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Suppression of Complement Regulatory Proteins (CRPs) Exacerbates Experimental Autoimmune Anterior Uveitis (EAAU)

Purushottam Jha,2* Jeong-Hyeon Sohn,2† Qin Xu,* Yali Wang,† Henry J. Kaplan,† Puran S. Bora,* and Nalini S. Bora*†

This study was undertaken to explore the role of complement regulatory proteins (CRPs) in experimental autoimmune anterior uveitis (EAAU). We observed that the levels of CRPs, Crry and CD59, in the eyes of Lewis rats increased during EAAU and remained elevated when the disease resolved. The in vivo role of these CRPs in EAAU was explored using neutralizing mAbs, antisense oligodeoxynucleotides (AS-ODNs), and small interfering RNAs against rat Crry and CD59. Suppression of Crry in vivo at days 9, 14, or 19 by neutralizing mAb or AS-ODNs resulted in the early onset of disease, the exacerbation of intraocular inflammation, and delayed resolution. Suppression of CD59 was only effective when the Abs and ODNs were given before the onset of disease. The most profound effect on the disease was observed when a mixture of Crry and CD59 mAbs or AS-ODNs was administered. A similar effect was observed with a combination of Crry and CD59 small interfering RNA. There was no permanent histologic damage to ocular tissue after the inflammation cleared in these animals. Increased complement activation as determined by increased deposition of C3, C5 activation fragments, and membrane attack complex was observed in the eyes of Lewis rats when the function and/or expression of Crry and CD59 was suppressed. Thus, our results suggest that various ocular tissues up-regulate the expression of Crry and CD59 to avoid self-injury during autoimmune uveitis and that these CRPs play an active role in the resolution of EAAU by down-regulating complement activation in vivo. The Journal of Immunology, 2006, 176: 7221–7231.

The importance of complement as a component of the innate immune system is well established, and complement activation has been reported to be crucial to the pathogenesis of various diseases (1). Several proteins tightly regulate the activation of complement cascade on the self-tissues and cells, thus preventing damage to host tissue during an inflammatory reaction (2).

Decay-accelerating activity and cofactor activity are two important mechanisms that down-regulate the complement cascade at the critical step of C3 activation (2–3). In humans these mechanisms are achieved by two membrane-bound regulatory proteins, namely decay acceleration factor (DAF,4 CD55) (3) and membrane cofactor protein (MCP; CD46) (4, 5). Failure of a cell to express DAF and MCP may result in increased deposition of complement and damage to host tissue during an inflammatory reaction (2).

DAF and MCP have been identified in rodents as well as in humans (7, 8). Crry (512 Ag) is a widely distributed complement regulatory protein in rodents and has both decay-accelerating and cofactor activities. It controls complement activation at the critical step of C3 convertase formation (9, 10). Two forms of Crry mRNA have been reported to be present in all rat tissues (10). These mRNAs are translated into Crry proteins of 55 and 65 kDa that are composed of six and seven consensus repeats, respectively (10). Rat CD59 (~19 kDa) has been isolated (11), and tissue distribution studies have shown that it is also a widely distributed protein (12).

Results from our laboratory suggest that the complement system is continuously active at a low level in the normal rat eye (13). RT-PCR analysis has demonstrated the presence of the alternative forms of Crry mRNA in the normal rat eye (13). Using immunohistochemistry, we have demonstrated that both Crry and CD59 proteins are expressed on various normal rat ocular tissues, including the iris and the ciliary body (13). We have also identified the soluble form of Crry and CD59 proteins in the normal rat aqueous humor (13). Thus, our observations provide evidence that a regulatory system exists in the eye to protect ocular cells from destruction by complement activating events during intraocular inflammation.

Idiopathic anterior uveitis (AU) is the most common form of intraocular inflammation in humans. The recurrent nature of the disease can lead to permanent visual loss from the secondary complications of cystoid macular edema, posterior subcapsular cataract, and glaucoma (14, 15). Experimental autoimmune anterior uveitis (EAAU) is an organ-specific inflammatory disease of the eye, which is an animal model of idiopathic human AU (16, 17). EAAU is characterized histologically by a lymphocytic infiltration in the iris and ciliary body. Ag-specific CD4+ T cells can adaptively transfer disease into naive syngeneic recipients and are the predominant inflammatory cells within the uvea (16, 17). EAAU is self-limiting, lasting ~2–3 wk. After the inflammation clears, the structures of the eye remain intact (16, 17). Recent results from our
CRPs in EAAU.

Materials and Methods

Animals

Pathogen-free male Lewis rats (5–6 wk old) were obtained from Harlan Sprague Dawley. This study was approved by the Institutional Animal Care and Use Committee, University of Louisville, Louisville, KY.

Antibodies

Anti-rat Crry/p65 (clone 512, mouse IgG1) and anti-rat MHC Ag RT1A (clone OX-18, mouse IgG1) were purchased from BD Biosciences, whereas anti-rat CD59 (clone T8H, mouse IgG1) was from Research Diagnostics. Rabbit- or mouse-antibody to CD59 (MCA-1737, mouse IgG1) was obtained from Serotec. Monoclonal β-actin (mouse IgG1), MOPC-21 (mouse IgG1), and Cy3-conjugated sheep anti-mouse IgM were purchased from Sigma-Aldrich. Mouse anti-GAPDH mAb (IgG1) was from Chemicon International. This Ab reacts with rat GAPDH. The IgG fraction of goat antiserum to rat C3 was from MP Biochemicals. This Ab recognizes C3 split products (C3b and iC3b) as well as intact C3 (13, 18). A polyclonal Ab against rabbit IgG (1/100) was used as the secondary Ab. Control stains were performed with non-immune rabbit IgG (Santa Cruz Biotechnology) were used as the secondary Abs for C3 and MAC. Rabbit anti-goat IgG (Sigma-Aldrich) and FITC-labeled goat anti-rabbit IgG (1/200) were used to detect mouse IgG. Samples were detected using the criteria previously reported (16).

Induction and Evaluation of EAAU

Melanin-associated Ag was purified from bovine iris and ciliary body as previously described by us (16, 17). Male Lewis rats were immunized with 100 μl of stable emulsion containing 75 μg of soluble melanin-associated Ag (MAA) emulsified (1:1) in CFA (Difco Laboratories) using a single-dose induction protocol in the hind footpad as previously described by us (16, 17). Purified pertussis toxin (1 μg/animal) was used as an additional adjuvant. Animals were examined daily between days 7 and 60 postinjection for the clinical signs of uveitis using slit lamp biomicroscopy. EAAU was graded in a mask fashion using the criteria previously reported (16). Eyes were also harvested at various time points for histological analysis to assess the course and severity of inflammation. EAAU was scored by an observer unaware of the experimental design. The intensity of uveitis was histologically scored on an arbitrary scale of 0–5 as follows: 0; normal; 1; dilated iris vessels plus thickened iris stroma exudates in the anterior chamber with protein or a few scattered inflammatory cells or both; 2; moderate infiltration of inflammatory cells in the stroma of the iris or ciliary body or both and a moderate number of inflammatory cells within the anterior chamber; 3; heavy infiltration of inflammatory cells within the iris stroma and the ciliary body and heavy infiltration of inflammatory cells within the anterior chamber; 4; heavy exudation of cells with dense protein aggregation in the anterior chamber and inflammatory cell deposits on the corneal endothelium; 5; presence of hemorrhage with extremely heavy infiltration of inflammatory cells within the iris, ciliary body, and the anterior chamber as well as dense inflammatory cell deposits on the corneal endothelium and lens epithelium.

Immunohistochemistry

Five micrometer thick paraffin-embedded tissue sections were immunostained for C3 and MAC using polyclonal Abs (1/100). Cy3-conjugated rabbit anti-goat IgG (Sigma-Aldrich) and FITC-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) were used as the secondary Abs for C3 and MAC staining, respectively. Sections were also stained for C3 and CD59 using mAbs (1/50). Cy3-conjugated sheep anti-mouse IgG (1/200) was used as the secondary Ab. Control stains were performed with non-relevant Abs of the same Ig subclass at concentrations similar to those of the primary Abs. Additional controls consisted of staining by omission of the primary or secondary Ab. Sections were examined under fluorescence microscope (Zeiss). This experiment was repeated three times with similar results.

Sample collection

Anesthetized animals were perfused through the heart with 200 ml of sterile pyrogen-free saline. Eyes were then immediately enucleated. Intraocular tissue from each eye was prepared using a previously described method (13). The intraocular tissues, which consisted of uvea, retina, lens, aqueous humor, and vitreous, were used in total RNA and protein extraction for RT-PCR and Western blotting, respectively.

RT-PCR analysis

Total RNA (0.1 μg) from pooled intraocular content (described above) isolated from Lewis rats sacrificed at different time points (three rats per time point) during EAAU was used to detect the mRNA levels of GAPDH, β-actin, Crry, and CD59 by semiquantitative RT-PCR using the reagents purchased from Applied Biosystems. Total RNA was prepared using the SV (spin or vacuum) total RNA isolation kit (Promega). This kit was used according to the manufacturer’s specifications. The sense (S) and antisense (AS) oligonucleotide primers for rat primers were synthesized at Integrated DNA Technologies. The primer sequences as well as the predicted sizes of amplified cDNA are as follows: GAPDH, 5′-TGAAGCTCGTTGTCAACAGGGATTGGC-3′ (forward) and 5′-CATGTAGGCCCATAGGTTCACTCATTTGCTCGAAGTGTC-3′ (reverse) (983 bp); β-actin, 5′-GTGGTGAGACCTTCACAAACC-3′ (forward) and 5′-TGCGGCTACCTTCGTCTCAGGTC-3′ (reverse) (318 bp); Crry, 5′-TGTCTTCTGGAGTGAAGACGTC-3′ (forward) and 5′-CTGAGGCGTGAACACAGCTGTC-3′ (reverse) (413 and 599 bp); and CD59, 5′-CTGCTTCTGGCTGCTCTTGCT-3′ (forward) and 5′-AGCGTGTCTCTCCAATAGGC-3′ (reverse) (302 bp).

Four different cycles, 25, 30, 35 and 43, were used for PCR, and all four cycles gave similar results. All reactions were normalized for GAPDH or β-actin expression. The negative controls consisted of the omission of the RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 2% agarose gel and quantitated by densitometry using Quantity One 4.2.0 (Bio-Rad). These experiments were repeated three times with similar results.

Western blot analysis

Intraocular content prepared as described above was pooled separately for each time point (six eyes per time point) and homogenized in 500 μl of ice-cold PBS containing protease inhibitors and 1% Nonidet P-40. The homogenate was centrifuged at 14,000 × g at 4°C for 15 min, and the supernatant was subjected to SDS-PAGE. After SDS-PAGE on a 12% linear slab gel, separated proteins were transferred to a polyvinylidene difluoride membrane using a Trans-Blot semidry electrophoretic transfer cell (Bio-Rad). After electroblotting, the gels were stained with Coomassie Blue to check the efficiency of transfer. Blots were stained at room temperature with 1/500 dilution of mouse anti-rat CD59 (clone MCA 1737) or a 1/300 dilution of mouse anti-rat Crry (clone 512), monoclonal β-actin, or anti-GAPDH Ab for 1 h at room temperature or overnight at 4°C. Control blots were treated with the same dilution of IgG isotype control. After washing and incubation with a HRP-conjugated secondary Ab, blots were developed using the ECL Western blotting detection system ECL Plus (Amersham Biosciences). Quantification of CD59, Crry, and β-actin was accomplished by analyzing the intensity of the bands using Quantity One 4.2.0 (Bio-Rad). These experiments were repeated three times with similar results.

In vivo Ab treatment

Neutralizing Abs against rat Crry (clone 512) and CD59 (clone TH9) were used for in vivo injections. MAA-immunized animals (n = 5) received a single i.v. injection of blocking Abs against rat Crry (250 μg) or CD59 (250 μg) or a combination of these two Abs on day 9, 14, 19, or 27 postimmunization. Controls were injected with same amount of isotype control or OX-18 (anti-rat MHC Ag RT1A). Clinical and histopathological examination (described above) was used to determine the onset and severity of EAAU. These experiments were repeated three times with similar results.

Oligodeoxynucleotide (ODN) synthesis and in vivo administration

The nucleotide sequences for full length rat Crry and CD59 were obtained from the GenBank data base. ODNs in the S and AS orientations were designed as previously described (21). Phosphorothioate-modified ODNs (21 nt) were used because of their increased resistance to nucleases and were synthesized at Integrated DNA Technologies. AS-ODNs for Crry (mixture of AS1 and AS2), CD59 (mixture of AS3 and AS4), or a combination of the two (mixture of AS1, AS2, AS3, and AS4) were dissolved in sterile saline and injected (3 mg/rat) once i.v. in the tail vein on day 9, 14, 19, or 27 after MAA sensitization (five rats for each time point). Controls received a similar treatment with S-ODNs. Clinical and histopathological examinations were used to determine the onset and severity
of the disease. To confirm the suppression of Crry and CD59 in vivo following AS-ODN treatment, naive Lewis rats were sacrificed 24 h after treatment, and eye, spleen and liver were analyzed by RT-PCR and Western blot analysis (described above). The sequences of rat Crry and CD59 ODNs used in this study are as follows: Crry-AS1, 5'-H11032-CAG AGG CGA AGA AGC CTC CAT-3' /H11032; Crry-AS2, 5'-H11032-TAC AAG GCG CCC CAC GGG GTC-3'/H11032; Crry-S, 5'-H11032-ATG GAG GCT TCT TCG CCT CTG-3'/H11032; CD59-AS3, 5'-H11032-CTA GAG GTT CTT CTT GCC TGC-3'/H11032; CD59-AS4, 5'-H11032-TCG CAG TCA GCT AGA TCT GTG-3'/H11032; and CD59-S, 5'-H11032-GCA GGC AAG AAC CTC TAG-3'/H11032. These experiments were repeated three times with similar results.

siRNA synthesis and administration

RNA interference was used to silence Crry and CD59 genes in vivo (22). For each gene, three target sequences were identified at different locations on the mRNA (GenBank accession numbers are NM_012925 for CD59 and L_36532 for Crry), and siRNAs were designed corresponding to those sequences using Invitrogen Life Technologies software (Block-iT RNAi Designer). siRNAs (duplexes of S and AS strands) were synthesized at Invitrogen Life Technologies and were 25-nt-long, double-stranded RNA. The S and AS strands of siRNAs were as follows: CD59 (sequence 1, beginning at nt 153), 5'-H11032-GUUAGCCUCAGAUGCUACAACUGUU-3'/H11032 (S) and 5'-H11032-AACAGUUGAUAGCAUCUGAGGCUAAC-3'/H11032 (AS); CD59 (sequence 2, beginning at nt 210), 5'-H11032-ACUUGCUCUCCUAACCUGGAUGCUU-3'/H11032 (S) and 5'-H11032-AAGCAUCCAGGUUAGGAGAGCAAGU-3'/H11032 (AS); CD59 (sequence 3; beginning at nt 218), 5'-H11032-UCCUAACCUGGAUGCUUGUCUUGUU-3'/H11032 (S) and 5'-H11032-AACAAGACAAGCAUCCAGGUUGAGGA-3'/H11032 (AS); Crry (sequence 1, beginning at nt 371), 5'-H11032-CCGGUUUGGAUCCUCUAUCUUAU-3'/H11032 (S) and 5'-H11032-TGUAAAUGUGAAUAGGAGAC-3'/H11032 (AS); Crry (sequence 2, beginning at nt 918), 5'-H11032-GGUGAGCUCUCCUAUUGGCAUGUAU-3'/H11032 (S) and 5'-H11032-AUCAUGGAUUGUGAUGUGUGCUAC-3'/H11032 (AS); Crry (sequence 3, beginning at nt 1046), 5'-H11032-GGAAAGCAUUUGGAGAUCAGCGU-3'/H11032 (S) and 5'-H11032-TGUUUUGUAAAGGAGUGAGAC-3'/H11032 (AS).
FIGURE 2. Effect of neutralizing Crry and CD59 mAbs on EAAU. MAA-injected rats received a single i.v. injection of anti-Crry mAb (A), anti-CD59 mAb (B), anti-RT1A (C), or PBS (D) at day 9 and were sacrificed at day 15 after MAA immunization. Extremely severe EAAU was observed in anti-Crry- (A) and anti-CD59-injected (B) Lewis rats at day 15. The anterior chamber (AC), the iris (I), and the ciliary body (CB) were infiltrated by inflammatory cells. No inflammation was observed at this time point in MAA-injected Lewis rats that received anti-RT1A (C) or PBS (D). MAA-injected Lewis rats received a single i.v. injection of anti-Crry and CD59 mixture at day 9 and were sacrificed at day 70 postimmunization with MAA (E). No histologic damage to ocular tissues was observed in these animals after the inflammation cleared. The data shown are representative of three different experiments. Objective magnification, ×20.

Table I. Effect of Abs against CRPs on EAAU

<table>
<thead>
<tr>
<th>MAA (μg)</th>
<th>Antibody</th>
<th>Day of Antibody Injection (i.v.)</th>
<th>Eyes with EAAUa</th>
<th>Duration of Disease (Days)</th>
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<tbody>
<tr>
<td>100</td>
<td>Crry</td>
<td>30/30 30</td>
<td>14.5 ± 1.5</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>0</td>
<td>CD59</td>
<td>0/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Crry + CD59</td>
<td>0/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Crry</td>
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<td>12.5 ± 1*</td>
<td>23 ± 4*</td>
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<td>27 30/30 30</td>
<td>13 ± 4</td>
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<tr>
<td>100</td>
<td>CD59</td>
<td>9 30/30 30</td>
<td>12.5 ± 1*</td>
<td>23 ± 3*</td>
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<td>Crry + CD59</td>
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<td></td>
<td></td>
<td>19 30/30 30</td>
<td>41 ± 4*</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>27 30/30 30</td>
<td>14 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

a Incidence of EAAU given as positive/total eyes following clinical examination. Severity of inflammation on histopathologic examination was grouped as severe (4+) or extremely severe (5+).

* <p >0.05.
Cry and CD59 suppression was done by RT-PCR (described above). These experiments were repeated two times with similar results.

Toxicity studies of intravenous Ab, ODN, and siRNA therapy

Various organs such as liver, heart, kidney, lung, and spleen, harvested at 24 h and on day 6 post i.v. injection of the Abs, ODNs, and siRNA, were analyzed histologically to assess the toxicity and adverse effect of systemic i.v. administration.

Statistical analysis

The data are expressed as the mean ± SD. Data were analyzed and compared using Student’s t test, and differences were considered statistically significant with p < 0.05.

Results

Expression of CD59 and Cry during EAAU

mRNA levels. To study the expression of CD59 and Cry mRNA, three independent studies were performed. MAA-immunized animals were sacrificed at four different time points: before the onset of inflammation (day 10), at the onset (day 14), at the peak (day 19), and after resolution (day 30) of EAAU. The eyes (six for each time point) were harvested, and semiquantitative RT-PCR was performed to study the expression of CD59 and Cry mRNA, respectively. Treatment with AS-ODNs resulted in ~95% inhibition of Cry and CD59 transcripts in the eye, spleen, and liver (B). The levels of GAPDH (B) protein were not affected in these tissues. The data shown are representative of three different experiments.

Table II. Effect of AS-ODNs against CRPs on EAAU

<table>
<thead>
<tr>
<th>MAA (µg)</th>
<th>ODN</th>
<th>Day of ODN injection (i.v.)</th>
<th>Eyes with EAAU</th>
<th>Duration of Disease (Days)</th>
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<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>AS-Cry</td>
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<td>14.5 ± 1.5</td>
</tr>
<tr>
<td>0</td>
<td>AS-CD59</td>
<td>0/30</td>
<td>0</td>
<td></td>
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<tr>
<td>0</td>
<td>AS-Cry + CD59</td>
<td>0/30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>AS-Cry</td>
<td>9 30/30</td>
<td>30</td>
<td>11.5 ± 1*</td>
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<td></td>
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<td>14 30/30</td>
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<td>9 30/30</td>
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<td>27 30/30</td>
<td>30</td>
<td>15 ± 2</td>
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</table>

* Incidence of EAAU given as positive/total eyes following clinical examination. Severity of inflammation on histopathologic examination was grouped as severe (4+) or extremely severe (5+).

*, p < 0.05.
Protein levels. The expression of CD59 and Crry proteins within the eye after MAA injection was studied by semiquantitative Western blot analysis. Protein bands corresponding to CD59 (19 kDa) and Crry (55 and 65 kDa) were identified in normal eyes and in the eyes of MAA-injected animals sacrificed at different time points (Fig. 1, E and F). CD59 protein levels increased dramatically on day 14, peaked on day 19, and remained at this level until day 30 (Fig. 1, E and G). Similar results were obtained for Crry protein (Fig. 1, F and H).

Effect of Crry and CD59 Inhibition on EAAU

We next blocked the function and/or expression of Crry and CD59 in vivo with blocking Abs, AS deoxynucleotide, and siRNA and examined the effects on EAAU.

Blocking mAbs. MAA-sensitized animals received a single i.v. injection of blocking mAbs against rat Crry (250 μg) or CD59 (250 μg) or combination of these two Abs on day 9, 14, 19, or 27 postimmunization. Blocking Crry function at day 9 caused an early onset of uveitis with increased severity and duration as compared with rats injected with MAA only (Table I and Fig. 2A). A similar increase in severity and duration was observed with the injection of anti-Crry at the onset (day 14) and at the peak (day 19) of EAAU but not during the resolution (day 27) of the disease (Table I). Blocking CD59 before the onset of the disease (day 9) resulted in a similar exacerbation of disease as anti-Crry (Table I and Fig. 2B). In contrast, anti-CD59 injection at the onset (day 14), the peak (day 19), and during resolution (day 27) did not have any significant effect on EAAU (Table I). Interestingly, simultaneous suppression of both Crry and CD59 by a mixture of monoclonal blocking Abs (512 and TH9) administered on day 9, 14, or 19 resulted in early onset, exacerbation of uveitis, and delayed resolution of the disease, with uveitis lasting for ~40 days (Table I). There was no obvious histologic damage to ocular tissue after the resolution of disease (animals sacrificed at day 70 after MAA injection) in the animals injected with anti-Crry, anti-CD59 (data not shown), or a combination of these mAbs (Fig. 2E). Injection of isotype-matched control Ab (data not shown), anti-rat MHC Ag RT1A (Fig. 2C), or PBS (Fig. 2D) at the above-mentioned time points had no effect on the onset, duration, or the severity of EAAU. Additionally, i.v. injection of these Abs (alone or in combination) in naive Lewis rats did not cause intraocular inflammation (Table I).

Samples of liver, heart, kidney, lung, and spleen were obtained from MAA-sensitized animals injected i.v. with the IgG fraction of 512 (anti-Crry) and/or TH9 (anti-CD59) for histologic analysis. These organs were well preserved, and no signs of damage or inflammation were noted (data not shown).

AS-deoxynucleotides. We assessed the suppression of mRNA and protein in the eye, spleen, and liver by i.v. administration of Crry (3 mg/rat) and CD59 (3 mg/rat) synthetic AS-ODNs in naive Lewis rats. Using RT-PCR and Western blot analysis, our results demonstrated that at 24 h after treatment Crry and CD59 transcripts and proteins in the eye, spleen, and liver were selectively inhibited (~95%) by the respective AS-ODNs, but S-ODNs had no such effect (Fig. 3, A and B). AS-ODNs did not affect the expression of GAPDH, thus confirming the specificity of ODNs used in this study (Fig. 3, A and B). Intravenous injection of AS-ODNs (alone or in combination) did not cause intraocular inflammation in naive Lewis rats (Table II).

Next, the effect of Crry and CD59 AS-ODNs on the development and evolution of EAAU was investigated. MAA-sensitized
Lewis rats were injected (3 mg/rat; i.v.) once only with AS-ODNs targeting rat Crry, rat CD59, or a combination of both on day 9, 14, 19, or 27 postimmunization. Treatment with AS-ODN targeting Crry transcripts on days 9 (Fig. 4B), 14, and 19, but not on day 27, had a significant impact on the onset, severity, and duration of uveitis (Table II) and was as effective as mAbs to Crry. CD59 AS-ODN on day 9 resulted in the early onset and exacerbation of EAAU (Fig. 4D) but had no effect when given on days 14, 19, and 27 postimmunization (Table II). Most impressively, a single i.v. injection of a mixture of Crry and CD59 AS-ODNs on day 9, 14, or 19 delayed the resolution of EAAU to almost 40 days (Table II). Histopathological analysis revealed that ocular tissues were well preserved after the inflammation resolved in the animals injected with AS-Crry, AS-CD59 (data not shown), or a mixture of Crry and CD59 AS-ODNs (Fig. 4E). Intravenous treatment with S-ODNs at all four time points did not alter the course of EAAU (Fig. 4A and C). Liver, heart, kidney, lung, and spleen harvested from these animals showed no toxic effect of AS-ODN i.v. injection (data not shown).

**Table III. Effect of Crry and CD59 siRNA on EAAU**

<table>
<thead>
<tr>
<th>MAA (μg)</th>
<th>siRNA</th>
<th>Day of siRNA injection (i.v.)</th>
<th>Incidence</th>
<th>Severe</th>
<th>Extremely Severe</th>
<th>Day of Onset</th>
<th>Duration of Disease (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>Control-siRNA</td>
<td>9 and 10</td>
<td>4/4</td>
<td>4</td>
<td></td>
<td>14 ± 1</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>100</td>
<td>siRNA-Crry + CD59</td>
<td>9 and 10</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>Control-siRNA</td>
<td>9 and 10</td>
<td>0/4</td>
<td>0/4</td>
<td></td>
<td>10</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>0</td>
<td>siRNA-Crry + CD59</td>
<td>9 and 10</td>
<td>10/10</td>
<td>10</td>
<td>10</td>
<td>11.5 ± 0.5*</td>
<td>26 ± 1*</td>
</tr>
</tbody>
</table>

*Incidence of EAAU given as positive/total eyes following clinical examination. Severity of inflammation on histopathologic examination was grouped as severe (4+) or extremely severe (5+).

*, p < 0.05.
siRNA. We examined whether i.v. injection of siRNA can inhibit expression of Cry and CD59. Naive Lewis rats ($n = 4$) were given two injections (24 h apart) of a mixture of Cry and CD59 siRNA via the tail vein. Animals were sacrificed 4 h after the second injection, and their eyes, spleen, and liver were harvested for RT-PCR. Our results, presented in Fig. 5A, demonstrated that injection of Cry and CD59 siRNA mix resulted in $\sim 95\%$ inhibition of Cry and CD59 transcripts within the eye. However, siRNA treatment resulted in $\sim 80\%$ inhibition of Cry and CD59 transcripts in spleen and liver (Fig. 5A). Similar treatment of naive Lewis rats with scrambled siRNA control did not affect the levels of Cry and CD59 mRNA in the eye, spleen, and liver (Fig. 5A). siRNA targeted to Cry and CD59 did not effect the expression of $\beta$-actin (Fig. 5A). These results demonstrated the specificity of message suppression by Cry and CD59 siRNA. Intravenous injection of Cry and CD59 siRNA mix or control siRNA alone did not cause intraocular inflammation in naive Lewis rats (Table III).

We then investigated the in vivo silencing effect of siRNA targeting genes for Cry and CD59 on EAAU as well as on their protein levels in MAA-sensitized animals. Animals ($n = 10$) received two injections (24 h apart) of Cry and CD59 siRNA mix (200 $\mu$g each) starting at day 9 after MAA sensitization. Immunofluorescence analysis of the eye and the liver harvested from these animals revealed that siRNA specific to Cry and CD59 genes markedly reduced the expression of Cry and CD59 proteins in the eye (Fig. 5B, b and f) and in the liver (Fig. 5B, d and h). In contrast, both Cry and CD59 proteins were very strongly expressed in the eye (Fig. 5B, a and e) and the liver (Fig. 5B, c and g) of control scrambled siRNA-treated animals. Sections stained without primary Ab did not show any staining (data not shown). This siRNA treatment resulted in an early onset, increased severity, and prolonged course of uveitis (Table III and Fig. 6). Histopathology did not show any abnormality, and there were no signs of damage to the ocular tissues after the resolution of EAAU in these animals (data not shown). Control siRNA had no effect on the course of disease in EAAU. Various organs such as the liver, lung, heart, kidney, and spleen were examined, and no toxic side effects due to i.v. administration of siRNA was observed (data not shown).

Deposition of C3, C3 activation products and MAC in mAb-, ODN-, and siRNA-treated animals

Eyes of MAA-immunized animals injected i.v. with anti-Cry mAb, anti-CD59 mAb, AS-Cry, AS-CD59, or a mixture of Cry and CD59 siRNA were stained for C3, C3 activation products, and MAC. Deposition of C9 was used as a marker of MAC deposition (19, 20). Immunofluorescence analysis revealed weak staining for C3 and its activation products (Fig. 7, B and C) and MAC (Fig. 7, F and G) in the eyes of Lewis rats that received PBS or control siRNA. In contrast, C3, C3 split product, and C9/MAC staining was very intense in the animals injected with a mixture of Cry and CD59 siRNA (Fig. 7, D and H). In these animals, deposition of C3, C3 activation products, and MAC was abundant on the iris and the ciliary body and in the anterior chamber (Fig. 7, D and H). Similar results were observed with Cry and CD59 mAbs as well as Cry and CD59 AS-ODNs (data not shown). These results showed a correlation between deposition of C3, C3 activation products, and MAC and the suppression of complement regulatory proteins (CRPs) during autoimmune uveitis. Thus, the inhibition of Cry and CD59 using neutralizing Abs, AS-ODN, and siRNA exacer-
bated EAAU and increased the deposition of C3, its activation products, and MAC in the eye, suggesting an important protective role in vivo for these CRPs in EAAU.

Discussion

AU is the most common form of uveitis and accounts for $\sim 75\%$ of the cases (14, 15). The inflammation in AU occurs within the anterior segment (the iris and/or ciliary body) of the eye. AU may be

![FIGURE 6. Histopathologic changes in the eye of MAA-immunized Lewis rats injected i.v. with PBS (A), control siRNA (B), or a mixture of CD59 and Cry siRNA (C) at days 9 and 10 and sacrificed at day 15 after MAA injection. At this time point, severe EAAU developed in the animals injected with a mixture of siRNA directed against CD59 and Cry (C). The iris (I), ciliary body (CB), and anterior chamber (AC) were infiltrated by inflammatory cells (C). MAA-immunized rats injected i.v. with PBS (A), and control siRNA (B) did not develop EAAU at this time point. Objective magnification, ×10.](image-url)
associated with systemic diseases or results from a variety of infections; however, the most common form of AU is of unknown (i.e., idiopathic) etiology (14, 15). EAAU is an organ-specific autoimmune disease of the eye that serves as an animal model of idiopathic human AU (16, 17). Recent results from our laboratory demonstrated that the inflammatory response in EAAU involved the activation of the complement, which is responsible for the sequence of events leading to intraocular up-regulation of cytokines, chemokines, and adhesion molecules and to the recruitment of monocytes to the eye (18). Thus, during EAAU the eye is under threat from homologous complement attack, and unregulated complement activation can be detrimental to ocular structures that are vital for vision. The present study was undertaken to explore the role of CRPs, namely, Crry and CD59, in the protection of ocular tissue from complement-mediated destruction and down-regulation of intraocular inflammation during EAAU. To the best of our knowledge, this study is the first to demonstrate that Crry and CD59 play a protective role in vivo during autoimmune uveitis.

The expression of Crry and CD59 in the eyes of Lewis rats with EAAU was investigated using semiquantitative RT-PCR and Western blot analysis. We observed that both Crry and CD59 are expressed constitutively in the rat eye and that the expression of both proteins is up-regulated during EAAU. CD59 mRNA and protein remained elevated even after the resolution of intraocular inflammation. A similar trend was observed for Crry (both mRNA and protein). This up-regulation of CD59 and Crry may be due in part to invading inflammatory cells; however, the fact mRNA and protein levels remained elevated when the disease resolved would argue against such a possibility.

Several studies have reported that CRPs are expressed on a wide variety of circulating cells and normal tissues in humans and rodents (7–12). The expression of these proteins has been reported to increase in a number of inflammatory conditions, including glomerulonephritis (23, 24), rheumatoid arthritis (25, 26), respiratory tract inflammation (27), and ulcerative colitis (28). Our results suggest that various ocular tissues up-regulate Crry and CD59 during
EAAU, probably to avoid self-injury from complement-mediated tissue damage and may play a role in down-regulating intracocular inflammation. We hypothesized that blocking Crry and CD59 should result in an exacerbation of EAAU. In the present study, we tested this hypothesis and examined whether Ab-, AS-, and siRNA-mediated blocking of CRPs will have such an effect.

We first blocked the function of Crry and CD59 with specific neutralizing mAbs during EAAU. We used purified IgG1 fractions of these mAbs and not the F(ab)2 fragments. Anti-Crry was more effective than anti-CD59 when given alone; however, combination treatment with both mAbs was most effective in worsening the inflammation in EAAU. In contrast, i.v. injection of MOPC-21 or an Ab that is not directed toward complement regulatory protein (i.e., anti-rat RT1A) had no effect on EAAU. Thus, we do not think that the exacerbation of EAAU by anti-Crry and anti-CD59 is due to complement activation triggered by the Fc portion of IgG1 or by immune complexes formed within the eye but rather by CRP inhibition.

We then inhibited the expression of Crry and CD59 genes with AS-ODNs and siRNA. Synthetic AS-ODNs are widely used to inhibit specific gene expression in vivo, and many studies have successfully validated the efficacy of AS technology in animal models (29–33). siRNA has also been used in vivo to inhibit various genes in several pathophysiological diseases (34–40).

Administration of AS-ODN designed to specifically inhibit Crry resulted in significant worsening of EAAU. Similar to the effect noted with neutralizing mAbs, CD59 AS-ODN only worsened EAAU when given before the onset of inflammation. Indeed, simuaneous suppression of both regulators by a mixture of Crry and CD59 AS-ODN was most effective. These results were confirmed using siRNA. Intravenous injections of a siRNA mixture targeting Crry and CD59 significantly worsened EAAU. These results of AS-ODN and siRNA further support our observation that the exacerbation of EAAU by anti-Crry and anti-CD59 mAbs was not due to complement activation by the Fc portion of these Abs. Furthermore, there was no obvious permanent histologic damage to ocular tissue after the inflammation resolved in all mAb-, AS-ODN-, or siRNA-injected MAA-sensitized rats.

Complement activation is essential for the generation of C3 split products and MAC (1, 2). C3 split products and MAC have previously been reported to be spontaneously and continuously deposited on self-tissue in small amounts under normal conditions and in larger quantities under various pathological conditions (13, 41–43). We observed that the eyes of MAA-injected Lewis rats in which the function and/or expression of CRPs was suppressed by neutralizing mAbs, AS-ODN, or siRNA stained very strongly for C3, C3 activation products, and MAC compared with control. These results demonstrated that the suppression of CRPs leads to increased intracocular complement activation as evidenced by the increased deposition of C3 activation products and MAC, as reported in various other inflammatory diseases (41–43).

Previous studies have reported a protective role of Crry and CD59 in normal animals (13, 44–47) as well as in animal models of human disease such as glomerulonephritis, ischemia-reperfusion injury, collagen-induced arthritis, and autoimmune hemolytic anemia (19, 48–56). Mice deficient in DAF have been reported to be more sensitive to glomerulonephritis (57) and dextran sulfate sodium-induced colitis (58). Miwa et al. (59) demonstrated that deletion of DAF exacerbates the development of autoimmune nephritis and dermatitis in MRL/lpr mice. A recent report in the literature (60) suggests that DAF and CD59 function synergistically to inhibit renal ischemia-reperfusion injury in mice.

In conclusion, our study is the first direct demonstration of the in vivo role of ocular CRPs in protecting autologous tissue from injury induced by complement activation during autoimmune uveitis. Collectively, our data demonstrated that the direct interference with the function and/or cell surface expression of Crry and CD59 by Ab, AS, or siRNA in vivo resulted in a significant reduction in Crry and CD59, increased complement activation, and the exacerbation of EAAU.

Disclosures
The authors have no financial conflict of interest.

References


