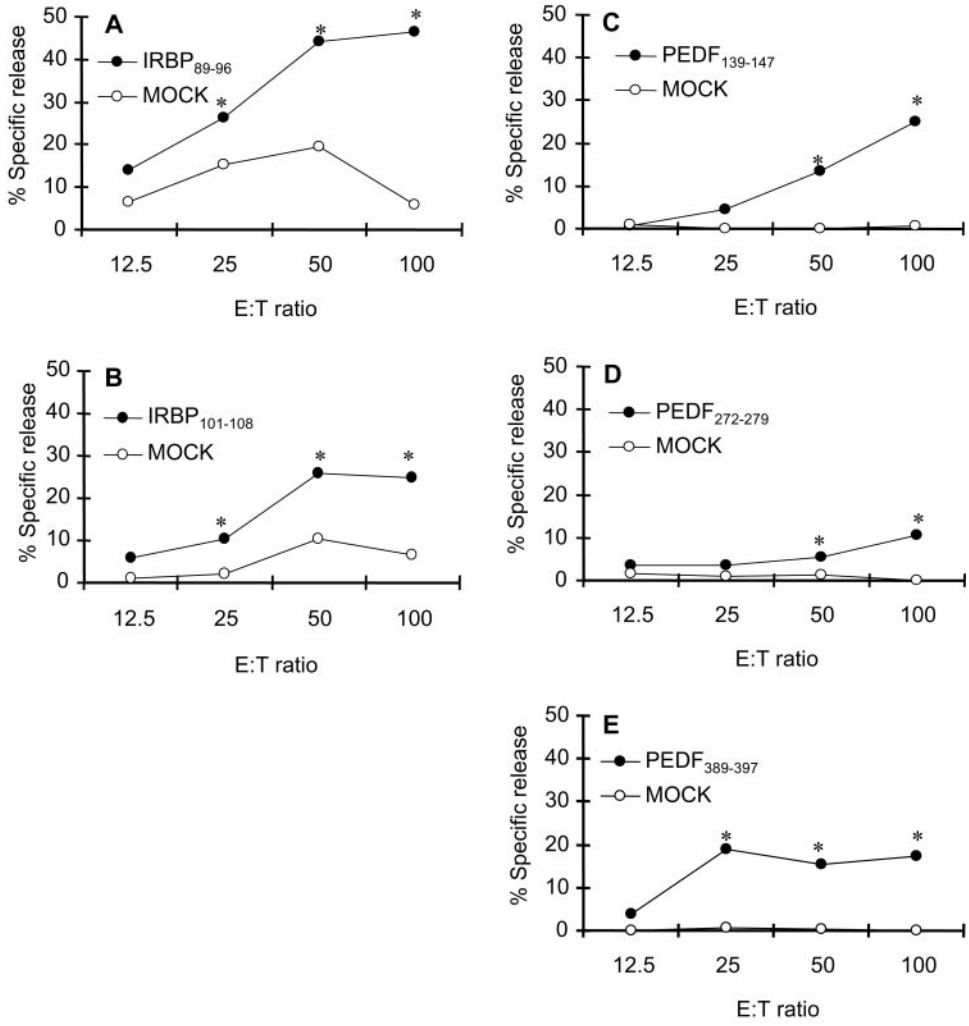

FIGURE 2. Induction of specific CTL responses against IRBP-derived peptides and PEDF-derived peptides. CTL responses against H-2 K^b-binding peptides $IRBP_{89\text{-}96}$ (**A**), $IRBP_{101\text{-}108}$ (**B**), $PEDF_{139\text{-}147}$ (**C**), $PEDF_{272\text{-}279}$ (**D**), and $PEDF_{389\text{-}397}$ (**E**) were detected after immunization with the peptides along with $HepBcore_{128\text{-}140}$ mixed in IFA. Effector cells were splenocytes from the immunized mice, which were cultured for 5 days in vitro with irradiated syngeneic spleen cells pulsed with the peptide, as indicated. ^{51}Cr-labeled RMA-S cells pulsed with appropriate peptide were used as target cells. A 4-hour ^{51}Cr release assay was used to detect CTL activities at different E:T ratios. MOCK is an irrelevant peptide and functions as negative control. Data are from a single representative experiment for each immunogenic peptide. Standard deviations are <10% of the mean ($^*P < 0.05$).

could be involved in the mechanisms of uveitis. We therefore decided to investigate whether MHC class I–restricted peptides derived from retinal antigens could be immunogenic. There are so far no reports on the uveitogenicity of PEDF, but it is a natural extracellular component of the retina, it has neuroprotective activity and can protect photoreceptors from degeneration.[32–34] Because of the high level of PEDF expression, peptides derived from PEDF could be expressed together with MHC-I and therefore be a potential target for $CD8^+$ T-cells; thus PEDF was included in this study besides IRBP, S-Ag, recoverin, and phosducin.

Nowadays, prediction strategies for peptides binding to MHC class I molecules are available,[35,36,45–47] so it is possible to predict MHC class I–restricted peptides according to different MHC class I– binding motifs. From the known autoantigens IRBP, S-antigen, recoverin, phosducin, and PEDF derived from the eye, 21 peptides were predicted to bind to $H\text{-}2^b$ with a computer-based program.[35,36] We were interested in whether these proteins contained putative CTL-inducing and therefore potentially pathogenic MHC class I–restricted peptides.

We found that all the predicted peptides with high (<50 nM) to intermediate (50 to 100 nM) affinity could upregulate the expression of MHC class I molecules on the TAP-deficient RMA-S cell line. The ability to upregulate MHC class I was in some instances comparable to the known good binder MOCK. These results indicate that the prediction program we used was reliable because all the peptides with predicted binding affinities less than 100 nM could upregulate H-2 K^b/D^b mole-

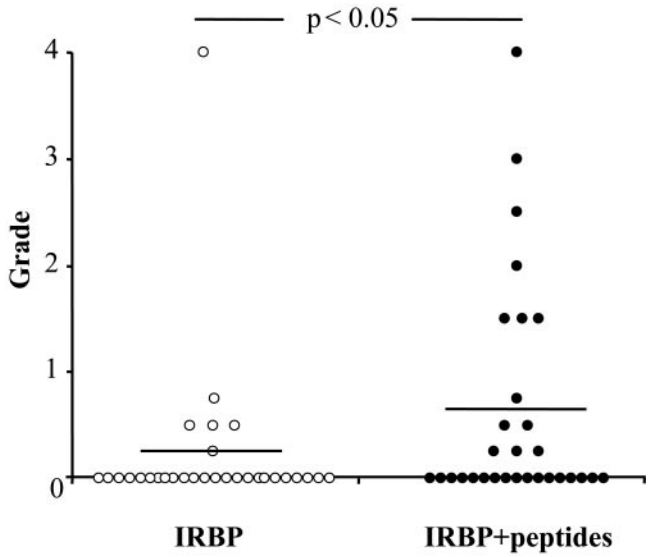

FIGURE 3. Histopathological evaluation of EAU. Comparison of histologic EAU severity between groups of mice treated with IRBP and IRBP plus five immunogenic peptides. Eyes of the mice immunized with IRBP or IRBP and peptides were removed on day 21. The EAU histologic scores were graded on a scale of 0 to 4. Each data point represents one mouse (average of both eyes). *Horizontal lines*: the average EAU score of the groups.

cules on RMA-S cells, and therefore this prediction program may also be useful to predict MHC-binding peptides from other ocular antigens.

Among the predicted peptides, the two IRBP-derived peptides $IRBP_{89-96}$ and $IRBP_{101-108}$ and the three PEDF-derived peptides $PEDF_{389-397}$, $PEDF_{139-147}$, and $PEDF_{272-279}$ induced specific CTL responses in vivo in up to half the immunized mice, whereas the remaining 16 peptides, including 5 IRBP-derived peptides, 5 S-antigen-derived peptides, 1 recoverin-derived peptide, 1 phosducin-derived peptide and 4 PEDF-derived peptides, did not induce specific CTL reactivity. More potential immunogenic peptides could have been identified by increasing the E:T cell ratio, but in the present study, we focused on those which most readily induced a CTL response.

Although 5 of 21 predicted peptides generated CTL responses, they did not induce CTL reactivities in all the immunized mice, as described earlier. For $IRBP_{89-96}$ and $PEDF_{139-147}$, three of six immunized mice generated cytotoxic responses, whereas only one of six mice immunized with $PEDF_{272-279}$ generated CTL activity. In a previous study, we found that self-peptide emulsified in IFA induces strong CTL responses in the regional lymph nodes.[43] This suggests that draining lymph nodes should also be included when testing for CTL reactivity.

Additional unspecific cytotoxic activity was generated in the presence of MOCK peptide, which most likely is due to bystander generation of natural killer cell–like activity in the system by IL-2 exogenously added or is produced by the specific CTL response.[48] The different unspecific response observed was due to variability of the individual cultures tested, but most of the cytotoxicity generated was shown to be specific against the immunized peptide.

Five MHC class I–restricted peptides generated specific CTLs, and we tested whether immunization with these peptides along with $HepBcore_{128-140}$ would induce uveitis. We found that immunogenic peptides alone did not induce uveitis (data not shown), but these peptides enhanced the severity of IRBP-induced uveitis, which means CTL may contribute to inflammation in the eyes, providing uveitogenic $CD4^+$ T cells are present. These data indicate that a threshold level exists for induction of uveitis. In the work by McPherson et al.,[30] it was found that only activated and not resting $CD8^+$ T cells specific for β-galactosidase induced uveitis by adoptive transfer in mice where β-galactosidase was expressed under the control of eye-specific promoters. In our study, a mixture of immunogenic peptides alone did not induce inflammation in the eyes, probably because of quiet specific $CD8^+$ T cells induced by the immunogenic peptides. It seems that additional stimulation of $CD8^+$ T-cells is needed and that this threshold level is achieved by simultaneous immunization with IRBP, because the MHC class II helper peptide $HepBcore_{128-140}$ together with the MHC class I peptides do not induce uveitis. Thus, how $CD8^+$ T cells cross-talked with $CD4^+$ T cells in EAU induction remains to be resolved.

In the present study, it was shown that a mixture of five peptides found to induce specific CTL in vivo could enhance the induction of uveitis when added together with IRBP. In the retina, a combination of each of these peptides would most likely be presented on the cell surface bound to MHC-I, and a larger number of clones could be generated because of there being more epitopes, in agreement with the approach used for peptide-based immunization to treat tumors.[49] It may be that the effect observed in our study is caused by some peptides in the mixture being immunodominant in the response. However, competition of peptides for MHC binding will not significantly reduce CTL recognition, even in the presence of limited antigen-presenting cells.[50] Because the number of antigen-presenting cells in vivo are not limited, all the peptides can be presented to induce specific CTL.

C57BL/6 mice were chosen for this study, as there are good computer-based algorithms for prediction of MHC class I–restricted peptides for the $H\text{-}2^b$ haplotype, and this mouse strain is known to be susceptible to induction of uveitis. Further information could have been obtained by using B10.RIII mice, which are highly prone to the induction of uveitis. However, the B10.RIII mouse is of the $H\text{-}2^r$ haplotype, and binding data do not currently exist for computer-based algorithms.

In conclusion, this is the first time that H-2 K^b-binding retinal protein-derived peptides have been reported to be immunogenic, suggesting that these peptides could function as autoantigenic epitopes, which play a role in the development of inflammatory eye diseases, such as uveitis.

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