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Autoimmune Responses against Photoreceptor Antigens during Retinal Degeneration and their Role in Macrophage Recruitment into Retinas of RCS Rats

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Abstract

Autoimmunity may contribute to retinal degeneration. The studies examined the evolution of autoimmune responses against retina in naïve dystrophic RCS rats over the course of retinal degeneration. We showed that anti-retinal autoantibodies and T cells are generated in response to the availability of antigenic material released from dying photoreceptor cells during retinal degeneration but with distinctive activation trends. Passive transfer of anti-retinal antibodies enhanced disease progression by disrupting the BRB, upregulating MCP-1, attracting blood macrophages into retina, and augmenting apoptotic photoreceptor cell death. Our findings directly link anti-retinal autoantibodies to activated macrophage entry and their possible role in neurodegeneration.

Keywords

retinal degeneration; passive transfer; autoimmunity; autoantibody; RCS rat; macrophages

1. INTRODUCTION

One common feature for retinitis pigmentosa (RP), age-related retinal degeneration (AMD), autoimmune retinopathy (AR), and related diseases is retinal degeneration that leads to incurable blindness. The mechanism involved in development of those various retinopathies is not completely understood, but it appears to be caused by a combination of factors, including inflammatory and autoimmune factors. While typical retinal degenerations are not classical inflammatory diseases, recent findings support the concept that the immune/autoimmune mechanisms may play an important role in the degenerative process (Penfold et al., 2001, Donoso et al., 2006, Morohoshi et al., 2009, Adamus, 2009, Anderson et al., 2010). The most common finding is the presence of serum anti-retinal autoantibodies (AABs), associated with a number of retinal diseases, including paraneoplastic retinopathies (Adamus, 2009). Notably, AABs persist over the progression of retinal degenerative and their levels can fluctuate (Saito et al., 2007, Adamus et al., 1998a). In addition, various leukocytes, including macrophages, CD4⁺ T cells, CD8⁺ T cells, and activated T suppressor

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cells were reported (Ezzat et al., 2008, Solomon et al., 1999, Penfold et al., 2001, Morohoshi et al., 2009, Adamus et al., 1993, Magrys et al., 2007, Maeda et al., 2001, Rong et al., 2002, Buchanan et al., 1984). Mild chronic intraocular inflammation with a few vitreous cells, aqueous flare, cystoid macular edema, infiltration of macrophages, and accumulation of microglia were reported in different forms of retinal degeneration (Zipfel et al., 2010, Chen et al., 2002). Waste products that accumulate in the retina, including drusen-containing complements factors, immunoglobulins, and pro-inflammatory cytokines/chemokines cause progressive loss of vision (Ding et al., 2009, Penfold et al., 2001, Xu et al., 2009). During neurodegeneration, activated microglial cells participate in the phagocytosis of debris and facilitate regenerative processes. In addition, microglia activated by photoreceptor death kill adjacent cells (Gupta et al., 2003). Therefore, they have dual functions in autoimmune retinal disease, including initiating inflammation of the retina and limiting subsequent inflammation (Chen et al., 2002).

Our laboratory has been interested in the pathogenic role of anti-retinal AAbs in retinal degenerative diseases. The past studies have shown that AAbs against retinal autoantigens such as recoverin, α -enolase and carbonic anhydrase II killed retinal cells *in vitro*, *ex vivo*, and *in vivo* (Adamus et al., 1998b, Ren and Adamus, 2004, Adamus and Karren, 2009). Those AAbs can be detected at onset of retinal degeneration and can persist as the disease progresses. Thus, we proposed that AAbs not only can initiate, but can also contribute to the degenerative process. The objective of the current studies was to examine autoimmune responses over the course of retinopathy in the dystrophic RCS rat, a model for inherited retinal degeneration, and determine whether adoptive transfer of anti-retinal AAbs collected from dystrophic rats contribute to ongoing retinal degeneration in the recipient RCS rats. We selected the RCS rat model of retinal degeneration because of similar pathology to human inherited retinal degeneration. Moreover, autoimmunity has long been suspected to be a factor in RCS retinal degeneration but has not been explored (Chant and Meyers-Elliott, 1982, Reid et al., 1987). We examined the natural immune responses for two retinal pathogenic autoantigens, arrestin (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), in those rats because both arrestin- and IRBP-reactive T cells, as well as specific AAbs have been found in diseased human serum (Gregerson et al., 1981, Wiggert et al., 1991). Our findings show that the autoimmune AAbs and T cell responses against both target autoantigens evolved in naïve RCS rats over the course of photoreceptor degeneration but showed different activation trends, suggesting their unique role in degeneration. Moreover, we demonstrated for the first time that adoptive transfer of anti-retinal antibodies causes macrophage recruitment into retina, which impact photoreceptor apoptotic death possibly triggered by arriving microglia.

2. METHODS

2.1. Animals

Dystrophic Royal College of Surgeons (RCS) rats were bred and housed at the Oregon Health Sciences University Animal Care Facility according to institutional and federal guidelines. Both genders were used for experiments in groups of 3 when they reached the appropriate postnatal age. All animal experimentation procedures adhered to the ARVO Resolution on the Use of Animals in Research and have been approved by the OHSU animal committee.

2.2. Adoptive Transfer of Serum to RCS rats

Randomized groups of female and male RCS rats (n=3) at day P26 (day 26 postnatal) or P30 received an intraperitoneal (i.p.) injection of 1 ml of anti-retinal serum obtained from donor RCS dystrophic rats, 1 mg/ml of monospecific affinity-purified rat anti-recoverin antibody

Rec-1 or anti- α -enolase antibody Enol-1, or vehicle (saline, 1ml). Both antibodies were produced by our laboratory (Adamus et al., 1998b, Adamus et al., 2006, Ren and Adamus, 2004). Eyes were harvested between day 1 and 7 post transfer, fixed in 2% paraformaldehyde for 1 hr, and frozen in OCT medium at -80°C before sectioning. Cryosections were used for immunolabeling (see below). Some of eyes were harvested from perfused rats (2% paraformaldehyde) to collect retinas for whole-mounting. Retinal wholemounts were blocked with 5% non-fat milk, 1% bovine serum albumin, and 0.1% Tween 40 in PBS for 30 min on a shaker followed by overnight incubation with anti-CD163 antibody (clone ED2; expressed on activated monocytes and macrophages, 1:1000, Serotec) at 4°C . The next day, after washing with PBS (3 times for 5 min with gentle shaking), isolectin B4 conjugated to Alexa Fluor 647 (1:2000, Invitrogen) was added for 5 hrs to label vasculature (Ernst and Christie, 2006). Experiment was repeated 2 times. The immunofluorescent labeling in cross-sections and retinal whole mounts was examined using an Olympus Fluoview1000 confocal microscope at 20x and 40x magnification and pseudocolor images were acquired for analysis.

2.3. Fluorescent Immunolabeling

Ten-micron RCS rat eye cryosections were post-fixed with 4% paraformaldehyde for 10 min followed by blocking with 10% normal goat serum with 1% bovine serum albumin in PBS for 60 min. The blocked sections were then incubated with specific primary antibodies overnight at 4°C as follows: anti-Iba-1 (allograft inflammatory factor 1, expressed in microglia/macrophages; 1:1000, Abcam), anti-CD11b (1:100, Abcam), anti-MCP-1 (1:60, PrepTech), anti-VEGF (1:1000, Abcam), anti-CD74 (1:100, Santa Cruz Biotechnology), and anti-CD3 (1:200, BD Pharmingen). After washing, appropriate fluorescent secondary antibodies conjugated to Alexa Fluor 488 (1:2000, Invitrogen) or Alexa Fluor 594 (1:1000, Invitrogen) were added for 1 hr incubation. The sections were washed in PBS and a mounting reagent with DAPI was added to seal the sections, inhibit fluorescence quenching, and stain nuclei. The immunofluorescent labeling was evaluated using an Olympus Fluoview1000 confocal microscope and pseudocolor images were acquired for analysis. A negative control contained secondary antibodies only.

2.4. ELISA

ELISA polystyrene plates were coated with $10\text{ }\mu\text{g}$ IRBP₁₁₇₇₋₁₁₉₁ peptide or $1\text{ }\mu\text{g}$ arrestin per well in 0.1 M Tris-HCl buffer, pH 9.0 (coating buffer) overnight at room temperature. After washing with coating buffer and blocking with 1% BSA in PBS for 1 h, diluted serum at 1:100 was added to each well and allowed to incubate for 1 h and then washed. Wells were then incubated 1 h with 1:2000 diluted biotinylated anti-rat IgG (H+L chain, Invitrogen) antibodies followed by 30 min incubation with streptavidin conjugated to peroxidase (1:5000; Invitrogen). A color reaction was developed for 30 min by incubation with peroxidase substrate (2,20-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)) in 0.1 M citrate-phosphate buffer, pH 4.5, containing 3% H_2O_2 and immediately measured at 415 nm using a Bio-Rad Microplate Reader.

2.5. Rat Antibody Isotyping Assay

Total rat immunoglobulin isotypes were measured using “Rat Ig Isotyping ELISA Ready-SET-Go” kit (eBioscience, Vienna, Austria). Briefly, ELISA polystyrene plates were coated with different antibodies against rat IgG and IgM isotypes according to the manufacturer’s procedure. After washing, 50 μl pooled sera diluted 1:50 from 3–5 naïve rats collected on days 15, 20, 30, 40, 60, and 90 was added to wells. Positive control was provided in the kit. A color reaction was measured at 415 nm using a Bio-Rad Microplate Reader.

In the second ELISA assay, specific anti-retinal IgG and IgM antibodies were determined as followed. A polystyrene plate was coated with 1 μ g arrestin or 10 μ g IRBP₁₁₇₇₋₁₁₉₁ peptide in 0.1 M phosphate buffer, pH 9 overnight. After washing and blocking, 50 μ l of diluted 1:50 pooled serum collected at different postnatal time points was added for 2 hr incubation followed by the incubation with 100 μ l of 1:10000 diluted biotinylated anti-rat IgG (γ chain specific, Brookwood Biomedical) or biotinylated anti-rat IgM (μ chain specific, Pierce) secondary antibodies. After 1 hr, plates were washed and incubated with 100 μ l of 1:10000 diluted streptavidin-HRP conjugate (Pierce) for 30 min. Finally, 100 μ l of tetramethylbenzidine (TMP) substrate was added and reaction was stopped by adding 50 μ l of stop solution. A color reaction was measured at 415 nm using a Bio-Rad Microplate Reader.

2.6. Lymphocyte Proliferation Assay (LPA)

The assay was performed in 96-well tissue culture plates in triplicates using RPMI medium supplemented with 10% FBS, 5×10^{-5} M 2-mercaptoethanol, and 50 μ g/ml gentamicin. Cells prepared from spleens were seeded at a density of 2×10^5 cell/well and incubated with 5 μ g IRBP₁₁₇₇₋₁₁₉₁ peptide, 5 μ g arrestin, or 1 μ g concanavalin A at 37°C in 5% CO₂ for 72 hr and then pulsed with 1 μ Ci ³H-thymidine/well for an additional 18 hr. The cells were harvested onto a glass fiber filter and the thymidine uptake was assessed by the liquid scintillation counting in a Betaplate counter (Wallac, model 1250). The data were expressed as a stimulation index (SI), which was calculated by dividing the proliferation (cpm incorporated) measured in the presence of antigen by the proliferation measured by medium alone. SI value below SI=1 is considered to be negative.

2.7. Terminal dUTP Nick End-labeling (TUNEL) of Fragmented DNA

Ten-micron RCS rat eye cryosections were post-fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 (10 min) for TUNEL nuclear staining of apoptotic photoreceptor nuclei with terminal deoxynucleotidyl transferase (rTdT) using DeadEnd Fluorometric TUNEL System (Promega) according to manufacturer's instructions. Retinal sections were further counterstained with DAPI (Molecular Probes) to identify the retinal cell layers. The immunofluorescent labeling was evaluated using an Olympus Fluoview1000 confocal microscope and pseudocolor images were acquired for analysis. The positive control was treated with DNAase I for 15 min and the negative control had no rTdT enzyme added.

2.8. Statistical Analyses

Statistical analysis was performed with Prism GraphPad software using a 2-tailed Student t test or the significance between antibody and vehicle-treated groups was determined by 1-way analysis of variance (ANOVA). Differences with a p value < 0.05 were considered significant and are denoted by an asterisk.

3. RESULTS

3.1. Autoimmune Responses during Retinal Degeneration

To determine the autoimmune responses in naïve RCS rats, we examined the immune responses over the course of 90 days for two pathogenic retinal autoantigens, potentially released from outer segments during photoreceptor degeneration, IRBP and Arrestin (S antigen). Sera from RCS dystrophic rats were collected before onset (P15) every 10 days until complete photoreceptor demise (P90). Figure 1A–D shows the levels of naturally occurring serum AAbs and T cells activities specific to IRBP₁₁₇₇₋₁₁₉₁ peptide (major pathogenic epitope) and arrestin (S-antigen). The peak of autoantibody response against IRBP and arrestin was determined to be the same at about P19. Antibody levels has dropped

by P40 and rebounded about 2 weeks later at P60 as measured by ELISA. To examine whether the immune responses were correlated with the progression of apoptotic cell death in the outer nuclear layer a TUNEL labeling was performed on normal RCS rat retina collected on day 19, 30, 40, 60, and 90. Figure 1E shows the presence of TUNEL-positive cells in the outer nuclear layer over a 90-day period. It was clear that the initial generation of anti-retinal AAbs coincided with photoreceptor apoptosis. These early rat sera (P19) strongly immunolabeled the photoreceptor outer and inner segments when applied on a non-dystrophic rat retina (Figure 1F). Later, P90 dystrophic serum still contained anti-retinal AAbs at lower titer that mostly labeled outer segments (Figure 1F). Figure 1B and D shows a measurable T cell response was detected later than AAbs response and showed a different activation trend – the highest recalled T cell proliferative activities to IRBP₁₁₇₇₋₁₁₉₁ peptide and arrestin was detected at P60 as determined by the lymphocyte proliferation assay, suggesting that those antigen-specific activated T cells were initially eliminated and only those that survived were detected in later time.

Given these finding we wanted to determine the origin of the early specific AAbs present in the rat sera. The unexpected decrease in AAbs on day 40 could suggest that these early antibodies were different from the AAbs detected in the sera collected at the later time points. Because the IgG in rats is transmitted mainly postnatally after birth and continued throughout the greater part of lactation (Pentšuk and Van Der Laan, 2009) the early antibodies is likely maternal origin. The mothers had fully degenerated retinas and their sera contained anti-retinal antibodies. Figure 2AB shows the results from the isotyping analysis of pooled serum antibodies present in the naïve RCS rat circulation. Of note, the pups were separated from their mothers on P21 (arrows on the graph). At P15, IgG2a (subclass associated with the Th2 immune response in rats) was present in the highest levels. IgG2b (subclass linked to the Th1 response) was initially present at about the same level but then dropped at P20 to regain the lost level after the rats were weaned. After P30 we observed an increase in the total IgM Abs, which indicates that these antibodies were generated by the pups. Moreover, both classes of specific anti-arrestin and IRBP peptide IgG and IgM antibodies were already detected in 2-week old rats (Figure 2CD). There was a slight decreased at P20 in IgG levels, suggesting that those early IgG autoantibodies originated from the mother's milk. At the same time there was an increase in IgM level, which then dropped at 30–40 postnatal days and later recovered.

The presence of retinal-specific CD3⁺ T lymphocytes was observed in degenerating retina after P40. Figure 3 shows immunofluorescent CD3⁺ T cells (red cells) in the degenerating retina at P40, frequently observed in the ganglion cell layer, inner plexiform layer, and RPE mostly around the retinal blood vessels (yellow arrows). CD3⁺ labeled T cells still persisted, even when most of photoreceptors were gone and a thick layer of debris emerged in the subretinal space (Figure 3B). Figure 3A shows that the retina was also infiltrated by activated microglial cells expressing CD74⁺ (the MHC class II invariant) phenotype (white arrow), which suggests that these cells may be involved in the generation of new anti-retinal immune responses. Overlapped immunofluorescence in cells labeled with both anti-CD3 and anti-CD74 antibodies (see pink arrow) were evident in the outer segment/debris zone, suggesting that these cells were involved in antigen presentation. The finding of CD74⁺ cells in the retina about the time of AAbs production rebound and T cells activation is in agreement with the previously published studies reporting the abundance of class II-positive cells between P28 and P42 in RCS rat retinas (Akaishi et al., 1998).

This data together demonstrates that autoimmune responses peak at P19 when photoreceptor apoptosis starts and antigenic material becomes available, as a consequence of photoreceptor cell death. However, we cannot exclude a possibility that some of the early antibodies were transmitted with the mother's milk. A decrease in AAbs titers, to almost undetectable in rats

around P40, could be related to the excess of the cognate antigen in the target tissue that absorbs specific AAbs. It seems that AAbs target the same IRBP and arrestin epitopes as T cells do (Figure 1A and D), which in effect may exacerbate the pathogenic process.

3.2. Rapid Recruitment of Macrophages/Microglia to Retina

Next, we examined the effects of dystrophic anti-retinal sera from donor RCS rats on naïve RCS recipient rats by intraperitoneal passive transfer to determine whether antibodies accelerate degenerative processes in the retina. In addition, we also adoptively transferred affinity-purified rat anti-recoverin monoclonal antibody Rec-1 and anti- α -enolase antibody Enol-1 that had been previously shown to produce pathogenic effects *in vitro* and *in vivo* on retinal cells (Adamus et al., 1998b, Adamus et al., 2006, Ren and Adamus, 2004). The dystrophic sera were collected on day P60, and P90, when specific AAbs titers rebounded (see Fig. 1). Then 1 ml of pooled sera from rats, including at least 3–5 animals, or vehicle was administered i.p. to a recipient RCS rat of age 26 or 30 days. Their effects on the recipient retina were examined for 7 days post administration by immunofluorescence confocal studies using retinal whole mounts or cross-sections (Fig. 4 and 5). The key unexpected finding from serum adoptive transfer experiments is that dystrophic serum and monospecific antibodies accelerated the migration of blood borne macrophages/microglia (m ϕ /MG) into the outer retina 24 hr after antibody injection. Figure 4 demonstrates that one day after Rec-1 or Enol-1 transfer there was a 2–3-fold increase in the counts of iba-1⁺ microglial cells in retinal cross-sections and such a high number of recruited cells sustained in following days (Fig. 4AB). The number of recruited m ϕ /MG in the antibody-treated groups showed a significant difference when compared with the vehicle-treated group (** p <0.001, ANOVA), which did not alter the background levels of iba⁺ immunolabeled cells. The highest counts of iba-1⁺ immunofluorescent cells reached, on average, 253 cells/retina after Rec-1 transfer, 302 cells/retina after Enol-1 transfer into P26 RCS rats, and 109 cells/retina in the vehicle group. To determine whether recruited cells influence survival of photoreceptor cells in recipient's retinas we analyzed loss of outer nuclear layers and the presence of apoptotic cells using a TUNEL system, detecting fragmented DNA in apoptotic cells. There was no apparent loss of photoreceptor cells one to three days post antibody transfer. However, there was a slight increase in apoptotic nuclei (>1%) in the outer nuclear layer of recipient retinas compared to the same age vehicle-treated controls. Fig. 4C shows TUNEL positive nuclei (arrows) in the outer nuclear layer analyzed 2 days after injection of antibodies.

Then, we analyzed the recruited microglia to the recipient retina after a single injection of dystrophic serum collected from P60 and P90 donor rats in comparison to Rec-1 and Enol-1 antibody transfer. Using macrophage specific antibodies for the CD163 phenotype we demonstrate a significant number of CD163⁺ macrophages in antibody-receiving retinas at 7 days post antibody transfer (Figure 5). We analyzed the distribution of round CD163⁺ labeled cells in retinal whole mounts obtained from one recipient eye and iba-1⁺ labeled cells in retinal cross-section of the second eye from the same recipient rat. CD163⁺ cells were seen both in the parenchyma and around vascular structures in the retina and had a round, foamy morphology. Numerous CD163⁺ labeled microglia of ramified shape were observed in the retinal parenchyma as well. Because the retinal vessels were perfused with fixative to wash out blood cells before enucleation, some migrating cells adhered within vasculature just before relocation from the circulation into retinal parenchyma. Passive transfer of dystrophic serum caused a significant increase in recruitment of CD163⁺ cells into recipient retinas receiving P60 and P90 dystrophic sera. Rec-1 and Enol-1 pathogenic antibodies recruited significant number of m ϕ /MG in contrast to the background m ϕ /MG seen in vehicle-treated retinas. The quantitative analysis of cells infiltrating recipient retinas (Fig. 5I–K) at 7 days post transfer revealed that CD163⁺ immunolabeled cells were in

considerably higher numbers and ranged from 117 cells/retina in the vehicle-group to 306 cells/retina after serum passive transfer ($p < 0.0001$ one-way ANOVA; $n = 4$). The mean number of iba-1^+ microglial cells across the retina varied from 90 cells/retina in the control group to 256 cells/retina in the P90 serum-treatment group, almost a 3 fold increase. The number of cells immunolabeled with anti- iba-1 and anti-CD163 antibodies was similar, suggesting that cells were recognized by both antibodies, although CD163^+ macrophages were small and round (Figure 4). Based on the phenotype we estimated that there was, on average, 1.5 more round cells (blood borne) than ramified cells (retinal resident microglia). The labeling with anti-CD11b/c antibodies showed a lesser number of immunolabeled cells compared to the CD163 phenotype and were mostly located in the ONL (not shown). In summary, the recruitment of $\text{m}\phi/\text{MG}$ into the retina from blood occurs rapidly, as early as 24 hours after antibody challenge and the increased number of recruited cells persists for several days, which causes apoptosis of the photoreceptor cells. This additional influx of microglia may have implication for long-term photoreceptor survival.

3.3. Upregulation of Inflammatory Mediators in Retina

To determine whether chemokine MCP-1, that regulates macrophage infiltration, was upregulated in the retina after the adoptive transfer of antibodies was performed immunostaining studies using anti-MCP-1 antibody on recipient retinal cross-sections were collected 24 hr post transfer. After passive transfer of P90 dystrophic serum, Enol-1 and Rec-1 antibodies, recipient retinas showed a positive MCP-1 cellular immunostaining in the retinal ganglion cells, and inner plexiform and outer plexiform layers as an indication of the MCP-1 production in contrast to vehicle-treated retinas (Fig. 6). Because we adoptively transferred *rat antibodies* into *rat donors* we could not directly immunodetect the leaking IgG presence in the retina. Nonetheless, the antibodies need to pass through the blood-retina barrier (BRB) in order to reach the retina to induce their effects. The increased production of vascular endothelial growth factor (VEGF) has been reported to induce disruption of the BRB and increased vascular permeability in some eye diseases (Witmer et al., 2003), thus we investigated whether passively transferred antibodies upregulate VEGF in the retinas. Fig. 7 shows representative retinal cross-sections of the recipient RCS rat retinas that received P90 serum, Rec-1 and Enol-1 antibodies. Immunostaining results using anti-VEGF antibodies in retinal cross-sections revealed a strong immunofluorescent labeling of VEGF leaking retinal vessels (arrows) as soon as 1 day after transfer. This observation was not seen in the vehicle-treated group. The BRB failure coincides with the increase in $\text{m}\phi/\text{MG}$ in recipient retinas and upregulation of MCP-1 (Fig. 4 and 5).

4. DISCUSSION

Our results corroborate the hypothesis that anti-retinal autoantibodies play a role in the pathogenesis of autoimmune retinopathy associated with retinal degeneration. We provide evidence that autoimmunity could be responsible for the progression of photoreceptor cell death, as autoantibodies are elicited by ongoing retinal degeneration. We showed that anti-retinal autoantibodies and T cells are generated during retinal disease in response to the availability of antigenic material released from dying photoreceptors. In effect, they may enhance disease progression by augmenting apoptotic photoreceptor cell death, disrupting the BRB, and attracting blood macrophages into the retina. Retinal autoantigens, which are secluded from the immune system under normal conditions, only become exposed to autoimmune mechanisms when retinal cells die (Taylor, 2009). Recruitment of inflammatory cells, such as macrophages and dendritic cells to the local injured area/retina is a crucial step in activating the immune cells and the release of pro-inflammatory cytokines to amplify the disease process (Murray and Wynn, 2011). Microglia can be activated by the initial rod cell death and then they can kill the adjacent photoreceptors (Gupta et al., 2003). We demonstrated that in the dystrophic RCS rat, the occurrence of

serum autoantibodies specific to the outer segment-specific autoantigens (arrestin, IRBP) correlated with early apoptosis of photoreceptors (Fig. 1), a process that is important in promoting cellular degeneration (Tso et al., 1994, Katai and Yoshimura, 1999). In naïve RCS rats, spontaneous anti-retinal autoimmune responses were present at the beginning of retinal degeneration, but the level of autoantibodies and T cells changed over the course of retinal death with a distinctive activation trend (see Figure 1A–D). At the same time, RCS pups born to mothers with retinal degeneration that were breast fed two weeks postnatally already had anti-retinal antibodies (IgG). A strong initial response in anti-photoreceptor antibodies declined about P40, but later there was a rebound of the second wave of antibody production caused by an additional antigenic re-stimulation as more cells died. Maternal anti-retinal antibody could play a supportive role in protection from degeneration as well high titers of anti-retinal antibodies in pups could likely represent an early stage of the autoimmune process or the initiation of the retinal disease. We believe that AAbs could be involved in the clearance of outer segments debris in subretinal space by opsonization thus their activities were not detectable (Baumgarth et al., 2005). We suggest that those early AAb can have two functions – an induction of apoptosis of photoreceptors cells (pathogenic) or clearance retinal debris by opsonization.

In contrast, T cells were not measurable for the first 3 weeks in RCS rats, suggesting they might undergo immunological elimination after primary activation, and only those that survived could be detected in the later time. Interestingly, around that time there was an increased in the level of IgG2b antibodies, a subclass linked to the Th1 response. It is also possible that antigen-specific memory T cells re-encounter the same self-antigens and become rapidly activated, as was observed in mice with similar RPE genetic defect in *Mertk* as is present in RCS rats (D'cruz et al., 2000, Ye et al., 2011, Strick and Vollrath, 2010). Similarities in mouse and rat retinal phenotypes and perhaps in humans (Duncan et al., 2003) with the RPE *Mertk* mutation, and in their spontaneous immunological activation, suggests that the RPE phagocytic defect caused by the loss of function of Mer tyrosine kinase could predispose those animals to activation of autoimmune responses. Therefore, when the immune system encountered self-antigens released from apoptotic photoreceptors promotes anti-retinal immune responses that could further contribute to undergoing retinal degeneration.

The key finding of this study is that passive transfer of serum from dystrophic rats caused an immediate increase in the number of CD163⁺ blood-borne macrophages of round shape recruited into the retina. Not only multi-specific anti-retinal dystrophic serum but also monospecific pathogenic anti-recoverin and anti-enolase antibodies attracted activated CD163⁺ cells into the retina. We postulate that those macrophages, in turn, are the first step in the induction/augmentation of pathology of retinopathy, including the induction of apoptosis. For AAbs to get into the retina they have to pass the BRB that restricts the entry of circulating anti-retinal antibodies or T cells into the retina under normal conditions (Caspi, 2006). The vascular endothelial cells, which form part of the BRB, can present chemokines to circulating lymphocytes and monocytes leading to their recruitment (Harkness et al., 2003). MCP-1 (CCL2), which is a potent chemoattractant of monocytes has been implicated in inflammatory retinal disease (Adamus et al., 1997). The upregulation of MCP-1 and VEGF correlated with macrophage recruitment in our animals. Our earlier passive transfer studies demonstrated that anti-recoverin antibody penetrated into the retina from the circulation better in the presence of cytokines such as IL-2 or pertussis toxin (PTx), factors known to increase BRB permeability (Adamus, 2006). Destruction of the BRB causing upregulation of VEGF and leakage of anti-retinal antibodies and cytokines into the retina may be an important step in retinopathy in RCS rats. VEGF has also been also linked to the pathogenesis of blood-brain barrier (BBB) breakdown in neuromyelitis optica, a disease mediated by AAbs against aquaporin 4 (AQP4) (Argaw et al., 2009). Similar to our

findings, anti-AQP4 AAbs reduced the expression of tight junction proteins and disrupted the BBB through upregulation of VEGF (Shimizu et al., 2011).

In rodent and human retinas, death of rods leads to activation of microglia, which migrate to the outer retina and phagocytize photoreceptor debris (Moore and Thanos, 1996, Ng and Streilein, 2001, Gupta et al., 2003). In addition to phagocytosis of dying cells, activated microglia secrete molecules (cytokine, chemokine) that kill normal neurons around the area of primary degeneration, producing a 'bystander' effect (Gupta et al., 2003). Importantly, retinal microglial cells can express markers of antigen-presenting cells (Penfold et al., 1993). We postulate that anti-retinal AAbs attract activate microglia/macrophages that produce pro-inflammatory cytokines/chemokines, in effect exacerbate retinal dysfunction and apoptosis. Our passive transfer studies support this hypothesis.

In some retinopathies, AAbs are generated in response to a distant malignancy and such anti-tumor AAbs that cross-react with retinal antigens can initiate the disease process, including anti-recoverin antibodies (Adamus et al., 2004, Adamus, 2009, Morohoshi et al., 2009). On the other hand, if AAbs do not initiate pathogenic processes they could influence their progression. Retinal autoantigens are exposed and are possibly accessible to the host immune system in the early stages of disease, but also in the later stages as more cells die, and therefore they can stimulate the immune responses. As our studies showed, in the later stages of retinal degeneration, a rebound in the antibody production and activation memory T cells was observed in RCS rats possibly to due to the occurrence of new autoantigens (proteins and peptides) from dying cells (epitope spreading). We speculate that secondary AAbs production contributes to the steady progression of retinopathy. Altogether, our new findings on the migration and activation of mφ/MG after passive transfer of dystrophic serum or pathogenic Rec-1 and Enol-1 antibodies further support the hypothesis that autoimmunity contributes to the progression of retinopathy.

In contrast to our findings, earlier studies showed that phagocytic cells during the early stages of dystrophy in RCS rat retinas are derived from resident microglial cells, not from peripheral macrophages of ED2/CD163 phenotype (Roque et al., 1996). We showed a significant number of CD163⁺ cells in the recipient retinas recruited from periphery after passive transfer. We have also found CD74⁺ activated microglial cells in late retinas along with CD3⁺ T cells. CD74 is the invariant chain directing the trafficking of MHC class II molecules in antigen presenting cells (Matza et al., 2003). The presence of CD74 microglia may indicate a predominant immune cell participating in antigen processing and presentation in the retina. The finding of CD74⁺ microglia in the retina about the time of rebound of AAbs production and T cells activation corresponds to the previously published studies reporting the abundance of class II-positive cells between P28 and P42 in RCS rat retinas (Akaishi et al., 1998).

In summary, these studies provide new insight into a possible mechanism, by which autoantibodies generate and contribute to retinal degeneration. Anti-retinal autoimmune responses fluctuate but are detectable until photoreceptor cells die and self-antigens released from dying cells are available. It is likely that early anti-retinal AAbs could contribute to the progression of retinal degeneration by recruiting activated macrophages/microglia into the retina. In fact, passive transfer of dystrophic anti-retinal antibodies enhanced disease progression by disrupting the BRB, attracting blood macrophages into the retina, and augmenting apoptotic photoreceptor cell death. These findings directly link anti-retinal autoantibodies to activated macrophages entry and their possible role in neurodegeneration.

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Figure 1.

Effect of retinal degeneration on anti-retinal autoimmune responses. Evolution of autoantibody and T cell responses against interphotoreceptor retinal binding protein (IRBP) and arrestin (S-antigen) in naïve RCS dystrophic rats during retinal degeneration over 90 days. **A to D:** Generation of autoantibodies occurred early P15 during degenerative process in contrast the proliferative T cells responses were not measureable. Rebound of immune activities occurred around P40 at what time both autoantibodies and T cells were low. Representative ELISA measurements of autoantibodies against IRBP₁₁₇₇₋₁₁₉₁ peptide (**A**) and arrestin (**C**) in pooled sera collected on 15, 20, 30, 40, 60, and 90 days of age (n=3, serum dilution 1:100); **B** and **D:** T cell proliferative responses against IRBP₁₁₇₇₋₁₁₉₁ peptide and arrestin in naïve splenocytes of RCS rats on days 15, 20, 30, 40, 60 and measured by lymphocyte proliferation assay. The bars represent mean $\pm$ SEM (n=3 rats for each time point). SI value below SI=1 is considered to be negative. **E:** Representative micrographs of TUNEL positive apoptotic nuclei (green color) in the outer nuclear layer during retinal

degeneration; note decreased number of labeled nuclei at P60 and P90 (arrows point at TUNEL⁺ nuclei), **F**: Immunolabeling of normal rat retina with dystrophic RCS serum collected at P19 and P90 (1:100); note strong labeling of the outer segments of photoreceptor (red color). Nuclei (blue) were stained with DAPI. OS- outer segments, ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer

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Figure 2.

Presence of IgG and IgM isotype autoantibodies in sera of RCS dystrophic rats collected over the period of 90 days and measured by ELISA. **A:** IgG1, IgG2a, IgG2b, IgG2c isotype antibodies; **B:** IgM class antibodies; **C:** Specific anti-arrestin and **D:** anti-IRBP₁₁₇₇₋₁₁₉₁ peptide autoantibodies of IgG (γ -specific) and IgM (μ -specific) classes. Data represent measurements in pooled sera collected on 15, 20, 30, 40, 60, and 90 days of postnatal rat age ($n=3-5$), serum dilution 1:50 in all assays. Arrows point at time of separation of pups from their mothers (P21).

Figure 3.

Confocal micrographs showing the presence of CD74⁺ microglia and CD3⁺ T lymphocytes in degenerating retina. **A:** P40 retina and **(B)** P90 retina; Yellow arrows point at positively-labeled cells with anti-CD3 (red color) and anti-CD74 antibodies (green color). Pink arrow points at overlapped fluorescence, suggesting interaction of CD3 T cell with CD74 microglia (yellow color) in the debris zone; Nuclei (blue) were stained with DAPI. OS- outer segments, ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer

Figure 4.

Rapid increased of microglia/macrophages recipient retinas after passive transfer via intraperitoneal injection of 1 mg affinity-purified anti-recoverin Rec-1 or anti- α -enolase Enol-1 antibodies into the RCS. **A.** Representative cross-sections of retinas from eyes were collected 1 day post transfer – arrows point at microglia (green color, arrows) immunolabeled with ionized calcium binding adaptor molecule 1 (iba-1); ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer **B.** Bar graphs represent quantitative analyses of iba⁺ microglia cells compared to vehicle treated rats over 3 day period. The cells were counted in the entire retina collected daily by a blind observer. Bars represent mean $\pm$ SEM (n=3); Significant difference between paired groups **p<0.001. **C:** Representative micrographs showing TUNEL⁺ nuclei in ONL in vehicle, Rec1-and Enol-1 treated recipient retinas; Nuclei were stained with DAPI (blue) and TUNEL⁺ apoptotic nuclei are in yellow (arrows).

Figure 5.

Macrophage/microglia distribution in RCS retinas at seven days after passive transfer via intraperitoneal injection of anti-recoverin antibody Rec-1 (1 mg/ml), Enol-1 (1mg/ml), P90 serum (1 ml), or vehicle (1 ml saline) into P30 recipient RCS rats.

A–D: Retinal whole mounts immunolabeled with anti-CD163 (ED2) antibodies (green) and with isolectin B4 to visualize the vasculature (grey), yellow arrows point at round or amoeboid looking mφ/MG cells in whole mounts from Rec-1 (**A**), Enol-1 (**B**) P90 dystrophic serum (**C**), vehicle (**D**). **E–H:** Representative retinal cross-sections from treated eyes at 7 days post antibody transfer as above. The sections were immunolabeled with anti-iba-1 antibodies to identify mφ/MG (green). White arrows point at ramified shape microglia cells, yellow arrows point at activate rounded/amoeboid looking mφ/MG; nuclei labeled with DAPI (blue). **I and J:** Graph bars represent the averaged total number of mφ/MG counted in the cross-sections (**I**) and whole mounts (**J**) of retinas collected on day 7 post transfer. **K:** Immunofluorescent cells were counted in 9 squares of all 4 flat portions from each retina as shown in the scheme. The bars represent mean ± SEM (n=3); significant difference when compared with vehicle-treated group, **p<0.001.

Figure 6.

Increased expression of MCP-1 chemokine after passive transfer of antibodies in recipient retinas. Immunofluorescent labeling of RCS recipient retinas with anti-MCP-1 (1:60) antibodies 7 days after passive transfer of P90 serum, Rec-1 and Enol-1 antibody. Arrows indicate MCP-1 expression by cells and diffuse immunostaining across the inner plexiform layer; Note no labeling in vehicle-treated retinas. Ph - photoreceptors, ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer.

Figure 7.

Passive transfer of antibodies induces expression of VEGF in recipient RCS retinas. Representative fluorescent images of recipient RCS rat retinal cross-sections immunolabeled with anti-VEGF antibodies (1:1000) after passive transfer of vehicle, P90 dystrophic serum, Rec-1 and Enol-1 antibodies. The eyes were collected on day 7 after antibody transfer. Note no immunofluorescent labeling in vehicle-treated rat retina. Arrows point at immunolabeled cellular structure mostly around the vessels. Ph - photoreceptors, ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer antibody.