Characterization of a New Epitope of IRBP That Induces Moderate to Severe Uveoretinitis in Mice With H-2[b] Haplotype

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PURPOSE. Experimental autoimmune uveitis (EAU) induced in mice using the retinal antigen interphotoreceptor retinoid binding protein (IRBP) is an animal model for posterior uveitis in humans. However, EAU induced by native IRBP protein or its widely used epitope amino acid residues 1 to 20 of human IRBP (hIRBP1-20) is inconsistent, often showing low scores and incidence. We found an urgent need to identify a better pathogenic epitope for the C57BL/6 strain.

METHODS. Mice were immunized with uveitogenic peptides or with native bovine IRBP. Clinical and histological disease and associated immunological responses were evaluated. Truncated and substituted peptides, as well as bioinformatic analyses, were used to identify critical major histocompatibility complex (MHC)/T cell receptor (TCR) contact residues and the minimal core epitope.

RESULTS. The new uveitogenic epitope of IRBP, amino acid residues 651 to 670 of human IRBP (LAQGAYRTAVDLESLASQLT [hIRBP651-670]) is uveitogenic for mice of the H-2b haplotype and elicits EAU with a higher severity and incidence in C57BL/6 mice than the previously characterized hIRBP1-20 epitope. Using truncated and substituted peptides, as well as bioinformatic analysis, we identified the critical contact residues with MHC/TCR and defined the minimal core epitope. This made it possible to design MHC tetramers and use them to detect epitope-specific T cells in the uveitic eye and in lymphoid organs of hIRBP651-670–immunized mice.

CONCLUSIONS. Data suggest that hIRBP651-670 is an epitope naturally processed from a conserved region of native IRBP, potentially explaining its relatively high uveitogenicity. This epitope should be useful for basic and preclinical studies of uveitis in the C57BL/6 model and gives access to genetically engineered mice available on this background.

Keywords: autoimmune, EAU, IRBP, H-2b mice

Autoimmune uveitis encompasses a group of diseases that affect 2 million Americans annually and is estimated to be responsible for 10% to 15% of cases of severe visual handicap.[1,2] Experimental autoimmune uveitis (EAU) induced in mice by immunization with the retinal antigen interphotoreceptor retinoid binding protein (IRBP) is an animal model used to represent these diseases and has been extremely useful to define basic mechanisms of disease as well as a template to evaluate new therapies. Susceptible animal strains are B10.RIII and C57BL/6. Although the former strain reproducibly develops high disease scores with native bovine IRBP or with its major uveitogenic peptide amino acid residues 161 to 180 of human IRBP (hIRBP161-180), the latter strain develops moderate disease scores when immunized with native bovine IRBP but only mild disease when immunized with its uveitogenic peptide hIRBP1-20, which was identified 15 years ago.[3]

Because C57BL/6 is the background on which most gene-manipulated mice are made and because native IRBP is difficult to obtain for many investigators, hIRBP1-20 is used by many laboratories out of necessity, for lack of a better epitope. However, in many laboratories, including our own, data generated in C57BL/6 mice using hIRBP1-20 are often uninterpretable due to low disease scores and low incidence. Crossing the genetically altered strains already available on the C57BL/6 background onto the more susceptible B10.RIII background requires considerable time and resources. Therefore, we recognized an urgent need to define an epitope that induced a more consistent disease in the C57BL/6 strain.

A recent study from our laboratory revealed several additional epitopes that were uveitogenic in C57BL/6 mice.[4] In the present study, we characterized one of these new uveitogenic epitopes and compared it to the previously characterized epitope hIRBP1-20.[3,5] This epitope, encoded by amino acid residues 651 to 670 of human IRBP (LAQGAYR-TAVDLESLASQLT [hIRBP651-670]), is completely conserved across human, mouse, and bovine species, although in bovine and mouse IRBPs the identical residues are in positions 666 through 685, due to amino acid changes that occurred in IRBP

TABLE 1. Truncations and Alanine Substitutions in the hIRBP651-670 Core Epitope*

Residues	Amino acids																									
648-662	R	A	K	L	A	Q	G	A	Y	R	T	A	V	D	L											
649-663		A	K	L	A	Q	G	A	Y	R	T	A	V	D	L	E										
651-670				**L**	**A**	**Q**	**G**	**A**	**Y**	**R**	**T**	**A**	**V**	**D**	**L**	**E**	**S**	**L**	**A**	**S**	**Q**	**L**	**T**			
651-668				L	A	Q	G	A	Y	R	T	A	V	D	L	E	S	L	A	S	Q					
651-666				L	A	Q	G	A	Y	R	T	A	V	D	L	E	S	L	A							
651-662				L	A	Q	G	A	Y	R	T	A	V	D	L											
651-660				L	A	Q	G	A	Y	R	T	A	V													
652-669					A	Q	G	A	Y	R	T	A	V	D	L	E	S	L	A	S	Q	L				
653-670						Q	G	A	Y	R	T	A	V	D	L	E	S	L	A	S	Q	L	T			
653-668						Q	G	A	Y	R	T	A	V	D	L	E	S	L	A	S	Q					
654-664							**G**	**A**	**Y**	**R**	**T**	**A**	**V**	**D**	**L**	**E**	**S**									
655-670								A	Y	R	T	A	V	D	L	E	S	L	A	S	Q	L	T			
655-668								A	Y	R	T	A	V	D	L	E	S	L	A	S	Q					
658-673											T	A	V	D	L	E	S	L	A	S	Q	L	T	A	D	L
659-670												A	V	D	L	E	S	L	A	S	Q	L	T			
661-670														D	L	E	S	L	A	S	Q	L	T			
G654A							**A**	A	Y	R	T	A	V	D	L	E	S									
Y656A							G	A	**A**	R	T	A	V	D	L	E	S									
R657A							G	A	Y	**A**	T	A	V	D	L	E	S									
T658A							G	A	Y	R	**A**	A	V	D	L	E	S									
V660A							G	A	Y	R	T	A	**A**	D	L	E	S									
D661A							G	A	Y	R	T	A	V	**A**	L	E	S									
L662A							G	A	Y	R	T	A	V	D	**A**	E	S									
E663A							G	A	Y	R	T	A	V	D	L	**A**	S									
S664A							G	A	Y	R	T	A	V	D	L	E	**A**									

* Full-length peptides, minimal core epitopes, and alanine substitutions are marked in bold text.

N-terminally to this peptide (Supplementary Fig. S1). We report that immunization with hIRBP651-670 in complete Freund's adjuvant followed by injection of pertussis toxin (PTX) resulted in EAU with consistently higher scores and incidence than that induced by hIRBP1-20. This new epitope should prove useful for basic studies of uveitis in addition to, or instead of, hIRBP1-20.

MATERIALS AND METHODS

Animals and Reagents

Inbred strains of C57BL/6J (stock 000664), C57BL/10J (stock 000665), and 129S1/SvImj (stock 002448) mice were pur-chased from Jackson Labs (Bar Harbor, ME, USA). Bovine IRBP was purified by concanavalin A Sepharose affinity chromatog-raphy and fast protein liquid chromatography.[6] Uveitogenic peptides of hIRBP1-20 (GPTHLFQPSLVLDMAKVLLD) and hIRBP651-670 (LAQGAYRTAVDLESLASQLT) were synthesized by GeneScript (Piscataway, NJ, USA), Anaspec (Fremont, CA, USA), or Shanghai Hanhong Chemical Co., Ltd. (Shanghai, China). The truncated peptides of hIRBP651-670 (Table 1) were synthesized by Shanghai Hanhong Chemical Company. Animal care and use conformed to institutional guidelines and to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of EAU and Disease Scoring

Animals were injected subcutaneously with bovine IRBP and/or human IRBP peptides, as indicated, emulsified 1:1 v/v in complete Freund's adjuvant containing 2.5 mg/mL *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). A total of 200 µL emulsion was injected subcutaneously (SC) distributed along the base of the tail (100 µL) and both thighs (50 µL each). In all immunizations, PTX (0.5–1 µg in 100 µL; catalog no. P7208; Sigma-Aldrich Corp., St. Louis, MO, USA) was injected intraperitoneally (IP) on the day of immunization.

Clinical disease was scored at regular intervals by fundus examination, using a binocular microscope, as described previously.[7] Eyes and lymphoid organs were harvested 21 days after immunization. Globes were immediately fixed with 4% glutaraldehyde (Thermo Fisher Scientific, Rockville, MD, USA) in phosphate-buffered saline (PBS) for 1 hour, followed by 10% neutral formaldehyde (Sigma-Aldrich Corp.) in PBS for 24 hours before grossing and embedding. Methacrylate blocks were cut into 6-µm sections and stained with hematoxylin and eosin (H&E) for histological analysis. Sections were evaluated in a blinded fashion by one of the authors (CCC) and were scored on a scale of 0 (no disease) to 4 (very severe disease) in half-point increments, according to the number and size of lesions, using criteria described previously.[7]

Quantitation of Antigen-Induced Immune Responses

Single cell suspensions of lymphocytes from lymph nodes draining the site of immunization were collected and cultured (0.5 million cells/200 µL/well of a 96-well plate) in the presence of graded doses (0.2 µg/mL, 2.0 µg/mL, and 20.0 µg/mL) of bovine IRBP or human IRBP peptides in HL-1 medium (Lonza, Inc., Walkersville, MD, USA) supplemented with methyl α-D-mannopyranoside (10 mg/mL) to neutralize the effect of residual concanavalin A in the IRBP preparation. Cells were incubated at 37°C and 5% CO_2 for a total of 72 hours and were pulsed with ^3H-labeled thymidine (1 µCi/well) during the last 18 hours of incubation. The level of [^3H]thymidine incorporation was measured using a Microβ scintillation counter (PerkinElmer, Waltham, MA, USA).

Antigen-induced cytokines were measured in culture supernatants of cells from draining lymph nodes (1 million/200 µL/well) of immunized mice, incubated in the presence or absence of immunizing peptide (20 µg/mL) for 48 hours. Cytokines were also measured in extracts of uveitic eyes as described previously.[8] Briefly, after removing external tissues and lens from enucleated eyes, the remaining eye tissue was minced with scissors, vigorously dispersed in HL-1 medium (50 µL/eye) with a pipette and centrifuged to collect the clarified supernatant for measuring cytokine levels. Multiplex cytokine analysis was done using Bio-Plex Pro mouse cytokine assay kit (Bio-Rad Lab, Inc., Hercules, CA, USA).

Expansion and Adoptive Transfer of hIRBP651-670–Specific T Cells

For expansion of primary cells, lymphocytes from draining lymph nodes and spleens from mice immunized with hIRBP651-670 were isolated on day 14 after immunization and cultured in vitro with 10 µg/mL hIRBP651-670 for 3 days at 37°C and 5% CO_2 as described previously.[9] On the third day, cells were collected and washed, and approximately 20 million cells/mouse were infused IP or intravenously (IV) in 0.5 mL HL-1 medium containing 1% normal mouse serum.

An antigen-specific T-cell line was derived from the cells infiltrating the eyes of EAU-affected mice immunized with hIRBP651-670, as described previously,[10] with modifications. At the end of each round of expansion in 12-well tissue culture plates (Costar; Sigma-Aldrich Corp.), resting cells (3–4 million cells/2 mL) were stimulated with 20 µg/mL hIRBP651-670 in the presence of naïve, irradiated (3000 rads) splenocytes as antigen-presenting cells (5 million cells/2 mL). Cells were collected at the end of 48 hours of antigenic stimulation, washed, and adoptively transferred by IP injection (number of cells/mouse are specified in the Fig. 5A legend).

Tetramer Staining of hIRBP651-670–Specific Cells

The I-A^b-hIRBP654-664 tetramer reagent labeled with streptavidin-phycoerythrin (PE) or streptavidin-allophycocyanin (APC; Prozyme, Hayward, CA, USA) was generated by Moon et al.,[11] using a method described previously.[11]

Eye-infiltrating cells were isolated from uveitic eyes as described previously.[8] Eye tissues were minced and dispersed in HL-1 medium containing 1 mg/mL collagenase D at the rate of 1 mL medium for 10 eyes and incubated at 37°C for 1 hour. Samples were then triturated, filtered through a 70-µm disposable sieve, washed, and resuspended in fluorescence-activated cell sorting buffer before being stained with antibodies and the tetramer reagent as described previously.[12] Briefly, For staining lymphocytes from lymph nodes and spleen, cells were stained with 10 µM of tetramer for 1 hour at room temperature and then washed and resuspended in 150 µL 1× PBS containing 1% fetal bovine serum. For enriching tetramer-positive cells, 50 µL anti-PE microbeads (product 130-048-801; Miltenyi Biotech, Gladbach, Germany) was added and samples were incubated for 30 minutes at 4°C. Cells were washed and PE-positive cells were sorted using a LS separation column (MACS; Miltenyi Biotech) according to the manufacturer's protocol. Cells were then surface-stained with the fluorochrome-labeled anti-mouse antibodies CD3 (BD Horizon V500 [V500]), CD4 (eFluor450 [eF450]), CD8 (phycoerythrin-cyanine7 [PE-Cy7]) and CD44 (allophycocyanin-cyanine7 [APC-Cy7]) in the presence of 1 µL Fc block, along with the antibodies for the dump channel (fluorescein isothiocyanate [FITC]) anti-mouse B220, CD11b, CD11c. For staining the cells infiltrating the eyes, after staining with the tetramer for 1 hour at room temperature, surface staining was done with one or more of the following antibodies: anti-mouse CD3 (V500), CD45 (FITC), CD8 (PE-Cy7), or CD4 (APC-Cy7) along with antibodies for the dump channel (eF450) anti-mouse CD11c, major histocompatibility complex (MHC) class II I-A^b, B220. Enrichment of tetramer-positive cells using microbeads was not done for eye samples. (Anti-mouse CD3 [V500] was purchased from BioLegend, San Diego, CA, USA; all other antibodies were purchased from eBioscience, San Diego, CA, USA.)

Protein Sequence Alignment and Epitope Prediction

Alignment of the IRBP (also known as RBP3) sequences in human (NCBI accession no. NP_002891.1), bovine (accession no. NP_776589.1), and murine (accession no. NP_056560.1) species was done using MultAlin tool interface (MultAlin/INRA, Paris, France; in the public domain at http://multalin.toulouse.inra.fr/multalin/).[13] MHC class II binding predictions for I-A^b molecule were done using IEDB analysis resource consensus tool (Immune Epitope Database and Analysis Resource, National Institute of Allergies and Infectious Diseases, Bethesda, MD, USA; in the public domain at http://tools.immuneepitope.org/mhcii/)[14,15] and RANKPEP software (in the public domain at http://imed.med.ucm.es/Tools/rankpep.html).[16]
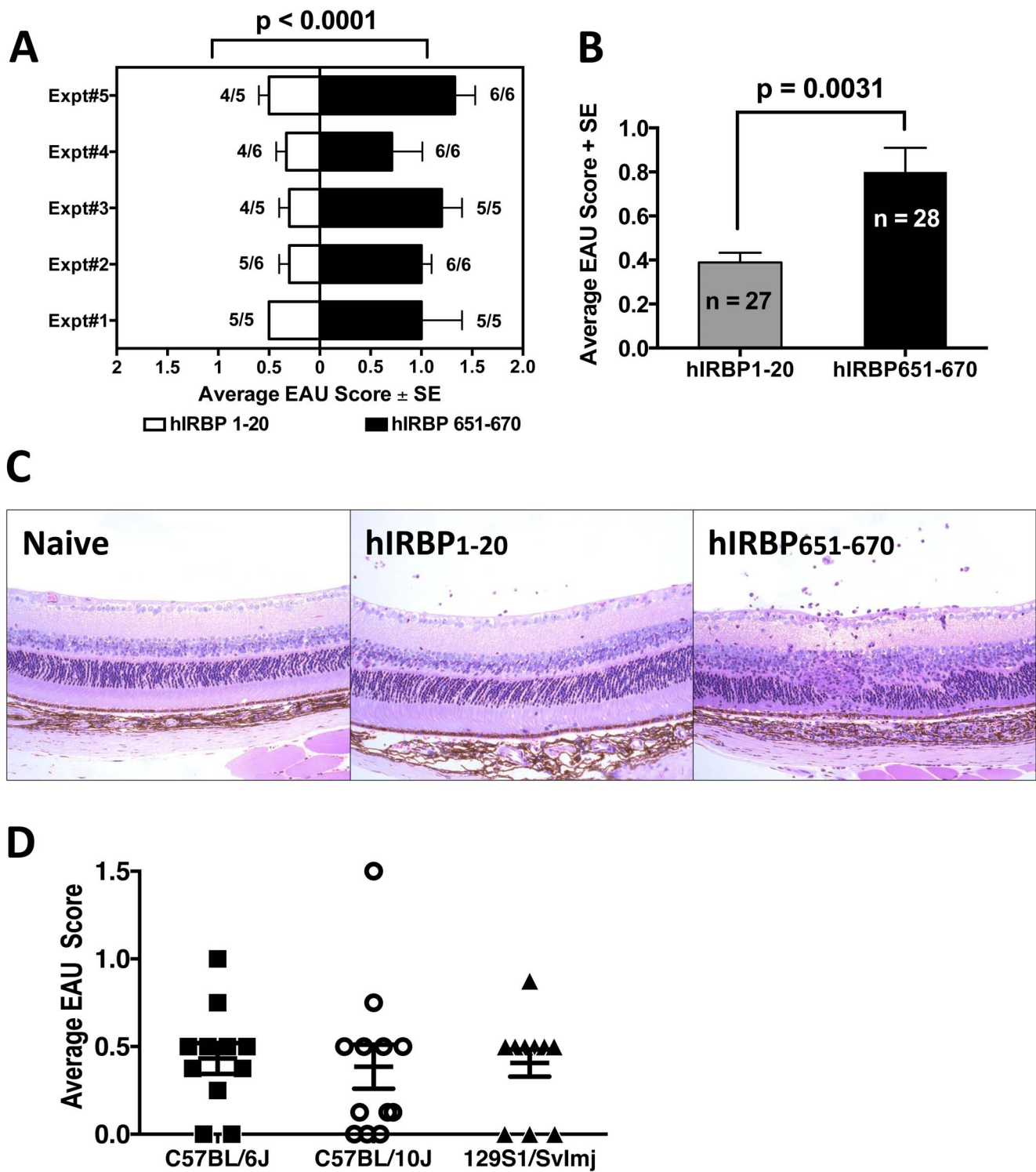
FIGURE 1. hIRBP651-670 induces more severe EAU than hIRBP1-20. C57BL/6J mice were immunized with peptide hIRBP1-20 or hIRBP651-670 (300 µg per mouse) and 0.5 µg PTX. Disease was scored by histopathological analysis of eyes collected 21 days after immunization. (A) Average (±SE) scores and incidence from five individual experiments are shown ($P < 0.0001$, two-way ANOVA). (B) Combined scores are shown from all five experiments shown in Figure 1A ($P = 0.0031$, Mann-Whitney U test). (C) Retinal pathology in C57BL/6J mice immunized with hIRBP1-20 or hIRBP651-670. (D) Average scores (±SE) of EAU in strains C57BL/6J, C57BL/10J, and 129S1/SvImj immunized with 200 µg hIRBP651-670 and 1 µg PTX in eyes collected on day 21. Data are from two independent experiments.

Statistical Analysis

Statistical differences between groups were analyzed using Prism version 6.0d software (GraphPad, San Diego, CA) as follows: differences in EAU scores (nonparametric), as determined by histology, were analyzed using Mann-Whitney U test. Differences in EAU kinetics were analyzed by two-way ANOVA or by two-tailed unpaired t test.
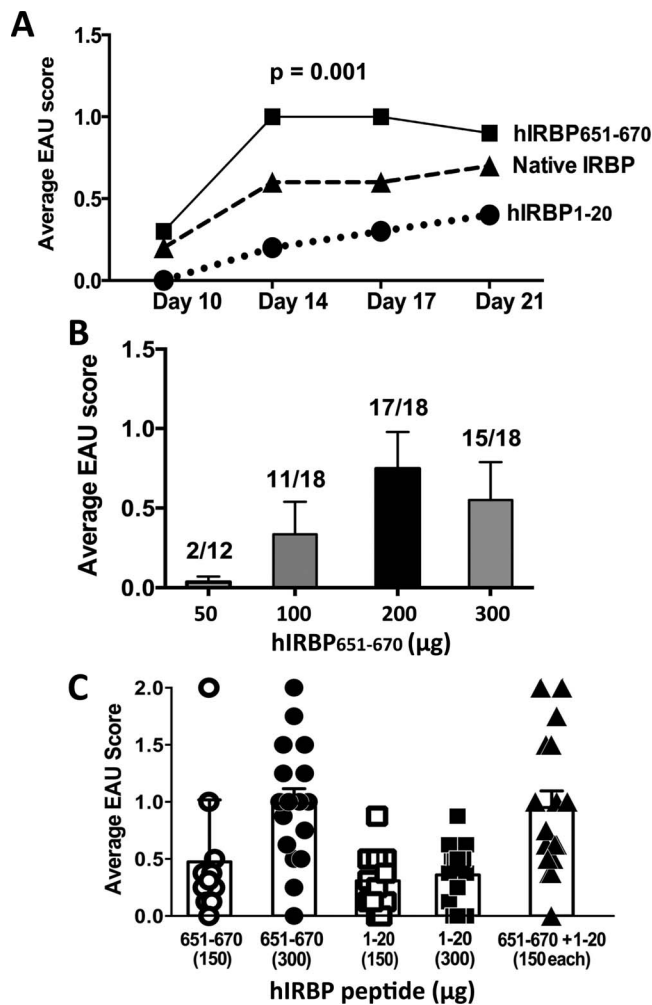
FIGURE 2. Kinetics and dose-response of EAU induced with hIRBP651-670. (A) Experimental autoimmune uveitis was induced in C57BL/6J mice by using 300 μg hIRBP651-670 ($n = 28$) or 300 μg hIRBP1-20 ($n = 28$) or 150 μg native bovine IRBP protein ($n = 12$) and 0.5 μg PTX IP. Shown are average clinical scores as evaluated on the indicated days. Incidence rate is given in Table 2. Disease scores of hIRBP651-670 and hIRBP1-20 are significantly different ($P = 0.0012$ between groups by two-way ANOVA). (B) Dose response of hIRBP651-670-induced EAU. C57BL/6J mice were immunized with specified doses of hIRBP651-670 plus 0.5 μg PTX. Shown is the severity of disease as average (±SE) EAU score by histological analysis on day 21. Data are combined from three repeat experiments. (C) Lack of synergy is shown by mixing both of the uveitogenic peptides. C57BL/6J mice were immunized with 300 μg each of the individual peptides or 150 μg hIRBP651-670 plus 150 μg hIRBP1-20 (total: 300 μg of both of the peptides) and 0.5 μg PTX. Shown are average (±SE) histopathology scores compiled from three repeated experiments on day 21 post immunization ($n = 17$ per group).

TABLE 2. Incidence of EAU During the Course of Disease*

Antigen	Days Post Immunization			
	10	14	17	21
hIRBP1-20	0%	57%	75%	82%
hIRBP651-670	86%	100%	100%	100%
IRBP protein	58%	92%	92%	100%

* Experimental autoimmune uveitis was induced as described in the legend to Figure 2A. Average EAU scores are shown in Figure 2A.

retina was more extensive and infiltration of inflammatory cells was higher in mice immunized with hIRBP651-670 than in those immunized with hIRBP1-20. In addition to C57BL/6J mice, other inbred strains of mice with H-2^b haplotype at the MHC gene loci are also susceptible to hIRBP651-670–induced EAU (Fig. 1D).

Kinetics and Dose Response of EAU Induced With hIRBP651-670

Mice were immunized with graded doses of hIRBP651-670 or hIRBP1-20. Disease was followed by fundus examination, and terminal scores were obtained by histopathology. Disease induced with hIRBP651-670 had an earlier onset and higher incidence rate than that induced with hIRBP1-20. The incidence of hIRBP651-670–induced disease reached 100% by day 14 after immunization (Fig. 2A; Table 2). A dose of 200 μg peptide per mouse appeared to yield maximal scores, and higher doses did not result in more disease (Fig. 2B). That said, we noted differences in uveitogenicity of different batches of peptide even from the same vendor. For that reason, in most of the experiments we preferred to use 300 μg/mouse to maintain a safety margin. Co-immunization with hIRBP1-20 and hIRBP651-670 was additive but not synergistic (Fig. 2C).
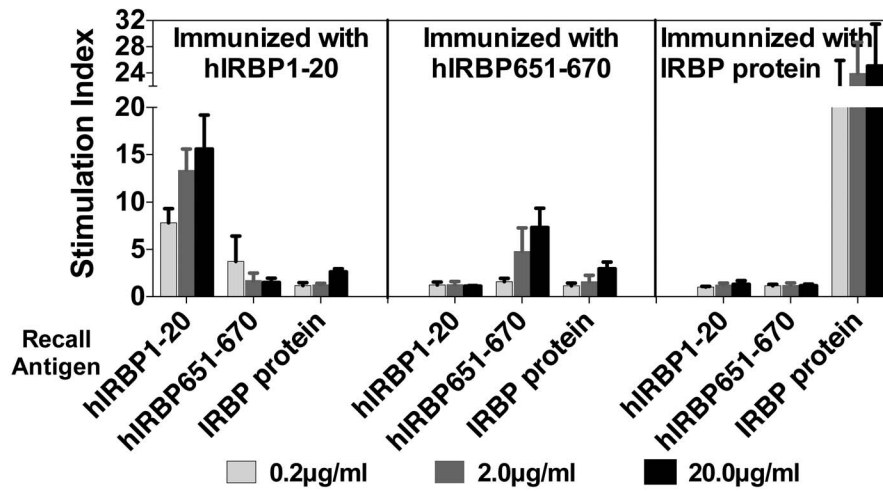
Immunological Responses Associated With hIRBP651-670–Induced EAU

When mice were immunized with either of the two peptides and lymphocytes from draining lymphoid organs were recalled in vitro with native IRBP protein, weak but consistent crossreactive responses could be detected (Fig. 3A). Cytokine levels measured in the extracts of enucleated eye preparations from EAU mice on day 14 after immunization showed increased proinflammatory cytokines in hIRBP651-670–immunized mice compared to those in hIRBP1-20–immunized mice (Fig. 3B). Clinical EAU scores for animals used in this experiment are shown in Figure 3C. In contrast to disease parameters, the in vitro recall response to the immunizing peptide was higher in mice immunized with hIRBP1-20 than in mice immunized with hIRBP651-670 (Fig. 3A). Furthermore, levels of proinflammatory cytokines in the culture supernatants of antigen-stimulated cells from draining lymph nodes were higher in hIRBP1-20–immunized mice than in hIRBP651-670–immunized mice (Fig. 3D). The reasons for this apparent discrepancy are discussed ahead.
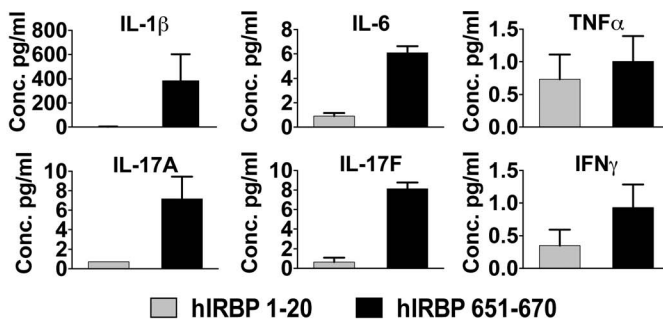
Identification of hIRBP651-670 Core Epitope and Critical Contact Residues by Using Truncated Peptides and Peptides With Alanine Substitutions

To identify the minimal uveitogenic epitope within the 20-amino acid–long epitope hIRBP651-670, we induced EAU with peptides truncated on both the N and C termini of the hIRBP651-670 sequence. Truncated peptides used to identify
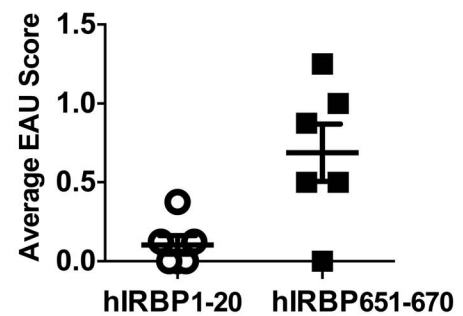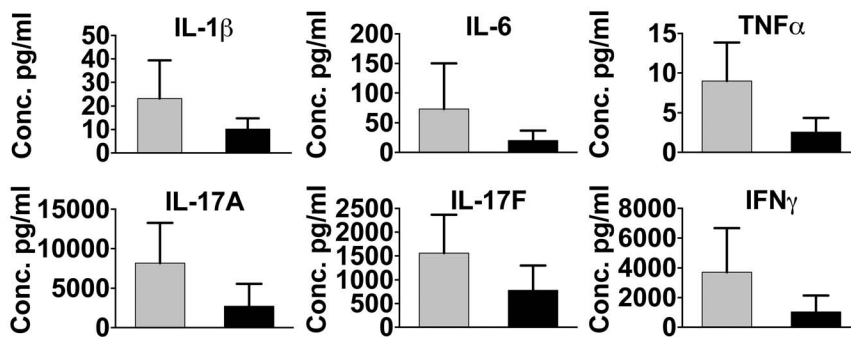
RESULTS

Comparison of Uveitis Induced With Human IRBP Peptide 651–670 to That Induced With Human IRBP Peptide 1–20

Mice were immunized with 300 μg hIRBP651-670 or hIRBP1-20, and disease was scored by fundus examination and by histopathology. hIRBP651-670 consistently induced higher disease scores with higher incidence than the previously described peptide hIRBP1-20[3,5] over multiple repetitions of the experiment (Figs. 1A, 1B). Figure 1C shows that damage to the

FIGURE 3. Disease-associated immune responses. (A) C57BL/6J mice were immunized with 300 μg of one of the peptides or 150 μg IRBP and 0.5 μg PTX. Lymphocytes from draining lymph nodes of six mice per group were pooled and cultured with graded doses of each of the peptides or IRBP protein. Data are average (±SE) stimulation indexes from three independent experiments. (B) Levels of proinflammatory cytokines in eye extracts of EAU mice on day 14 after immunization. (C) Clinical scores of EAU by fundus examination of mice (those used in [B]) on day 13 post immunization. (D) Proinflammatory cytokine production by draining lymph node cells collected on day 9 or day 14 post immunization in response to immunizing peptide (20 μg/mL) measured in the culture supernatants after 48 hours of cell culture. Data are average (±SE) titers of cytokines from two independent experiments.
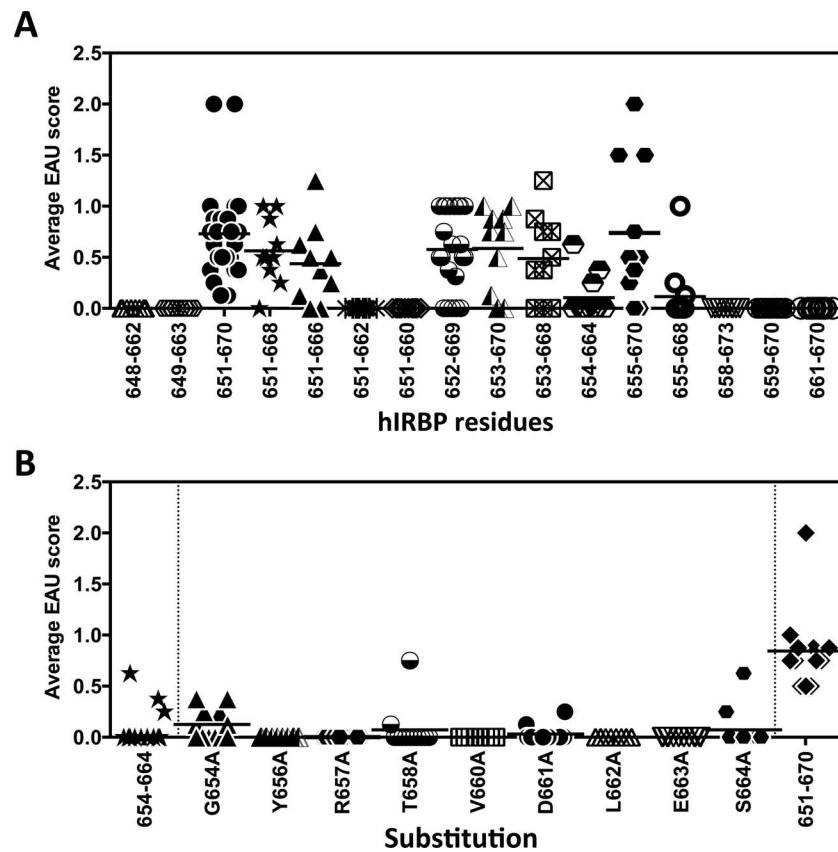
FIGURE 4. Identification of the minimal core epitope of hIRBP651-670 (residues 654–664). (**A**) C57BL/6J mice were immunized with 300 µg truncated versions of peptide hIRBP651-670 (listed in Table 1) plus 0.5 µg PTX. Average EAU scores of both of the eyes on day 21 are shown. Minimal epitope required for the induction of uveitis was residues 654 through 664. (**B**) Critical residues within the minimal core epitope were identified by inducing EAU in C57BL/6J mice, using 300 µg peptide hIRBP654-664 with alanine substitutions at each position (Table 1) plus 0.5 µg PTX. Shown are average EAU scores from both eyes of individual mice on day 21 after immunization compiled from two independent experiments ($n = 12$).

the core epitope are listed in Table 1. Based on the disease scores obtained by immunizing with truncated peptides (Fig. 4A) and the I-A^b MHC class II peptide binding predictions obtained using the IEDB analysis resource consensus tool (Supplementary Fig. S2A) and RANKPEP tool (Supplementary Fig. S2B), we defined the minimal core epitope region essential for binding to the I-A^b molecule and inducing EAU as residues 654 to 664 (GAYRTAVDLES).

Key residues within this core epitope involved in MHC or TCR binding were identified by inducing EAU with alanine replacements at positions where nonalanine residues were present in the native sequence (Table 1). Amino acid residues at positions 656Y, 657R, 660V, 662L, and 663E were found to be critical for inducing EAU (Fig. 4B). The 9-mer peptide that was subsequently shown to efficiently bind to I-A^b molecule was predicted to be the hIRBP656-664 sequence YRTAVDLES (Supplementary Fig. S2B) by IEDB, and RANKPEP tools. Positions P1, P4, P6, and P9 are known to be MHC contact residues for MHC class II, and positions P2, P5, and P8, and in some cases also P7, are TCR contact residues.[17] Loss of pathogenicity as a result of replacing 657R (P2), 660V (P5), 662L (P7), and 663E (P8) with alanine supports the finding that these are likely to be the TCR contact residues of this epitope. Tyrosine at position P1 of this epitope (656Y) is a previously known MHC contact residue for the I-A^b molecule.[18] Because the native sequence at P4, a known MHC class II contact residue, was already alanine (659A), no substitution was made at this position. In contrast, substitution of alanine at positions 661D (P6) and at position 664S (P9) did not affect induction of

EAU (Fig. 4B), suggesting that these amino acids do not affect the ability of hIRBP651-670 to bind to I-A^b and be presented.

EAU Can Be Induced by Adoptive Transfer of hIRBP651-670–Specific T Cells

Experimental autoimmune uveitis is a T cell–mediated disease.[5,19] An antigen-specific T cell line was therefore established from the infiltrating cells isolated from uveitic eyes, as described in Materials and Methods. Adoptive transfer of 10 million or more line cells by IP or IV injection consistently induced moderate to severe EAU in naïve C57BL/6J mice (Figs. 5A, 5B). Adoptive transfer of activated primary cells (20 million/mouse, IP) from hIRBP651-670–immunized mice stimulated in vitro for one round with the immunizing peptide induced mild disease (EAU score ≤ 0.5) with low incidence (Supplementary Fig. S3).

Detection of Epitope-Specific T Cells in Uveitic Eyes by Using hIRBP654-664–Loaded I-A^b Tetramers

We previously demonstrated in the B10.RIII mouse model of EAU that T cells specific to hIRBP161-180 accumulate in large numbers in uveitic eyes of B10.RIII mice with a polyclonal T-cell repertoire.[20] We therefore examined the eyes of uveitic C57BL/6 mice immunized with hIRBP651-670 for the presence of T cells able to bind to this peptide in the context of I-A^b MHC class II molecule. Fluorescently labeled MHC class II
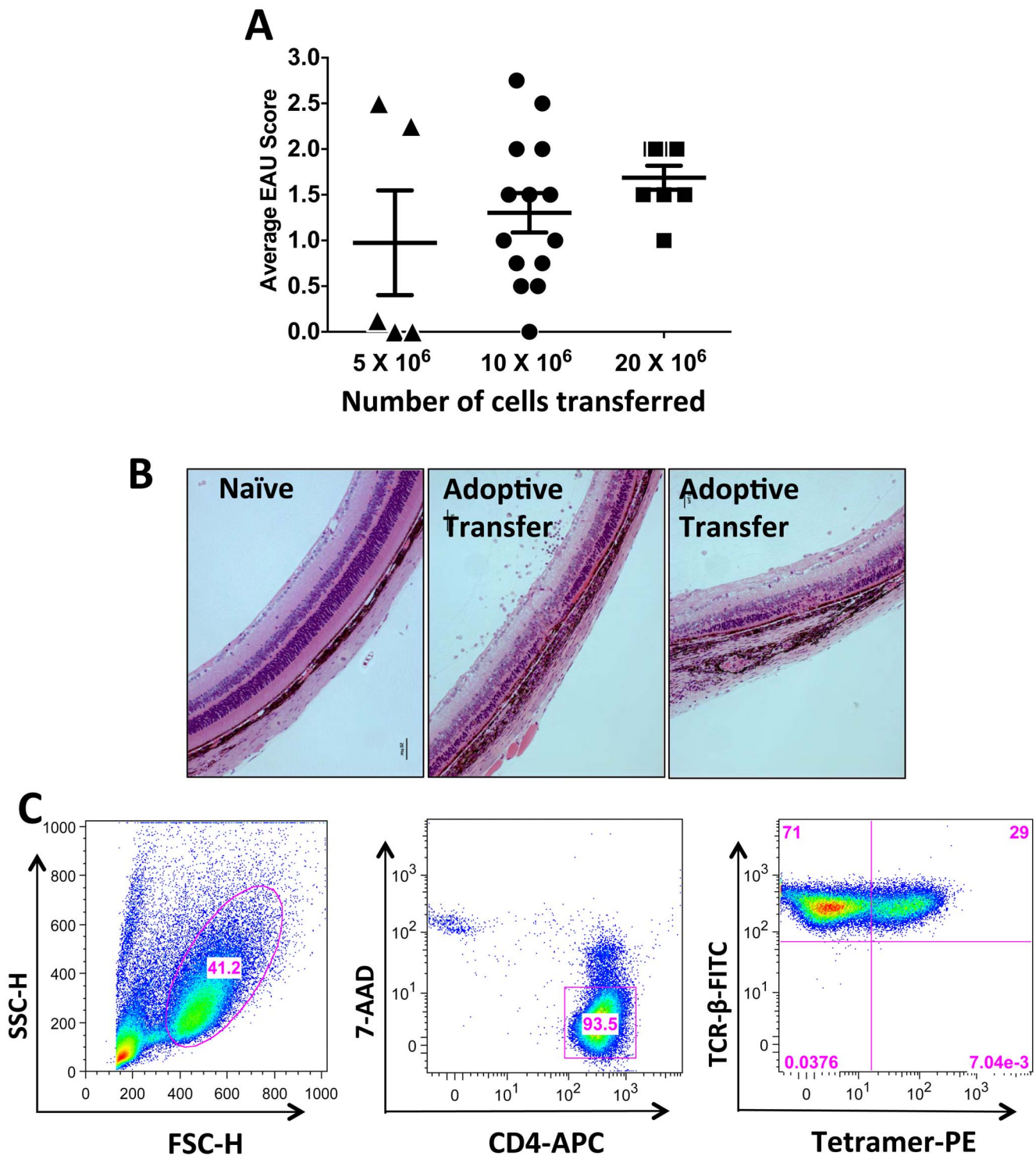
FIGURE 5. hIRBP651-670–specific T-cell line induces EAU in naïve mice. (A) Average (±SE) EAU scores of mice infused with the indicated numbers of cells from an hIRBP651-670–specific T-cell line after eight rounds of stimulation (eyes were collected on day 14 after transfer). (B) Histopathology of the eyes of mice that received 10×10^6 activated hIRBP651-670–specific T-cell line on day 14 after adoptive transfer. Note destruction of photoreceptor layer, infiltration of inflammatory cells into the vitreous cavity, hemorrhage into subretinal space, and choroiditis compared to the healthy retinas in naïve mice. (C) hIRBP651-670–specific T-cell line stained with the fluorescently tagged tetramer I-A^b-hIRBP654-664. Approximately 29% of the live (7AAD-negative) CD4$^+$ TCRα/β cells in the culture were tetramer-positive.

(I-A^b) tetramers loaded with the minimal hIRBP654-664 core epitope sequence (GAYRTAVDLES [I-A^b-hIRBP654-664 tetramer]) detected antigen-specific T-cell clones in the T-cell line (Fig. 5C) derived from uveitic eyes of mice immunized with hIRBP651-670. The same tetramer was used to detect epitope-specific T cells infiltrating eyes and draining lymphoid organs (Figs. 6A, 6B) of EAU mice immunized with either hIRBP651-670 or native IRBP protein. Notably, tetramer-positive cells
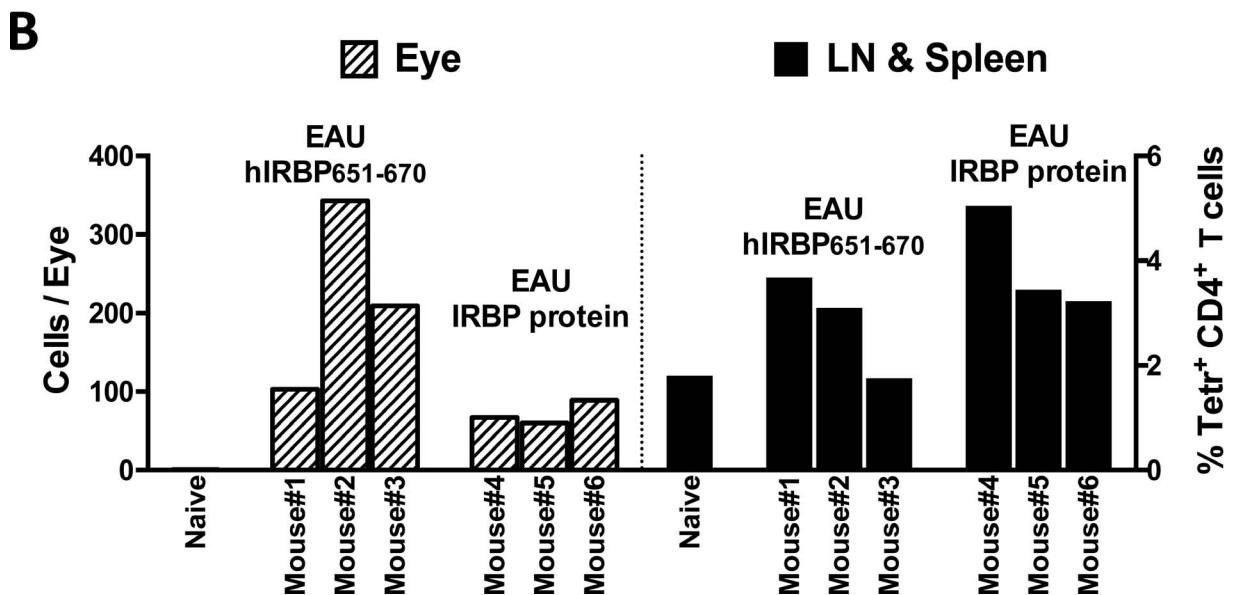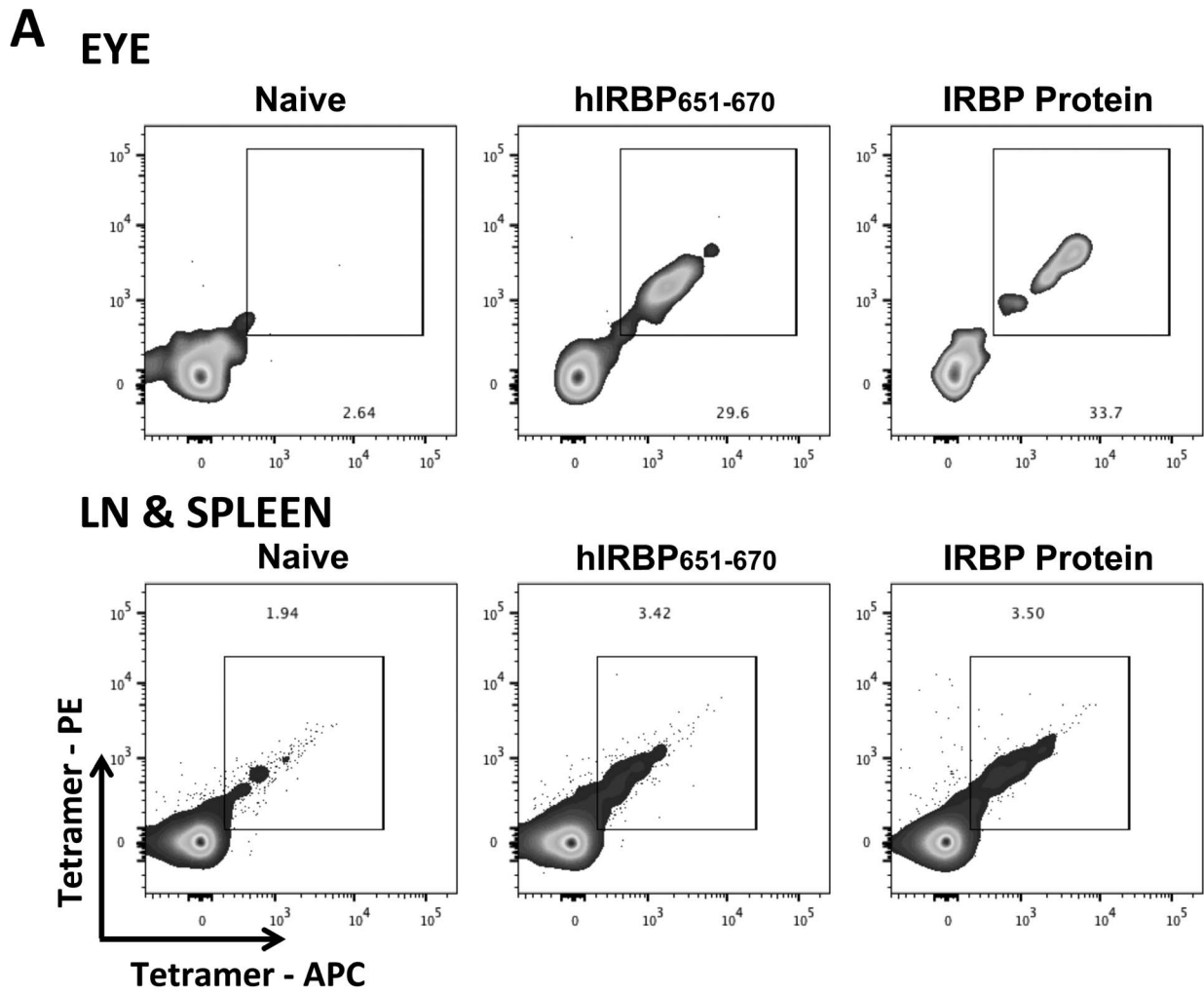
FIGURE 6. Identification of the I-A^b-hIRBP654-664 tetramer-positive cells in uveitic eyes of mice immunized with peptide hIRBP651-670 or the whole IRBP protein is shown. (**A**) Representative plot of CD4 T-cell population in the eyes (*top*) and peripheral lymphoid organs (*bottom*) of naive or immunized mice (day 21 after immunization). (**B**) Total number of tetramer-positive cells within the eyes (*left*) and percentage of tetramer-positive cells within the CD4 T-cell population in the peripheral lymphoid organs (*right*) of naive and immunized mice on day 21. Tetramer-positive cells could be detected in both the peptide-immunized and the native IRBP protein-immunized mice, showing that this sequence includes naturally processed epitope from IRBP protein and that epitope-specific T cells infiltrate the eyes of EAU mice.

could be detected not only in eyes of mice immunized with hIRBP651-670 but also in eyes of mice immunized with native bovine IRBP protein. Thus, I-A^b-hIRBP654-664 tetramers can be used to detect antigen-specific uveitogenic T cells in C57BL/6 mice.

DISCUSSION

In the present study, we characterized hIRBP651-670, a conserved fragment of IRBP that induces uveitis in H-2^b haplotype mice and demonstrated that it causes more consistent and more severe EAU than the previously reported peptide hIRBP1-20. This form of disease is accompanied by higher inflammatory cytokine levels in uveitic eyes and demonstrable presence of hIRBP651-670–specific T cells, detectable by I-A^b-hIRBP654-664 tetramers both in uveitic eyes and in lymphoid tissues. Interestingly, despite being more pathogenic, hIRBP651-670 elicited lower immunological responses peripherally (as measured by in vitro proliferative and cytokine responses of draining lymph node cells) than hIRBP1-20. This apparent discrepancy could be due to several reasons. Unlike hIRBP651-670, hIRBP1-20 is not fully conserved among species; however, the single amino acid substitution at position 17 is a conservative one (isoleucine versus valine) and is outside the minimal core epitope. Nevertheless, we cannot exclude the possibility that this affects processing and creates a heterologous epitope in hIRBP1-20 that is not present in mouse IRBP. Although it would elicit a response, such an epitope would not contribute to disease. Additionally, Shao et al.[21] reported multiple epitopes within the hIRBP1-20 sequence, whereas we previously showed that a higher CD8 T-cell response was seen in hIRBP1-20–immunized mice than in hIRBP651-670–immunized mice.[4,22] Because the contribution of CD8 T cells to pathology of EAU is in question (depletion of CD8 T cells does not reduce disease scores),[23] this again could contribute to immunological responses without contributing to disease scores.

Knowledge of the core epitope, as defined by truncated peptides, allowed us to design MHC tetramers which can be used to detect hIRBP651-670–specific cells and track them. It is notable that even in IRBP-immunized mice it was possible to detect hIRBP651-670–specific T cells by in vitro proliferation of lymphocytes from (bovine) IRBP-immunized mice and by in vivo detection of T cells with TCRs having this specificity with I-A^b-hIRBP654-664 tetramers in the uveitic eye and in lymphoid organs. Detection of this cross-reactivity with the native IRBP molecule was made possible by the conserved nature of this epitope, which, unlike the hIRBP1-20 sequence, is completely conserved across human, mouse and bovine species. Importantly, elicitation of responses to human IRBP651-670, bovine IRBP666-685, and mouse IRBP666-685 specificity after immunization with the native IRBP molecule, supports the notion that hIRBP651-670 is a naturally processed epitope from the whole IRBP molecule and is recognized as a product and target resulting from processing of the endogenous IRBP. The finding that hIRBP651-670 is in all likelihood a naturally processed epitope may also be connected to the reason why this sequence is more pathogenic than hIRBP1-20, which is incompletely conserved across species[3] and may not be as efficiently processed from endogenous murine IRBP.

Using truncated and alanine-substituted peptides, as well as publicly available bioinformatics tools, we then defined the residues that were critical for pathogenicity and therefore likely to be the contact residues that interact with MHC and TCR. A consensus sequence pattern for the I-A^b molecule had been defined previously as YYAPWCNNA,[24] but other amino acids have been seen to occasionally replace some of these

positions,[18] Accordingly, although proline is the amino acid most frequently found at P4 of self-peptides that bind to the I-A^b molecule, alanine (in our case 659A) and serine can also occupy this position.[18] Also, alanine was shown to bind efficiently to I-A^b at positions P6 and P9,[18] which potentially explains why substitution of alanine for 661D and 664S did not abrogate EAU induction. Defining the critical contact residues is important for designing altered peptide ligands, which can modulate the response of T cells specific to this epitope and protect from uveitis, as we demonstrated in a previous study.[25]

We anticipate that the identification and characterization of hIRBP651-670, which can elicit uveitis in C57BL/6 mice, with scores and incidence of a magnitude similar to IRBP, will allow investigators to take better advantage of the large selection of genetically manipulated mice available on the C57BL/6 background. This should prove extremely valuable for studies of basic mechanisms and for evaluation of new therapeutic approaches to uveitis.

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