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Suppression of Complement Activation by Recombinant Crry Inhibits Experimental Autoimmune Anterior Uveitis (EAAU)

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Abstract

This study was initiated to explore the effect of recombinant rat Crry linked to the Fc portion of rat IgG2a (Crry-Ig) on the induction of experimental autoimmune anterior uveitis (EAAU) and on established disease. EAAU was induced in Lewis rats by immunization with bovine melanin associated antigen (MAA). MAA sensitized animals received Crry-Ig, rat IgG2a (isotype control) or PBS separately before the onset of EAAU or after the onset of clinical disease. Administration of Crry-Ig suppressed the induction of EAAU while all animals injected with IgG2a or PBS developed the normal course of EAAU. Treatment with Crry-Ig resulted in suppression of ocular complement activation as well as the functional activity of complement in the peripheral blood. At the peak of EAAU, levels of IFN- γ , IP-10, ICAM-1 and LECAM-1 were significantly reduced within the eyes of Crry-Ig treated Lewis rats. Importantly, administration of Crry-Ig even after the onset of EAAU resulted in a sharp decline in the disease activity and early resolution of EAAU. Collectively, the evidence presented here demonstrate that inhibition of complement by Crry-Ig results in low levels of inflammatory molecules - C3 activation products, MAC, cytokines, chemokines and adhesion molecules in the eye. Down-regulation of these molecules affects the infiltration and recruitment of inflammatory cells to the eye resulting in inhibition of EAAU.

Keywords

Ocular autoimmunity; complement system; C3; Crry (complement- related gene/protein y, 512 antigen); membrane attack complex (MAC); uveitis

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1. Introduction

Idiopathic anterior uveitis (AU), inflammation of the anterior segment of the eye, is the most common form of intraocular inflammation in humans and the recurrent nature of the disease can lead to permanent loss of vision (Bloch-Michel and Nussenblatt, 1987; Bora and Kaplan, 2007; Gritz and Wong, 2004;). Experimental autoimmune anterior uveitis (EAAU) in rats is a model of organ-specific autoimmune inflammatory disease of the eye that bears close resemblance to human idiopathic AU (Bora et al., 1995, 1997, 2004; Broekhuysen et al., 1991; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010;). Over the past 15 years, the EAAU animal model has been extensively studied in our laboratory and we demonstrated that in EAAU, following injection of melanin associated antigen (MAA) in the hind foot-pad of Lewis rats, severe inflammation occurs in the anterior segment of the eye which includes the iris, the ciliary body (CB) and the anterior chamber (Bora et al., 1995, 1997, 2004; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010).

Evidence from the EAAU animal model led us to propose that the presence and activation of the complement system is central to the pathogenesis of idiopathic AU (Jha et al., 2006a). In recent years it has become increasingly evident that the complement system, a major component of innate immunity, is also involved in the modulation of antigen specific immune and inflammatory responses (Carroll, 2004; Morgan, 1995; Morgan and Harris, 1999; Sohn et al., 2003). A delicate balance exists between complement activation and complement inhibition and disruption of this balance contributes to various inflammatory diseases (Bora et al., 2005, 2006, 2008; Copland et al., 2010; Gaede et al., 1995; Hietala et al., 2002; Jha et al., 2006a, 2006b, 2007; Kaya et al., 2001; Marak et al., 1979; Pasinetti, 1996; Singhrao et al., 1999; Tsokos, 2004; Tran et al., 2002; Vriesendorp et al., 1998). We further demonstrated that various ocular tissues up-regulate complement regulatory proteins (CRegs) including Crry (complement-related gene/protein y, 512 antigen) to avoid self injury during autoimmune uveitis (Jha et al., 2006b). We showed that suppression of CRegs exacerbates EAAU and that these CRegs play an active role in the resolution of EAAU by down-regulating complement activation in vivo (Jha et al., 2006b). Current studies in our laboratory are directed towards exploring the possible use of CRegs as novel antiuveitic agents in the treatment of idiopathic AU. In the present study, we utilized recombinant Crry linked to the Fc fragment of rat immunoglobulin G (Crry-Ig) to investigate if the induction of EAAU as well as already established (on-going) EAAU can be inhibited by this long-lived recombinant protein. Crry, a trans-membrane glycoprotein is widely expressed in rodents (Kim et al., 1995; Quigg et al., 1995). It is expressed on various rat ocular tissues including the iris and the CB (Funabashi et al., 1994; Sohn et al., 2000). Crry regulates the activation of complement system by inhibiting the formation of C3 convertase (Funabashi et al., 1994; Kim et al., 1995; Morgan and Harris, 1999; Quigg et al., 1995; Sohn et al., 2000). The recombinant Crry-Ig was designed and generated to provide a powerful fluid-phase complement inhibitor with a long half-life in vivo and has proven effective in other rat models of autoimmune diseases (Hepburn et al., 2008; Mizuno et al., 2009).

2. Materials and Methods

2.1. Animals

Pathogen-free male Lewis rats (5–6 wk old) were obtained from Harlan Sprague Dawley (Indianapolis, IN). This study was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences, Little Rock, AR.

2.2. Induction and Evaluation of EAAU

Melanin-associated antigen (MAA) was purified from bovine iris and ciliary body as previously described by us (Bora et al., 1995, 2004). Male Lewis rats were immunized with 100 μ l of stable emulsion containing 100 μ g of MAA emulsified (1:1) in complete Freund's adjuvant (Sigma, St. Louis, MO) using a single-dose induction protocol in the hind footpad as previously described (Bora et al., 1995, 1997, 2004; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010). Animals were examined daily between days 7 and 30 post immunization for the clinical signs of uveitis using slit lamp biomicroscopy. EAAU was scored by an observer unaware of the experimental design using the criteria previously reported (Bora et al., 1995, 1997, 2004; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010). Eyes were also harvested at various time points for histological analysis to assess the course and the severity of inflammation using the criteria previously described by us (Bora et al., 1995, 1997, 2004; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010).

2.3. Crry-Ig Administration

Crry-Ig was generated as described previously by the fusion of amino-terminal domain of rat Crry containing four short-consensus repeats that are required for the regulatory and C3b-binding activities of Crry to the Fc portion of rat IgG2a (Hepburn et al., 2008). In our initial experiments MAA sensitized Lewis rats received a single intraperitoneal (ip) injection of Crry-Ig (2 mg/kg) at days 9 and 12 post-immunization or single ip injection of Crry-Ig (5 mg/kg) on days 9, 11 and 13 post-immunization. Total inhibition of EAAU was observed with ip - 5 mg/kg given on days 9, 11 and 13 post-immunization and was used in subsequent experiments. MAA sensitized animals were divided into three groups, each comprising three rats. MAA sensitized rats in group 1 received a single ip injection of Crry-Ig before the onset of EAAU on days 9, 11 and 13 post-immunization. Control animals received similar treatment with purified rat IgG2a (isotype control, group 2; R&D systems, Minneapolis, MN) or PBS (group 3) separately. This experiment was performed three times and data from all three experiments are expressed as the mean $\pm$ SD. In another set of experiments, MAA sensitized Lewis rats (n=3 rats) received intravenous (iv) injection of Crry-Ig (20 mg/kg) after the onset of EAAU on days 14 and 15 post MAA immunization. Control animals received similar treatments with purified rat IgG2a (n=3 rats) or PBS (n=3 rats) separately. In these experiments we used iv injection and higher dose because our pilot experiments demonstrated that after the onset of EAAU 5 mg/kg of Crry-Ig injected via ip route was not effective in suppressing EAAU. This may be due to the fact that complement activation in the eye is very high at the onset of EAAU (Jha et al., 2006a, b). This experiment was performed two times and data from both experiments are expressed as the mean $\pm$ SD.

2.4. Sample collection

Anesthetized animals were perfused through the heart with 200 ml of sterile pyrogen-free saline. Eyes were immediately enucleated. Intraocular tissue was prepared as previously described (Jha et al., 2006a; Sohn et al., 2000). The intraocular tissue was used for total RNA and protein extraction.

2.5. Total Hemolytic Complement Activity

Blood was collected by cardiac puncture, and total hemolytic complement activity in serum was determined using sensitized sheep erythrocytes (Diamedix, Miami, FL) as previously described by us (Jha et al., 2006a). Hemolytic activity in the serum obtained from Lewis rats that were not immunized with MAA and did not receive any treatment (naïve rats) was taken as 100%. Hemolytic activity in the serum obtained from Lewis rats that were immunized with MAA and received Crry-Ig, IgG2a or PBS was compared with the hemolytic activity in

the serum of naïve rats. The data are expressed as the mean $\pm$ SD. Data were analyzed and compared using Student's *t* test, and differences were considered statistically significant with $P < 0.05$.

2.6. Histology

Freshly enucleated rat eyes were fixed in neutral buffered 10% formalin solution (Sigma-Aldrich, St. Louis, MO) for 24 hours at room temperature, dehydrated in ethanol through ascending series of ethanol concentrations and embedded in paraffin. Five-micron thick sections were stained with hematoxylin and eosin (H&E) purchased from Fisher (Fair Lawn, NJ). Sections were examined using a light microscope (Olympus, Center Valley, PA).

2.7. Immunohistochemistry

Five micron thick paraffin-embedded tissue sections of eyes were immunostained for MAC, C3, ICAM-1 and LECAM-1 using a polyclonal antibody (raised in rabbit) reactive with rat/mouse C9 (1:1000, prepared in Dr. B. Paul Morgan's Laboratory), rabbit anti rat C9 (1:1000), IgG fraction of goat antiserum to rat C3 (1:500, MP Biomedicals, Solon, OH), purified goat anti rat CD54 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti rat CD62L (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) respectively. Cy3-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) and FITC labeled rabbit anti-goat IgG (Zymed, San Francisco, CA) were used as the secondary antibodies for MAC and C3 staining. Cy3 labeled rabbit anti goat (Sigma) were used as the secondary antibody for both ICAM-1 and LECAM-1 staining. Control stains were performed with irrelevant antibodies of the same Ig subclass at concentrations similar to those of the primary antibodies. Additional controls consisted of staining by omission of the primary or secondary antibody. The sections were covered with mounting medium with DAPI (ProLong Gold Mounting Medium; Invitrogen) and were examined under fluorescence microscope (Olympus, Center Valley, PA).

2.8. Semi-quantitative RT-PCR

Total RNA (0.1 μ g) from pooled intraocular tissue (described above) was used to detect the mRNA levels of β -actin, IFN- γ , IP-10, ICAM-1 and LECAM-1 by semi-quantitative RT-PCR using the reagents purchased from Applied Biosystems (Foster City, CA). Total RNA was prepared using the SV total RNA isolation kit (Promega) used according to the manufacturer's specifications. The forward and reverse oligonucleotide primers for rat proteins were synthesized at Integrated DNA Technologies (Coralville, IA). The primer sequences and the predicted size of amplified cDNA are presented in Table 1. Polymerase chain reaction was performed using 25, 30 and 35 cycles and all three cycles gave similar results. All reactions were normalized for β -actin expression. The negative controls consisted of omission of RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 1.5% agarose gel and visualized using GelDocXR and Quantity One 4.2.0 program (Bio-Rad Laboratories, Hercules, CA).

2.9. ELISA

Intraocular tissue prepared as described above was homogenized in 500 μ L of ice-cold PBS containing 1% protease inhibitors and 1% NP40. After centrifugation, the supernatant was assayed (in triplicate) for rat IFN- γ protein using rat ELISA kit from BD Biosciences (San Diego, CA). ELISA was performed according to the manufacturer's recommendations. The concentration of IFN- γ was calculated by computer software using the standard curves obtained from known concentrations (ELISA kit) and was expressed as the mean concentration (picograms per milligram of total protein) of cytokine $\pm$ SD. Data were

analyzed and compared using Student's *t* test, and differences were considered statistically significant with $P < 0.05$.

2.10. Semi-quantitative Western Blot Analysis

Pooled intraocular tissue was homogenized in 500 μ l of ice-cold PBS containing 1% protease inhibitors and 1% NP40. The homogenate was centrifuged at 14,000x *g* at 4°C for 15 min, the supernatant was subjected to SDS-PAGE on 12% linear slab gel and separated proteins were transferred to a polyvinylidene fluoride membrane. Blots were blocked in 5% BSA for 1 hr at room temperature and were probed with purified IgG fraction of rabbit anti rat interferon-inducible protein - IP-10 (1:5000, Torrey Pines Biolabs, Inc., Houston, TX) and monoclonal β -actin antibody (mouse IgG1, Sigma-Aldrich, St.Louis, MO) at 4°C overnight. For Crry-Ig, SDS-PAGE was run under reducing conditions and the resultant blots were incubated with 1: 300 dilution of purified mouse anti-rat Crry/p65 (clone 512; BD Biosciences, San Jose, CA) at 4°C overnight. Control blots were treated with the same dilution of appropriate IgG isotype control. After washing and incubation with HRP-conjugated secondary Ab (1:5000 dilution), blots were developed using the ECL Western blot analysis detection system (ECL Plus; Amersham Biosciences). Quantification of proteins was accomplished by analyzing the intensity of the bands using Quantity One 4.2.0 (Bio-Rad Laboratories, Hercules, CA).

3. Results

In the present study we explored the effect of Crry-Ig on the induction of EAAU and on established disease in MAA sensitized Lewis rats.

3.1. Effect of Crry-Ig on the induction of EAAU

3.1.1. Disease activity—To study the effect of Crry-Ig on the induction of EAAU, MAA sensitized Lewis rats received intraperitoneal (ip) injection of Crry-Ig (5 mg/kg) on three occasions before the onset of EAAU (at days 9, 11 and 13 post MAA immunization). This treatment with Crry-Ig resulted in complete inhibition of EAAU both clinically and histologically (Table 2, Fig. 1A). In contrast, all animals that received a similar treatment with IgG2a (isotype control) or PBS developed the normal course of EAAU. In these animals, severe anterior uveitis occurred in both eyes between days 16 to 20 (Table 2). Histopathologic examination of the harvested eyes at day 19 post-immunization demonstrated massive infiltration of inflammatory cells in the iris, the ciliary body (CB) and the anterior segment of the eye of rats injected with IgG2a (Fig. 1B) or PBS (data not shown). In EAAU maximum inflammation typically occurs between days 16 to 20 post MAA-immunization (Bora et al., 1995,1997,2004; Jha et al., 2006a, 2006b, 2007,2009;Kim et al., 1995;Matta et al., 2008,2010).

3.1.2. Localization of Crry-Ig in the eye—Lewis rats injected separately with Crry-Ig (n=2 rats), IgG2a (n=2 rats) or PBS (n=2 rats) via ip route on days 9, 11 and 13 post MAA immunization were sacrificed at day 19 post-immunization (the peak of EAAU). Total protein extracted from harvested ocular tissue was analyzed on 12% SDS-PAGE under reducing conditions. Representative Coomassie stained gel presented in Fig. 2A shows the presence of Crry-Ig in the eye of the Lewis rats which received this recombinant protein via ip route (lane 2). In contrast, recombinant Crry-Ig was not detected in the eye of PBS (lane 3) and IgG2a (lane 4) injected Lewis rats. Pure Crry-Ig protein was used as the positive control and was loaded in lane 1 (Fig. 2A). The Crry-Ig fusion protein has a molecular weight of 70 kDa under reducing conditions (Hepburn et al., 2008). These results were confirmed by Western blot analysis using anti-rat Crry antibody (Fig. 2B). The band with a molecular weight less than that of Crry-Ig present in lanes 2-4 may be Crry protein

constitutively expressed by the resident ocular cells (Figure 2B). These experiments were performed three times.

3.1.3. Serum hemolytic activity—Blood was collected by cardiac puncture on day 19 post-immunization (the peak of EAAU) from MAA sensitized animals injected separately with Crry-Ig (n=3 rats), IgG2a (n=3 rats) or PBS (n=3 rats) on days 9, 11 and 13 post MAA immunization. Serum was isolated and inhibition of complement following ip injection of Crry-Ig was confirmed by measurement of complement dependent serum hemolytic activity (Jha et al., 2006). At day 19 post-immunization MAA sensitized PBS injected and MAA sensitized IgG2a injected animals had increased serum hemolytic activity (Fig. 2C). These animals had severe EAAU at this time point and elevated serum hemolytic activity is due to acute phase response (our unpublished data). An increase in hemolytic activity in rats with experimental autoimmune myasthenia gravis was noted in a previous study (Hepburn et al., 2008). In contrast, MAA sensitized Crry-Ig injected animals had significantly ($P<0.05$) reduced serum hemolytic activity relative to MAA sensitized PBS injected and MAA sensitized IgG2a injected animals at this time point (Fig. 2C). Together, our findings demonstrate that Crry-Ig in treated animals keeps excessive complement activation under check in MAA sensitized Lewis rats and brings the serum complement activity at a level similar to that in naïve animals (Fig. 2C). This experiment was performed three times.

3.1.4. Local complement activation—Formation of C3 split products and membrane attack complex (MAC) in the eye was used as a measure of local complement activation. MAA sensitized Lewis rats received ip injections of Crry-Ig (5 mg/kg, n=2 rats), IgG2a (5 mg/kg, n=2 rats) or PBS (n=2 rats) separately on days 9, 11 and 13 post MAA immunization. Rats were sacrificed at day 19 post-immunization and harvested eyes were stained for C3 and MAC using goat anti-rat C3 and rabbit anti-rat C9 respectively. Goat anti-rat C3 recognizes C3 split products (C3b and iC3b) as well as intact C3 (Jha et al., 2006a, 2006b; Sohn et al., 2000). Paraffin sections of rat eyes were immunofluorescence stained to detect the differences in the ocular level of C3 (including C3 activation products) and MAC staining between Crry-Ig injected and control animals. Immunofluorescence analysis of the eyes revealed increased deposition of both C3 (including C3 split products) and MAC in IgG2a injected (Fig. 3, B and D respectively) and PBS (data not shown) injected control animals. In contrast, C3 (Fig. 3A) and MAC (Fig. 3C) were markedly suppressed in Crry-Ig injected rats at this time point. This experiment was performed three times.

3.1.5. Intraocular expression of IFN- γ , IP-10 and adhesion molecules—We next investigated if there was a difference in the levels of intraocular IFN- γ , IP-10, ICAM-1 and LECAM-1 between Crry-Ig injected and control animals because we previously demonstrated that systemic complement depletion using cobra venom factor (CVF) altered the levels of these molecules within the eye during EAAU (Jha et al., 2006a). MAA sensitized Lewis rats received ip injections of Crry-Ig (5 mg/kg), IgG2a (5 mg/kg) or PBS separately on days 9, 11 and 13 post MAA immunization. Animals were sacrificed at day 19 post-immunization and the eyes were harvested.

We analyzed the expression of IFN- γ , IP-10, ICAM-1 and LECAM-1 at mRNA and protein level using semi-quantitative RT-PCR (IFN- γ , IP-10, ICAM-1 and LECAM-1; n=2 rats), Western blotting (IP-10; n=2 rats), ELISA (IFN- γ ; n=2 rats) and immunofluorescence staining (ICAM-1 and LECAM-1; n=2 rats). At the peak of EAAU (day 19 post-immunization) using semiquantitative RT-PCR, we detected increased levels of IFN- γ , IP-10, ICAM-1 and LECAM-1 transcripts in the eye of IgG2a and PBS injected Lewis rats (Fig. 4A). In contrast, IFN- γ , IP-10, ICAM-1 and LECAM-1 mRNA were drastically reduced in the eyes of Crry-Ig injected rats at this time point (Fig. 4A). Levels of IFN- γ protein as determined by ELISA were significantly ($P<0.05$) reduced in the eyes of Crry-Ig injected

rats compared with IgG2a and PBS injected animals (Fig. 4B). Administration of Crry-Ig resulted in decreased ocular levels of IP-10 protein compared to IgG2a and PBS injected animals at the peak of EAAU as demonstrated by Western blot analysis (Fig. 4, C and D). Using immunofluorescence analysis extremely weak staining for ICAM-1 (Fig. 4E) and LECAM-1 (Fig. 4G) was observed in the eyes of MAA sensitized Lewis rats injected with Crry-Ig. In contrast, strong staining for ICAM-1 and LECAM-1 was observed within the eyes of MAA sensitized animals injected with IgG2a (Fig. 4, F and H) or PBS (data not shown). These experiments were performed three times.

3.2. Effect of recombinant soluble Crry-Ig on established EAAU

We also explored if established EAAU can be inhibited by administration of Crry-Ig. MAA sensitized Lewis rats received intravenous (iv) injection of Crry-Ig, IgG2a or PBS separately on two occasions after the onset of the clinical disease (on days 14 and 15 post-immunization) and the disease progression was monitored. A sharp decline in the clinical disease activity and early resolution of EAAU was observed in all animals that received Crry-Ig (20 mg/kg) via iv route on days 14 and 15 post immunization (Fig. 5A). Similar treatment with PBS or IgG2a did not alter the course or the severity of EAAU. Two animals were sacrificed at day 20 post-immunization and the severity of ocular inflammation was determined by histology. Histopathologic analysis of the harvested eyes revealed that the disease had almost completely resolved by day 20 post-immunization in the animals injected with Crry-Ig. In these animals only few inflammatory cells could be detected in the iris (I), the CB (CB) and the anterior chamber (AC) of the eye (Fig. 5B). In contrast, heavy infiltration of inflammatory cells was observed in the iris, the CB and the anterior chamber of the eye of Lewis rats that received similar treatment with IgG2a (Fig. 5C) or PBS (data not shown).

4. Discussion

Idiopathic anterior uveitis (AU), an intraocular inflammatory disease that causes serious loss of vision when not treated properly, is a leading cause of blindness in the United States (Bloch-Michel and Nussenblatt, 1987; Bora and Kaplan, 2007; Gritz and Wong, 2004). It is the most common form of intraocular inflammation in humans and is characterized by the inflammation of the iris and the ciliary body (CB). Experimental autoimmune anterior uveitis (EAAU) is an organ specific autoimmune inflammatory disease of the eye (Bora et al., 1995, 1997, 2004; Broekhuysen et al., 1991; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010). In this model, Lewis rats experimentally injected with melanin associated antigen (MAA) in the foot-pad develop ocular disease that mimics human idiopathic AU (Bora et al., 1995, 1997, 2004; Jha et al., 2006a, 2006b, 2007, 2009; Kim et al., 1995; Matta et al., 2008, 2010). Our studies using EAAU animal model have provided evidence that the presence and activation of the complement system plays a central role in the pathogenesis of idiopathic autoimmune anterior uveitis (Jha et al., 2006a). Furthermore, our published results demonstrated that various ocular tissues up-regulate the expression of complement regulatory proteins (CRegs) to avoid self-injury during autoimmune uveitis and these proteins play an active role in the resolution of EAAU by down-regulating complement activation in vivo. We reported that interference with the function and/or cell surface expression of CRegs in vivo resulted in increased complement activation and the exacerbation of EAAU (Jha et al., 2006b). Collectively, our published data support an important role of CRegs in controlling EAAU and suggest that recombinant CRegs might be of benefit to reduce inflammation and ameliorate disease in EAAU. Therefore, in the present study we explored the role of recombinant Crry-Ig in EAAU. To our knowledge, this study is the first to demonstrate that recombinant CReg can inhibit autoimmune uveitis.

Recombinant Crry-Ig was generated by the fusion of amino-terminal domain containing four short-consensus repeats that are required for the regulatory and C3b-binding activities of Crry to the Fc portion of rat IgG2a, providing extended plasma half-life. This recombinant protein was shown to prevent experimental autoimmune myasthenia gravis and peritonitis in rats (Hepburn et al., 2008; Mizuno et al., 2009). Recombinant forms of the naturally occurring CRegs (both human and rodent) have been exploited in the treatment of several diseases (Bora et al., 2007; Goodfellow et al., 2000; Linton et al., 2000; McGrath et al., 1999; Mizuno et al., 2000; Mizuno et al., 2002; Piddlesden et al., 1994; Quigg et al., 1998; Rehrig et al., 2001; Williams et al., 2004;).

In this study we first investigated the effect of Crry-Ig on the induction of EAAU. Our results demonstrated that systemic administration of Crry-Ig to MAA sensitized Lewis rats before the onset of EAAU inhibited disease assessed both clinically and histologically. Agent was given on three occasions on alternate days - days 9, 11 and 13 post-immunization with MAA. These time points were selected based on our previous study (Jha et al., 2006a) which demonstrated that a single ip injection of cobra venom factor (CVF) at day 9 post-immunization suppressed EAAU and the fact that recombinant Crry-Ig has a half life of 53 hours in the circulation (Hepburn et al., 2008). A single ip injection of CVF in Lewis rats depletes the complement system of the host for five days (Jha et al., 2006a; Sohn et al., 2000). Importantly, administration of Crry-Ig even after the onset of EAAU resulted in a sharp decline in the disease activity and early resolution of EAAU. This is a critical observation because it mimics the usual clinical situation in humans where a uveitis patient presents with an active disease and suggests that anti-complement agents can suppress established disease.

To confirm that the effect of systemically injected Crry-Ig was on ocular complement system, eyes were examined for C3, C3 activation products and MAC deposition because activation of the complement system is necessary for the generation of these molecules (Carroll, 2003; Morgan, 1995; Morgan and Harris, 1999; Sohn et al., 2003). The animals that received Crry-Ig showed extremely low C3 and MAC deposition at the peak of EAAU where as in the control animals intense staining for C3 and MAC was observed in the anterior chamber of the eye. We also performed the total hemolytic assay in the serum samples from MAA sensitized animals injected with Crry-Ig, IgG2a and PBS and observed that Crry-Ig injection resulted in significantly decreased complement activation compared to complement activation in the control animals. Taken together, these results suggest that in MAA sensitized Lewis rats systemic administration of Crry-Ig inhibited the complement system both locally (in the eye) and systemically (in the blood). Furthermore, the result presented in the current manuscript demonstrated that Crry-Ig injected systemically was able to reach the rat eye.

We have previously reported that at the peak of EAAU levels of intraocular cytokines, chemokines and adhesion molecules were up-regulated in complement sufficient rats but not in complement deficient (CVF treated) animals (Jha et al., 2006a). Therefore, we next examined the intra-ocular expression of cytokines, chemokines and adhesion molecules during EAAU post Crry-Ig treatment. At the peak of EAAU, ocular cytokine (IFN- γ), chemokine (IP-10) and adhesion molecules (ICAM-1 and LECAM-1) were up-regulated at both mRNA and protein level in control animals but not in the Crry-Ig injected group at this time point. Studies reported in the literature have emphasized that the expression of various cytokines, chemokines and adhesion molecules is affected by the presence and activation of the complement system (Fava et al., 1993; Hartwig et al., 2003; Kilgore et al., 1995; Tramontini et al., 2002; Tedesco et al., 1997; Vaporciyan et al., 1995;). Additionally, our published data clearly demonstrated that in EAAU recruitment of T cells to the eye as well

as ocular expression of cytokines, chemokines and adhesion molecules requires the presence and activation of complement system (Jha et al., 2006a).

In summary, we report two novel observations in this paper. First, induction of EAAU can be inhibited by the recombinant complement regulatory protein Crry-Ig; second, Crry-Ig was effective in suppressing EAAU in Lewis rats after the disease had already developed. Our results further suggest that inhibition of complement activation by Crry-Ig leads to down-regulation of inflammatory molecules such cytokines, chemokines and adhesion molecules in the eye. Thus, our data demonstrate a strong relationship between inhibition of complement by Crry-Ig with disease activity as well as ocular expression of cytokines, chemokines and adhesion molecules during EAAU. Importantly, disease modifying effects of Crry-Ig observed in the present study suggest that complement inhibition may be a promising therapeutic target for idiopathic anterior uveitis, a sight-threatening human disease which is associated with significant morbidity and can currently only be treated symptomatically (Bloch-Michel and Nussenblatt, 1987; Bora and Kaplan, 2007; Gritz and Wong, 2004).

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Fig. 1.

Effect of Crry-Ig on the induction EAAU. MAA sensitized Lewis rats received a single intraperitoneal (ip) injection of Crry-Ig (**A**) or IgG2a (**B**) on days 9, 11 and 13 post-immunization. Animals were sacrificed at day 19 post-immunization (peak of EAAU) and harvested eyes were stained with hematoxylin and eosin (H & E) for histopathologic analysis. EAAU did not develop in Crry-Ig treated Lewis rats as no inflammation was noted in the eyes of these animals (**A**). Severe EAAU was observed in IgG2a injected animals with heavy infiltration of the inflammatory cells in the iris (I), the ciliary body (CB) and the anterior chamber (AC) of the eye (**B**). Objective magnification x 20

Fig. 2.

Localization of Crry-Ig in the eye and effect of Crry-Ig on serum complement activity. (A) MAA sensitized rats that received Crry-Ig, IgG2a or PBS separately were sacrificed at the peak of EAAU. Total protein (30 μ g) extracted from the harvested eyes was separated on 12% SDS-PAGE under reducing conditions. Representative Coomassie stained gel shows the presence of Crry-Ig within the eyes of the Crry-Ig injected Lewis rats (**lane 2**) and not in the eyes of rats treated with PBS (**lane 3**) and IgG2a (**lane 4**). Pure Crry-Ig (70 kDa) was loaded in **lane 1**. (B) Western blot analysis using mouse anti-rat Crry/p65 and ocular protein samples from rats treated ip with Crry-Ig (**lane 2**), PBS (**lane 3**) and IgG2a (**lane 4**). Note that 70 kDa protein band representing Crry-Ig was detected only in Crry-Ig injected rats (**lane 2**) and not in rats treated similarly with PBS (**lane 3**) and IgG2a (**lane 4**). Purified Crry-Ig was loaded in lane 1 and served as the positive control. (C) Serum collected on day 19 from MAA sensitized animals injected separately with Crry-Ig, IgG2a or PBS was used in complement hemolytic assay. At the peak of the EAAU (day 19 post-immunization) serum complement activity in MAA sensitized Crry-Ig injected animals was significantly low compared to MAA sensitized PBS or IgG2a injected animals and was similar to those in naïve rats. Hemolysis observed in the serum of naïve Lewis rats was considered as 100% hemolytic activity. * $p < 0.05$

Fig. 3.

Immunofluorescence micrographs showing staining for C3 and C3 split products (**A** and **B**) and membrane attack complex (MAC; **C** and **D**). MAA immunized Lewis rats injected ip with Crry-Ig (**A** and **C**) and IgG2a (**B** and **D**) on days 9, 11 and 13 post MAA immunization were sacrificed at day 19 post-immunization. Extremely weak green fluorescence for C3 and C3 split products (**A**) and extremely weak red fluorescence for MAC (**C**) was observed within the eyes of Crry-Ig injected animals. However, intense green fluorescence for C3 and C3 split products (**B**) and intense red fluorescence for MAC (**D**) was observed within the eyes of IgG2a injected animals. Blue color in each panel represents DAPI. The data shown are representative of three separate experiments. I, iris; CB, ciliary body; AC, anterior chamber. Objective magnification: x10 for **A** and **B**; x 20 for **C** and **D**.

Fig. 4.

Effect of systemic Crry-Ig on intraocular IFN- γ , IP-10, ICAM-1 and LECAM-1. At day 19 post-immunization, eyes were harvested from MAA sensitized Lewis rats injected ip with Crry-Ig, IgG2a or PBS. Panel A shows ethidium-bromide-stained PCR products (after UV exposure) for IFN- γ , IP-10, ICAM-1 and LECAM-1 within the eyes of Crry-Ig, IgG2a and PBS injected Lewis rats. PCR products were generated using semi-quantitative RT-PCR and were analyzed on a 1.5% agarose gel. A strong band at 335 bp for β -actin indicated equal amount of total RNA in each lane. (B-F) Effect of Crry-Ig on intraocular IFN- γ , IP-10, ICAM-1 and LECAM-1 protein production at day 19 post-immunization. IFN- γ , production was assessed by protein specific ELISA and data are reported as mean $\pm$ SD for triplicate determinations, * p <0.05 (B). Representative semi-quantitative Western blot (C) and densitometric analysis of the blot (D) for IP-10 protein in Crry-Ig, IgG2a and PBS injected rats is shown. Paraffin sections were prepared from eyes harvested from MAA sensitized Crry-Ig (E and G) and IgG2a (F and H) injected Lewis rats at day 19 post-immunization (the peak of EAAU). Sections were stained with antibodies against ICAM-1 (E and F) and LECAM-1 (G and H). Red fluorescence in E and F represents ICAM-1 while the red fluorescence in G and H represents LECAM-1. Blue color in panels E-H represents DAPI. Each micrograph is representative of three separate experiments. I, iris; CB, ciliary body; AC, anterior chamber. Objective magnification x 20

Fig. 5. Inhibition of ongoing EAAU by Crry-Ig. (A) MAA sensitized Lewis rats received Crry-Ig via iv route at days 14 and 15 post-immunization (after the onset of EAAU). This resulted in early resolution of EAAU and blocked the progression of the disease. MAA sensitized Lewis rats treated similarly with IgG2a or PBS alone developed the normal course of EAAU. Lewis rats injected with Crry-Ig (B) and IgG2a (C) as described above were sacrificed at day 20 post immunization. H and E staining of harvested eyes demonstrated heavy infiltration of the inflammatory cells within the iris (I), the ciliary body (CB) and the anterior chamber (AC) of IgG2a injected rats (C). In contrast, only few inflammatory cells could be detected in the eye of Lewis rats injected iv with Crry-Ig (B). Objective magnification x 20

Table 1

Primer sequences used in RT-PCR

Gene	Primer	Sequence	PCR product size (bp)
ICAM-1	Forward	5'- AGGAACACCATGCTTCCTCTGACA- 3'	215
	Reverse	5'- TGGAGAAGCCCAAACCCGTATGAT- 3'	
LECAM-1	Forward	5'- AACGAAAGGCAGCTCTCTGCTACA- 3'	376
	Reverse	5'- TGACTGCATTCCATAGTGCCCA- 3'	
IFN- γ	Forward	5'- ATCTGGAGGAACTGGCAAAGGACG-3'	288
	Reverse	5'- CCTTAGGCTAGATTCTGGTGACAGC-3'	
IP-10	Forward	5'- TTCCTGCAAGTCTATCCTGTCCGC-3'	560
	Reverse	5'- TTTGCCATCTCACCTGGACTG-3'	
β -actin	Forward	5'- GCGCTCGTCGTCGACAACGG-3'	335
	Reverse	5'- GTGTGGTGCCAAATCTTCTCC-3'	

The sequences of all oligonucleotides are shown in the 5' to 3' direction.

Table 2

Effect of Cry-Ig on the Induction of EAAU

Treatment in vivo	Days of injection	Eyes with EAAU*				Day of onset	Peak of Disease (days)	Duration of Disease (days)
		incidence	mild	moderate	severe			
Cry-Ig	9, 11, 13	0/18	-	-	-	-	-	-
IgG2a	9, 11, 13	18/18	-	-	18	14±0.4	16-19	13±1
PBS	9, 11, 13	18/18	-	-	18	15±0	17-20	12.8±0.5

Experimental autoimmune anterior uveitis (EAAU) was induced by immunizing Lewis rats with 100 µg of melanin associated antigen (MAA).

* Incidence of EAAU given as positive/total eyes following clinical examination. Data are presented as the mean ± SD for day of onset and duration of disease. Severity of inflammation on histopathologic examination was grouped as mild (1+), moderate (2+ to 3+) or severe (4+).