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Uveitis-Associated Epitopes of Retinal Antigens Are Pathogenic in the Humanized Mouse Model of Uveitis and Identify Autoaggressive T Cells

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Noninfectious uveitis is a leading cause of blindness and thought to involve autoimmune T cell responses to retinal proteins (e.g., retinal arrestin [soluble-Ag (S-Ag)]). There are no known biomarkers for the disease. Susceptibility is associated with HLA, but little is known about susceptible class II alleles or the potentially pathogenic epitopes that they present. Using a humanized HLA-transgenic mouse model of S-Ag–induced autoimmune uveitis, we identified several susceptible and resistant alleles of HLA-DR and -DQ genes and defined pathogenic epitopes of S-Ag presented by the susceptible alleles. The sequences of these epitopes overlap with some previously identified peptides of S-Ag (“M” and “N”), known to elicit memory responses in lymphocytes of uveitis patients. HLA-DR–restricted, S-Ag–specific CD4+ T cells could be detected in blood and draining lymph nodes of uveitic mice with HLA class II tetramers and transferred the disease to healthy mice. Importantly, tetramer-positive cells were detected in peripheral blood of a uveitis patient. To our knowledge, these findings provide the first tangible evidence that an autoimmune response to retina is causally involved in pathogenesis of human uveitis, demonstrating the feasibility of identifying and isolating retinal Ag-specific T cells from uveitis patients and may facilitate their development as biomarkers for the disease. The Journal of Immunology, 2011, 187: 1977–1985.

Uveitis is a major cause of blindness and the primary manifestation in numerous ocular and systemic diseases. Strong HLA associations have been identified in various ethnic groups for a number of these diseases that include both HLA class I and class II alleles (1–17) (Table I).

PBMCs from uveitis patients respond to various ocular autoantigens in culture (18), with responses most often being directed against retinal arrestin (also known as soluble Ag [S-Ag]) and occasionally against interphotoreceptor retinoid binding protein (IRBP). A role for these responses in pathogenesis of uveitis is supported by a clinical study in which patients fed with S-Ag to induce oral tolerance had a positive clinical outcome (19). Responses to peptide fragments of human S-Ag have been studied using PBMCs from uveitis patients (20); however, presence of in vitro-proliferative or cytokine responses, although suggestive, is not sufficient to establish a causal relationship with disease. Furthermore, the patients had not been HLA-typed, so there is no information regarding restriction by particular HLA haplotypes.

Experimental autoimmune uveitis (EAU) is an animal model that mimics human uveitis (21). EAU can be induced in rodents by immunization of genetically susceptible strains with various retinal proteins (22). S-Ag as well as IRBP are pathogenic in Lewis rats, whereas IRBP is the stronger uveitogen in mice (23). Although the classical EAU model in mice and rats has helped to study the cellular and molecular mechanisms of uveitis, it cannot help to identify the antigenic epitopes that might be involved in human disease because rodent MHC molecules bind and present different antigenic epitopes as compared with humans. To address this, we developed a humanized HLA-Tg model of EAU in mice that are transgenic (Tg) for human class II molecules and deleted for mouse class II and showed that, in contrast to their parental wild-type strains, mice expressing HLA-DRB1*0301 were highly susceptible to uveitis caused by S-Ag (24).

In the current study, we have not only identified DRB1*0402 and DQB1*0302 (DQA1*0301/DQB1*0302) as additional susceptible HLA class II alleles, but also demonstrated that some HLA alleles that confer higher risk for other autoimmune diseases are protective in uveitis [e.g., HLA-DR2 alleles and the -DR4(0401) allele]. Importantly, we have defined the uveitogenic S-Ag epitopes recognized by each of these HLA alleles using bioinformatic predictions and biological testing. It is of note that the HLA-associated uveitogenic epitopes identified in our study overlap with the sequences of “peptide M” and “peptide N” of the bovine
S-Ag that were previously reported to elicit recall responses in lymphocytes from uveitis patients (25, 26). HLA-DR3 tetramers loaded with a pathogenic epitope of S-Ag could detect Ag-specific autoreactive T cells in draining lymph node (LN) cells of uveitic mice, and sorted tetramer-labeled cells could transfer EAU to unimmunized mice. Importantly, as proof of concept, the same tetramer could specifically detect Ag-reactive T cells in the PBMCs from an HLA-DR3+ uveitis patient. These findings provide tangible evidence that autoimmune responses to retina restricted by human MHC class II are pathogenic and provide new insights into the biology of uveitis. Our data also suggest that epitope-specific HLA class II tetramer reagents could be developed as a biomarker for diagnosis and follow-up of human uveitis and could potentially be useful in development of personalized immunotherapies adapted to particular MHC haplotypes.

Materials and Methods

Animals

HLA Tg mice expressing alleles DRB1*1501, -DRB1*1502, -DRB1*1503, -DRB1*0301, -DRB1*0401, -DRB1*0402, -DQA1*0103/DQB1*0601 (DQ6), and DQA1*0301/DQB1*0302 (DQ8) were developed at Mayo Clinic (Rochester, MN), as previously described (27–30). Tg lines were bred and maintained at the Mayo Clinic or at the National Institutes of Health under specific pathogen-free conditions. The care and use of the animals complied with guidelines of the Institutional Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology.

Patients

Research performed in this study was in compliance with guidelines of the National Institutes of Health Institutional Review Board, and all procedures conformed to the tenets of the Declaration of Helsinki. Patients with well-defined clinical diagnosis of noninfectious uveits enrolled into the National Eye Institute Institutional Review Board protocol number 03-EL-02-01 to 02 were selected on the basis of their HLA type. Informed consent was obtained from all patients for blood sampling. HLA typing was performed at the Department of Transfusion Medicine, National Institutes of Health, using standard molecular typing techniques.

Ags

Native S-Ag and IRBP were purified from bovine retinal tissue using published protocols (31, 32) with minor modifications (33). Twenty residue peptides overlapping by 10 residues and corresponding to the entire length of human S-Ag sequence (Supplemental Table I), as well as peptide “M” (DTNLASSTIIEKGIDKTV), peptide “N” (LLANNRERRGIALDGKIKHE), and truncated versions of peptide “N” (N281–N290; Table II) were developed as a biomarker for diagnosis and follow-up of human uveitis and could potentially be useful in development of personalized immunotherapies adapted to particular MHC haplotypes.

Induction of EAU and disease scoring

Mice were immunized s.c. with 150 μg of S-Ag emulsified 1:1 v/v with CFA (Sigma-Aldrich) containing 2.5 mg/ml Mycobacterium tuberculosis H37RA (Sigma-Aldrich) in a total volume of 200 μl, divided among both thighs and base of tail, and were given 0.4 mg/ml pertussis toxin (#P7208; Sigma-Aldrich) i.p. in 100 μl RPMI 1640 plus 1% mouse serum. The progression of disease was monitored by fundus examination, and eyes were collected for histopathological confirmation at the end of the experiment (days 30–32). Severity of EAU was scored on a scale of 0–4 to 5 million cells/well and expanded for 7 d with partial medium changes every other day. Additional rounds of stimulation were performed in the same way. At the end of the fourth cycle, cells were stained with peptide-loaded HLA-DR3 tetramer-PE, anti-TCRβ–FITC, and anti-CD4–allophycocyanin Abs as described below. Tetramer and TCR double-positive cells from the CD4+ population were sorted using a BD FACSAria (BD Biosciences, Mountain View, CA) into DMEM containing 20% FCS and antibiotics. The purity of sorted cells was at least 70% tetramer-positive cells out of the CD4+ lymphocyte population. Sorted cells were re-stimulated with 2 d with peptide N282 (NRRRGLDGIKHKE) and 5 million cells (after removing irradiated splenocytes and cell purifications) were i.p. into the recipient HLA-DR3 Tg mouse. Animals were evaluated by fundus examination. Eyes were collected for histopathology on day 15 after transfer.

Quantification of Ag-specific lymphocyte proliferation and cytokine release

Cells from spleen and LN draining the site of immunization (iliaic and inguinal) were collected from immunized animals 32–34 d after immunization and cultured with the immunizing Ags as described (23). Briefly, 5 × 10^6 cells/well were cultured in 200 μl RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 1% normal mouse serum, mercaptoethanol, antibiotics, glutamine, and nonessential amino acids with native S-Ag (20 μg/ml or as specified) or with synthetic peptides (25 μg/ml) for a total of 72 h, and pulsed with [³H]thymidine during the last 16–18 h of incubation. Isotope uptake was determined by liquid scintillation counting. Cytokine production was measured in 48-h culture supernatants (1 × 10^5 cells/well in a total of 200 μl HL-1 medium; Lonza, Walkersville, MD) by multiplex ELISA using the Search Light Technology (Aushon Biosystems, Billerica, MA).

Protein sequence alignment and epitope prediction

The S-Ag (retinal S-Arrestin) protein sequences from human (NP_000532), bovine (NP_851343), and murine (NP_033144) species were aligned by global multiple sequence alignment method using “MultiAlin” (35) and by ClustalW2 (36). T cell epitopes of the human S-Ag sequence for the HLA-DRB1*0301 (DR17), -DRB1*0402, and -DQ8 alleles were predicted using the online epitope prediction tools PROPRED (37) and RANKPEP (38).

Expansion and adoptive transfer of T cells

HLA Tg mice were immunized with S-Ag in CFA as described above for EAU. After 10 d, a booster dose of 150 μg S-Ag emulsion was given in IFA (Sigma-Aldrich; 100 μl on the nape and 50 μl on either forelimb). Ten to 12 d later, peripheral blood and draining LN were collected. Single-cell suspensions of lymphocytes were made from draining LN and peripheral blood, the latter after lysing RBCs using ACK lysis buffer (Lonza). LN cells were purified using the CD4⁺ T Cell Isolation kit on an autoMACS Pro (Miltenyi Biotec). Cells (4 to 5 million/ml) were stimulated for 2 d in 24-well Linbro plates (Hampton Research, Aliso Viejo, CA) with 20 μg/ml full-length peptide N (LLANNRERRGIALDGKIKHE) or peptide N282 (NRRRGLDGIKHKE) in DMEM (Lonza) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml IL-2 (Protektin; Novartis Pharmaceuticals, East Hanover, NJ), 10% FCS (Lonza), 10% rat spleen-conditioned medium (supernatant from Con-A–stimulated rat splenocytes), 10 ng/ml IL-12 (PeproTech, Rocky Hill, NJ), and 10 μg/ml anti–IL-4 (clone 11B.11; Biological Research Branch, National Cancer Institute, Frederick, MD). Five million irradiated (3000 rad) naïve syngeneic splenocytes were added as APCs. Cultures were incubated at 37°C and 10% CO₂. Following 2-d antigenic stimulation, cells were split using medium with 10% spleen-conditioned medium without peptide to maintain a concentration of ~4 to 5 million cells/well and expanded for 7 d with partial medium changes every other day. Additional rounds of stimulation were performed in the same way. At the end of the fourth cycle, cells were stained with peptide-loaded HLA-DR3 tetramer-PE, anti-TCRβ–FITC, and anti-CD4–allophycocyanin Abs as described below. Tetrarmer and TCR double-positive cells from the CD4⁺ population were sorted using a BD FACSAria (BD Biosciences, Mountain View, CA) into DMEM containing 20% FCS and antibiotics. The purity of sorted cells was at least 70% tetramer-positive cells out of the CD4⁺ lymphocyte population. Sorted cells were re-stimulated with 2 d with peptide N282 (NRRRGLDGIKHKE) and 5 million cells (after removing irradiated splenocytes and cell purifications) were i.p. into recipient HLA-DR3 Tg mice. Animals were evaluated by fundus examination. Eyes were collected for histopathology on day 15 after transfer.

DRB1*0301 tetramer staining of HLA-DR3 Tg mouse cells

The generation of soluble HLA-DRB1*0301/DRB1*0301 molecules and the procedure for loading the peptides have been described (39). Peptide N282 of S-Ag that was uveitogenic in the HLA-DR3(0301) strain (Table II) was used to generate HLA-DR3 tetramers (Beneroya Research Institute at Virginia Mason, Seattle, WA). Cells were stimulated with tetramers (0.5 μg/0.5 × 10⁶ cells in 50 μl HL-1 medium [Lonza]), in the presence of 1 μg Fc block (BD Biosciences) for 1 h at 37°C, followed by surface staining for 20 min on ice with anti-TCRβ–FITC and anti-CD4–allophycocyanin Abs (BD Biosciences). To exclude dead cells, 5 μl 7-aminoactinomycin D (7-AAD; BD Biosciences) was added to each sample 10 min before analysis. Seventy-five-thousand events in the live lymphocyte gate were collected using FACS Diva flow cytometry software (BD Biosciences) and further analyzed for tetramer⁺, TCRβ⁺ CD4⁺ T cells using FlowJo 8.8.6 software (Tree Star).

Detection of tetramer-positive T cells in patient PBMCs

PBMCs were isolated from peripheral blood of DR3⁺ uveitis patients using Histopaque–1077 (Sigma-Aldrich), washed, and resuspended in RPMI 1640 medium (Lonza) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated human AB serum (Sigma-Aldrich). Cells were cultured in a 24-well Linbro plate (Hampton Research) at 4 million cells/ml for 7 d. PBMCs were then washed, resuspended in 200 μl CFA (Sigma-Aldrich), and injected i.p. into HLA-DR3 Tg mice. Mice were killed and eyes were collected for histopathology on day 15 after transfer.

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CO₂ for 7 d. Cells from confluent wells were split using fresh culture medium. After 7 d, cells were collected, washed, and restimulated with the peptide and costimulants as above. After day 10, culture medium was refreshed with medium supplemented with 10 IU/ml recombinant human IL-2 (Proleukin; Novartis Pharmaceuticals) every other day. The cells were stained with peptide N282-loaded DR3 tetramers as described above after 17 d in culture. Staining for T cell markers was done with anti-CD3 (FITC) and anti-CD4 (allophycocyanin) (BD Biosciences).

Results
Susceptibility of HLA class II Tg mice to uveitis
HLA class II Tg mice deleted for murine MHC class II molecules and expressing different HLA-DR or -DQ alleles were immunized with the uveitogenic retinal proteins IRBP and S-Ag. Among the eight HLA-Tg strains tested for susceptibility, most developed EAU after immunization with IRBP at varying degrees of severity (Fig. 1A). However, susceptibility to S-Ag–induced uveitis was restricted to HLA-DR3(0301), HLA-DR4(0402), and HLA-DQ8 strains, with the HLA-DQ8 strain being the least susceptible of the three (Fig. 1B). Interestingly, a minor difference in the amino acid sequence (three residues) between the two allelic forms of HLA-DR4 was associated with a major difference in their susceptibility to S-Ag–induced EAU. In contrast to HLA-DR4(0402), HLA-DR4(0401) Tg mice were resistant to EAU induced with S-Ag, although both were equally susceptible to EAU induced with IRBP (Fig. 1). None of the three HLA-DR2 (DRB1*1501, *1502, and *1503) alleles conferred susceptibility to either IRBP or S-Ag. Because uveitis patients tend to have immunological responses to S-Ag rather than to IRBP (20), further studies concentrated on S-Ag–induced responses.

S-Ag–specific proliferation and cytokine profile
Draining LN cells of HLA-DR3, HLA-DR4(0402), and HLA-DQ8 Tg mice immunized with a uveitogenic regimen of S-Ag proliferated in vitro in response to S-Ag in a dose-dependent manner (Fig. 2A). Notably, Ag-specific proliferative responses were considerably higher in the susceptible strains, namely HLA-DR3 and HLA-DR4(0402), than in resistant strains such as HLA-DR4(0401) and HLA-DQ6(0601).

Th1 and Th17 cytokines have been reported to play a pathogenic role in various EAU models (40, 41), and both types of responses have been reported in human uveitis (42–44). We therefore evaluated the S-Ag–specific cytokine production by LN cells from susceptible and resistant strains of HLA Tg mice with S-Ag–induced uveitis. As with proliferation, susceptible strains produced higher levels of both Th1- and Th17-type cytokines as compared with the resistant strains (Fig. 2B). LN cells from HLA-DR4(0402) Tg mice also produced higher amounts of IL-27 and as well as the inflammatory chemokines MCP-1 and RANTES.

Epitopes of human S-Ag restricted by HLA-DR3, -DR4, and -DQ8 alleles
To identify S-Ag epitopes presented by susceptible HLA class II alleles, we used epitope prediction tools (PROPRED and RANK-PEP) to identify the regions in the human S-Ag protein molecule that could potentially bind to HLA-DR3(DRB1*0301), -DR4 (DRB1*0402), and -DQ8(DQA1*0301 × DQB1*0301) alleles with higher affinity. The results from these predictions are shown in Supplemental Table II. It is notable that multiple registers from a given region of the S-Ag protein were predicted for all three alleles.

The immunogenicity of various regions of S-Ag in the context of uveitis-susceptible HLA class II alleles was examined using a standard peptide-mapping strategy based on immunizing mice with the native S-Ag protein and recalling responses in culture with overlapping 20 residue peptides representing the entire sequence of human S-Ag (Supplemental Table I). Because of the unavailability of native human S-Ag, bovine S-Ag was used for immunization. We recognize that despite the very high interspecies homology, we may inevitably have missed some epitopes that are not shared between the bovine and human proteins. However, because the target for uveitis in the HLA Tg mice is the murine protein, uveitogenic epitopes detected in this model are likely to represent sequences that are conserved among species.

The response profile of the susceptible HLA class II alleles, -DR3(0301), -DR4(0402), and -DQ8, to different regions of human S-Ag protein sequence are shown in Fig. 3. Dominant epitopes recognized by each of these alleles were distinct. The two dominant epitopes of HLA-DR3 spanned overlapping residues 281–300 and 291–310, whereas dominant epitopes of HLA-DR4(0402) were two distinct nonoverlapping regions between residues 311–330 and 331–350. Notably, these regions corresponded to the predicted epitopes for -DR3(0301), -DR4(0404), and -DQ8 alleles shown in Supplemental Table II.

Although DR4(0401) differs from the DR4(0402) only by three residues, mice carrying this allele were resistant to S-Ag–induced EAU. We therefore examined epitope recognition in these two strains of HLA Tg mice. Results of a peptide scan performed as above showed that resistant DR4(0401) mice recognized a distinct immunodominant peptide than did the susceptible DR4(0402) mice, spanning residues 301–320 (Supplemental Fig. 1).

Uveitogenicity of the immunodominant epitopes
To determine the uveitogenic potential of the immunodominant peptides identified for each of the HLA class II alleles, we immunized each of the susceptible strains of HLA Tg mice with their corresponding immunodominant peptides to induce EAU. Because several of these immunodominant peptide sequences overlap with the previously defined peptide M and peptide N that elicit responses...
in humans as well as in other species (45), the original peptide M and peptide N sequences (based on bovine S-Ag) were also used. Histological scores of the severity of EAU lesions induced with the various peptides are shown in Fig. 4.

Although in HLA-DR3 Tg mice peptide #29 (residues 281–300) and #30 (residues 291–310) were highly immunogenic (Fig. 3), these peptides elicited at best only marginal EAU at the doses used in this study (Fig. 4). Notably, the 20 aa peptide N (residues 287–306, LLANNRERRGIALDGKIKHE), which is fully conserved among murine, bovine, and human S-Ag (Supplemental Fig. 2), induced very severe uveitis in HLA-DR3 Tg mice. Shorter peptides were synthesized to identify the minimal core region of peptide N for the HLA-DR3(0301) allele (Table II). The results showed that there could be multiple HLA-DR3(0301) registers between aa positions 287–306 that could induce uveitis in the context of this allele (Table II), which may underlie the high pathogenicity of this peptide.

In HLA-DR4(0402) Tg mice, two adjacent but nonoverlapping peptides, #32 (aa 311–330) and #34 (aa 331–350), induced severe disease (Fig. 4). The amino acid sequences of one of these peptides, #32 (ASSTIIKEGIDRTVLGILVS), overlapped with the sequence of “peptide M”, which spans residues aa 303–320 of the bovine S-Ag (DTNLASSTIIKEGIDKTV), elicits responses in PBMCs of some uveitis patients (26) and is pathogenic in Lewis rats (45). However, peptide M did not induce uveitis in HLA-DR4(0402) Tg mice (Fig. 4). The human “peptide M” equivalent (aa 307–325, DTNLASSTIIKEGIDRTVL) also did not elicit disease in this strain (data not shown).

Specific binding of S-Ag peptide-loaded HLA-DR3 tetramer to primary T lymphocytes of DR3 Tg mice with uveitis

MHC class II tetramers loaded with specific epitopes have been used as biomarkers to detect and to study Ag-specific autoreactive CD4+ T cells present in peripheral blood and in tissues of patients.

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** S-Ag–specific T cell responses of immunized HLA class II Tg mouse. EAU-susceptible and -resistant HLA class II Tg strains were immunized with a uveitogenic regimen of S-Ag. Proliferative and cytokine responses of cells from LN draining the site of immunization were examined. A. Draining LN cells collected 30 d after immunization were cultured with serial dilutions of S-Ag. Average background [³H]uptake by the cells in the absence of Ag was ~4000 cpm and is subtracted (representative data from one of two experiments). B. Cytokine production was measured by multiplex ELISA in cell-culture supernatants collected 48 h after in vitro antigenic stimulation. Ag-specific cytokine production is plotted after subtraction of the background levels in the absence of Ag (representative data from one of three experiments).

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** Immunodominant epitope profiles of human S-Ag protein for the susceptible HLA class II alleles. Spleen cells from S-Ag–immunized mice were used in an in vitro lymphocyte proliferation assay in the presence of 25 μM each of 20 aa long overlapping synthetic 20-mer peptides spanning the human S-Ag protein sequence, peptide M, peptide N, and whole S-Ag protein (20 μg/ml). [³H]thymidine uptake by cells was plotted after subtracting the background counts in cells cultured in the absence of Ag (average background counts ≤5000 cpm). Data shown are the average of five individual mice for each strain.
Identification of the uveitogenic epitopes of S-Ag specific to different HLA class II alleles could have direct application to the study of Ag-specific T cells in uveitis.

To determine whether S-Ag–specific CD4 T cells could be detected using MHC class II tetramers in blood and lymphoid tissues of mice primed to native S-Ag (to mimic the clinical situation), we used HLA-DR3 tetramers loaded with peptide N282 (NRERRGIALDGKIKHE, Table II). To increase the frequency of epitope-specific T cells for detection, the cells were first expanded in culture with peptide N or its shorter version N282 for one or more stimulation cycles. Several-fold higher frequencies of S-Ag–specific CD4+ T cells were detected in the PBMCs and draining LN cells already after a single stimulation cycle (Fig. 5A), and the proportion of tetramer-positive cells continued to increase with further stimulations, demonstrating specificity of the response (Fig. 5B, 5C).

**S-Ag–specific HLA-DR3 tetramer+ T cells are uveitogenic in HLA-DR3 Tg mice**

To examine whether the tetramer-positive T cells that bound the N282-loaded HLA-DR3 tetramers represent uveitogenic effector T cells, we infused 5 million sorted CD4+ TCRβ+ HLA-DR3 T cells into HLA-DR3 Tg mice.
tetramer+ T cells after four cycles of expansion into naive HLA-DR3 Tg recipient mice. Severe inflammation with cellular infiltrate was detected in the eyes of recipient mice by fundus examination as early as 5 d after cell transfer. Severity of inflammation and retinal damage induced by tetramer-positive cells was considerably higher than EAU induced by active immunization of HLA-DR3 Tg mice with whole S-Ag protein or peptide N, or its truncated version N282 (Fig. 6A). Histopathological analysis of eyes collected 15 d after adoptive transfer showed cellular infiltrate in the posterior segments of the eyes and a partial or complete destruction of the photoreceptor cells (Fig. 6B).

**HLA class II tetramers detect S-Ag–responsive cells in uveitis**

As proof of concept that Ag-reactive cells could be detected in the circulation of S-Ag–responsive uveitis patients, HLA-DR3 tetramer staining was next tested using patient samples. HLA-DR3 tetramers loaded with peptide N282 could successfully detect a population of CD4+ T cells specific to this peptide in an HLA-DR3–positive patient (Fig. 7) after selective expansion of Ag-specific cells in culture. Close to 6% of the live CD4+ cells were CD3 and tetramer double-positive cells in this patient. PBMCs from an HLA-DRB1*0401–positive patient not expressing the HLA-DR3 allele did not stain for this specific epitope under similar culture and staining conditions.

**FIGURE 6.** T cells positive for HLA-DR3 tetramer loaded with S-Ag epitope are pathogenic in HLA-DR3 Tg mice. HLA-DR3 tetramer loaded with the peptide N282 was used to sort CD4+ TCRβ+tetramer+ cells from cultures expanded in vitro with the same peptide. Sorted cells were stimulated with the same peptide and expanded in vitro to obtain sufficient numbers of tetramer-positive T cells for adoptive transfer into recipient HLA-DR3 Tg mice. After expansion for 7 d, cells were restimulated with the peptide 48 h before adoptively transferring 5 million live cells/mouse i.p. Eyes from the recipient mice were collected for histopathological evaluation 15 d after adoptive transfer of cells. Average disease scores of both eyes in four individual mice that received tetramer-sorted cells (A) and histopathological lesions in the eyes of one representative mouse (B) in comparison with the severity of disease in mice actively immunized with peptide N or peptide N282 or normal eye are shown. Original magnification ×200.

**FIGURE 7.** HLA class II tetramers detect S-Ag–responsive cells in PBMCs of uveitis patient. PBMCs from HLA-DR3 or HLA-DR4 uveitis patients were stimulated with peptide N282 for 7 d as described in Materials and Methods. Cells were restimulated on day 7 and expanded in IL-2–containing medium. On day 17 of culture, cells were stained with HLA-DR3 tetramer (PE) loaded with peptide N282 (NRERRGIALDGKIKHE) or with unloaded HLA-DR3 tetramer (PE) followed by anti-CD4 (allophycocyanin) and anti-CD3 (FITC). Dead cells were excluded from analysis (7-AAD). Percentages of tetramer+ CD3+ cells are shown after gating on CD4+7-AAD+ cells.

**Discussion**

In the current study, we analyze the immunodominant and pathogenic epitopes of human arrestin (S-Ag), an autoantigen recognized by lymphocytes of uveitis patients, which is believed to have a role in driving progression of uveitis. Using available HLA Tg mouse strains, we identify permissive and nonpermissive HLA class II alleles and characterize the immunodominant and uveitogenic epitopes that they recognize. Importantly, we find that the pathogenic regions that elicit EAU in HLA class II Tg mice overlap or are identical with previously identified sequences known as peptide M and peptide N, which elicit lymphocyte responses in experimental animals and in human uveitis patients.

Although in human uveitis the initial triggers are unknown, some types of uveitis show a clear class II association. Furthermore, effector responses in the EAU model and proliferative responses to S-Ag in patients are primarily CD4 restricted, as expected for responses to a soluble protein. We therefore used class II Tg mice for the present studies. The choice of strains was dictated by published MHC class II associations and the availability of HLA class II Tg strains. It is interesting that minor differences in the amino acid sequence between two related allelic forms of HLA-DR4 (29) drastically changed the susceptibility to S-Ag–induced EAU. This is reminiscent of observations in type 1 diabetes, for which DR0401 and DR0405 confer increased risk, whereas DR0403 is protective (49). The immunodominant epitopes of S-Ag presented by DR4(0401) and DR4(0402) alleles are adjacent, but do not overlap with each other. Unlike in the arthritis model (29), HLA-DR4(0401) is a nonpermissive allele for S-Ag–induced uveitis. However, the strain itself is not inherently incapable of developing uveitis, as the disease can be induced with IRBP and is even more severe than in DR4(0402) mice (Fig. 1A). In contrast, the HLA-DR3(0301) allele is permissive to both IRBP- and S-Ag–induced disease (Fig. 1). Association of various forms of uveitis and HLA genes in different populations is not well explored, and it is possible that the importance of HLA-DR3 may have been...
Both peptides were examined for their ability to induce disease known to elicit immune responses from PBMCs of uveitis patients by HLA-DR4(0402). Overlapped with peptide M, these are both immunodominant sequences of the human S-Ag presented in humanized uveitis models similar to those reported in this paper. However, it cannot be predicted whether the immunogenic epitopes are also pathogenic (Supplemental Table II). Current bioinformatic tools can accurately pinpoint the immunogenic epitopes of S-Ag in the context of particular HLA class II alleles. Various algorithms can accurately pinpoint the immunogenic epitopes of S-Ag in the context of particular HLA class II alleles. Because there is clustering of multiple autoimmune diseases in families, we speculate that HLA-DR2 could be an important gene for uveitis susceptibility as well. HLA-DR2 has been reported to be associated with pars planitis (17, 55, 56) or uveitis associated with MS (Table I). Even though all three alleles of HLA-DR2 examined in this study were resistant to IRBP as well as to S-Ag–induced uveitis (Fig. 1), it is possible that other allelic forms of HLA-DR2 could be susceptible. As well, S-Ag– and IRBP-resistant DR2 alleles could be permissible to uveitis induced with other retinal proteins, such as RPE-65, rhodopsin, recoverin, phosphducin, etc. The finding that both DR4 and DQ8 alleles in the Tg mice are permissible to S-Ag–induced EAU may perhaps help to explain the relatively higher risk reported for individuals with the HLA-DR3 in the respective strains. The peptide N sequence, which is completely conserved among the mouse, human, and bovine species (Supplemental Fig. 2A), induced severe uveitis in HLA-DR3 Tg mice (Fig. 4). However, neither the original peptide M (based on the bovine S-Ag sequence; Fig. 4) nor its human equivalent (data not shown) elicited disease in the HLA-DR4(0402) Tg mice. It should be noted that due to the nature of the experimental system and its logistics, three kinds of S-Ag are players in this study. Because we are interested in human peptides binding to human HLA molecules, the overlapping synthetic 20-aa peptides were derived from the human S-Ag sequence. Autoimmunization resulting in uveitis occurs to the whole protein; therefore, we immunized mice with native S-Ag, which had to be bovine, as native human S-Ag cannot be obtained in sufficient quantities. Finally, the target Ag in the uveitic eye is the mouse protein. This experimental design will necessarily tend to select for evolutionarily conserved epitopes that remain immunologically cross-reactive among these species (i.e., ~85% of the sequence) (Supplemental Fig. 2B). Thus, it is possible that some epitopes that are not conserved among species may have been missed.

In conventional mice, EAU development appears not to be restricted to a particular type of effector response. We recently reported that EAU in C57BL/6 and B10.RIII mice can develop in the context of either a Th1 (IFN-γ) or Th17 (IL-17) effector response (58, 59), and others have demonstrated that in immunodeficient hosts a Th2 response can also be permissive to EAU development (60). In uveitis patients, increased levels of both IFN-γ and IL-17 have been reported (42–44). These observations are directly supported by the present data showing that HLA-DR3 and HLA-DR4(0402) Tg mice develop S-Ag–induced EAU with equal severity and that both Th1 and Th17 cytokines are part of the immunological response profiles (Fig. 2B).

Table I. HLA Gene Associations in Autoimmune Uveitic Syndromes

<table>
<thead>
<tr>
<th>Disease</th>
<th>Ethnic Group</th>
<th>HLA Genes (Relative Risk)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birdshot retinocchoroidopathy</td>
<td>White</td>
<td>A29 (49.9–224.35)</td>
<td>(3–5)</td>
</tr>
<tr>
<td>Behcet’s disease</td>
<td>White, Asian</td>
<td>B51 (4.0–18.2)</td>
<td>(6–8)</td>
</tr>
<tr>
<td>Anterior uveitis in ankylosing spondylitis</td>
<td>White</td>
<td>B27 (3.8–20.0)</td>
<td>(9–11)</td>
</tr>
<tr>
<td>Vogt–Koyanagi–Harada disease</td>
<td>Asian</td>
<td>DR8 (34.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DR4 (46.7–74.5)</td>
<td>(12, 13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DQA3 (12.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DQ4 (18.9–41.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP5 (3.8)</td>
<td></td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>Various</td>
<td>DR4 (5.6–13.7)</td>
<td>(14, 15)</td>
</tr>
<tr>
<td>Intermediate uveitis (unrelated to MS)</td>
<td>White</td>
<td>DR3 (Not reported)</td>
<td>(16)</td>
</tr>
<tr>
<td>Pars planitis in MS</td>
<td>White</td>
<td>DR2 (3.2)</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DR51 (4.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DR17 (14.3)</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Amino Acid Sequences of Truncated Versions of Peptide N and the Average Score of EAU Induced by These Peptides in HLA-DR3 Tg Mice

<table>
<thead>
<tr>
<th>Peptide Identification</th>
<th>Amino Acid Sequence</th>
<th>Average EAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-hSA 287–306</td>
<td>L L A N R R G I A L D G K I K H E</td>
<td>1.14 ± 1.06</td>
</tr>
<tr>
<td>N281-hSA 289–306</td>
<td>A N R R R G I A L D G K I K H E</td>
<td>0.48 ± 0.27</td>
</tr>
<tr>
<td>N282-hSA 291–306</td>
<td>N R R R G I A L D G K I K H E</td>
<td>1.38 ± 1.64</td>
</tr>
<tr>
<td>N283-hSA 293–306</td>
<td>E R R G I A L D G K I K H E</td>
<td>0.83 ± 1.16</td>
</tr>
<tr>
<td>N284-hSA 295–306</td>
<td>R G I A L D G K I K H E</td>
<td>0.62 ± 0.88</td>
</tr>
<tr>
<td>N285-hSA 297–306</td>
<td>I A L D G K I K H E</td>
<td>0.09 ± 0.19</td>
</tr>
<tr>
<td>N286-hSA 291–300</td>
<td>N R R R G I A L D G</td>
<td>ND</td>
</tr>
<tr>
<td>N287-hSA 290–301</td>
<td>N R R R G I A L D G</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>N288-hSA 289–302</td>
<td>A N R R R G I A L D G K</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>N289-hSA 288–303</td>
<td>L A N R R R G I A L D G K I</td>
<td>0.02 ± 0.07</td>
</tr>
<tr>
<td>N290-hSA 287–304</td>
<td>L L A N R R R G I A L D G K I K</td>
<td>0.89 ± 1.03</td>
</tr>
</tbody>
</table>
Multiple genes contribute to the susceptibility to human uveitis, but by far the strongest associations have been reported with HLA (2). Previous studies documented responses in a proportion of the patients to S-Ag and some of its peptides, but detection of the responding cells and their quantitation as a biomarker of disease has not been possible. To our knowledge, the current study demonstrates for the first time the specificity of uvetogenic epitopes in the context of susceptible HLA allele using an MHC class II tetramer reagent (Fig. 5). The observed frequency of positive T cells in the peripheral blood and primary LN cells of uveitic mice is comparable to what has been reported earlier in similar systems (61, 62). Our results show that it is possible to select, identify, and characterize rare populations of HLA allele-specific uvetogenic CD4* T cells for diagnosis and study. Adoptive transfer of uveitis in HLA Tg mice can be also achieved with T cells restricted by IRBP, to which these mice are also susceptible as a uveitis-inducing Ag by active immunization. In this study, we specifically concentrated on peptide N282 tetramer-positive T cells because we wished to show relevance of this T cell population to human disease, in which responses to S-Ag are more frequently encountered than to IRBP.

Importantly, our data provide proof of concept that detection of such cells is possible in human uveitis. Out of nine DR3 patients tested, all already on immunosuppressive therapy, one showed presence of autoreactive T cells specific to S-Ag epitope detectable with HLA-DR3 tetramer loaded with peptide N282. Absence of tetramer-positive cells in the HLA-DR4 uveitis patient, whose cells also proliferated to S-Ag, reveals the specificity of this reagent. The frequency of tetramer-positive cells is comparable to that of patients with arthritis and type 1 diabetes to their respective disease-associated Ags (47, 48). This tetramer-positive patient was in an active inflammatory stage of the disease at the time when blood was obtained for testing. Notably, after 20 d of immunosuppressive therapy, we could no longer detect tetramer-positive cells in this patient. The other eight patients were already under prolonged immunosuppressive therapy at the time of blood collection, and their proliferative responses not only to S-Ag but also to tetanus toxoid, purified protein derivative of tuberculin, and PHA were several-fold lower than those of healthy donors (data not shown). Thus, presence of tetramer-positive cells appears associated with disease activity. Although clearly much additional work needs to be done with patients during different clinical stages of uveitis and expressing different HLA allele/epitope combinations, our data provide a proof of principle that this technology has the potential to be developed into the first disease biomarker for clinical diagnosis and follow-up as well as for isolation and study of uveitis-relevant T cells in the S-Ag-responsive patient population.

In conclusion, we have identified alleles of HLA class II genes that confer susceptibility or resistance to uveitis-associated Ags in humanized mice and have identified pathogenic epitopes that they bind and present in the context of human MHC class II molecules. We further provide proof of concept that these findings may lead to development of the first disease biomarker(s) for uveitis. Knowledge of the risk associated with HLA alleles and of the pathogenic epitopes that they present promises to contribute toward the improved understanding, diagnosis, and treatment of uveitis.

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Disclosures

The authors have no financial conflicts of interest.

References
