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A Disease-Causing Mutation in C1QTNF5 Shows Altered Affinity for
Complement Factor H and the Y402H Polymorphism, Which Is Associated With
Increased Risk of Age-Related Macular Degeneration
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Purpose: A late-onset retinal macular degeneration (L-ORMD), an autosomal dominant disease with features that are similar to age-related macular degeneration (AMD), is caused by a St6SR mutation in the C1QTNF5 gene product. The Y402H polymorphism in combination with the Cw*0701 allele (CH) is associated with increased risk of AMD. An interaction between CHF and C1QTNF5 was investigated, with the aim of identifying biochemical pathways involved in both L-ORMD and AMD.

Methods: C1QTNF5 was expressed in mammalian cells and purified using Ni-NTA affinity chromatography. Full length CHF was obtained commercially or purified from human plasma, whilst its short consensus repeat (SCR) modules 7-8 were produced using a Pichia pastoris expression system and purified by SP-Sepharose ion exchange chromatography. Interactions between C1QTNF5, CHF and SCR 7-8 were investigated using plate binding assays and surface plasmon resonance (SPR). MALDI-TOF analysis of C1QTNF5 was also carried out.

Results: Plate binding assays and SPR show an interaction between C1QTNF5 and CHF. CHF shows a higher affinity for mutant C1QTNF5 compared to wildtype, with the interaction kinetics suggesting a two-state binding model involving a conformational change. CHF modules SCR7-8 402V and 402H both interact with C1QTNF5, and show similar affinities for wildtype but altered affinities for mutant C1QTNF5. The SCR7-8 module interactions with C1QTNF5 suggest a 1:1 binding model. MALDI-TOF analysis of native C1QTNF5 showed that the wildtype protein has slightly greater mass (around 260Da). Analysis of trypsin digested C1QTNF5 also revealed differences between wildtype and mutant.

Conclusions: Full length CHF interacts with C1QTNF5 and shows a higher affinity for the 163K mutant. Mutant C1QTNF5 also shows higher affinity for SCR 7-8 402H. Differences between wild type and mutant C1QTNF5 shown by MALDI-TOF could be the result of altered post-translational modifications, which may be responsible for the altered CHF binding.

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201 - A431
Inflammatory Response and Involvement of Immune Components in a Laser
Induced CNV (Choroidal Neovascularization) Mouse Model
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Purpose: To investigate the nature and kinetics of the involvement of the immune response using a laser induced CNV mouse model.

Methods: C57/B6 and SCID or Rag-1 transgenic mice were used for analysis. A previously established laser induced CNV mouse model was used to generate CNV lesions. Three additive and complimentary cellular and immunological techniques were applied to quantitatively and qualitatively analyze the involvement of immune responses, including: 1) immunohistochemistry staining for confocal microscopic analysis for CNV lesions on either flat mount or vibrotome tissue preparations; 2) flow cytometry analysis for immune cell infiltration and 3) antibody-based depletion (for CD25 + Treg) or blocking strategies (for LFA-1) for a functional analysis.

Results: Flat-mount tissue preparation enables quantitatively and qualitatively measuring CNV lesions while vibrotome tissue preparation is best for visualizing the infiltration of inflammatory cells. Flow cytometry is suitable for qualitatively assessing and quantitatively assessing globally the infiltrating immune cells during CNV development. There is an early influx of neutrophils, macrophages, NK cells and microglia within 72 hours. Early CNV lesions (within 72hrs) are marked by edema and a random infiltration of immune cells, while lesions at a later stage (7 days) are characterized by a much reduced edema but very organized infiltrating immune cells along the region of laser disruption. Microglial cells are also among the earliest to arrive to the laser lesion and persist along with the development of CNV lesions. While no significant differences for CNV development were observed in comparing wild type mice to SCID, or Rag-1 or CD25- Treg cells-depleted mice, there was a significant increase of CNV lesion volume when an LFA-1 blocking antibody was administered.

Conclusions: We have successfully applied several strategies to qualitatively and quantitatively analyze the kinetic involvement of immune cells during a typical laser induced CNV. Our data revealed a complicated but very well orchestrated infiltration of immune components, mostly innate immune cells, during CNV. It appears that LFA-1 may play a beneficial role for the reversal of CNV. Our data suggest a pivotal role of immune response during CNV development.

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Production of Anti-CEP Autoantibodies in Immune Mediated Retinal
Degeneration
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Purpose: We have previously reported a novel murine model of immune mediated retinal degeneration induced by immunization with carboxyethylpyrrole (CEP)-modified albumin (CEP-MA). CEP albumin adducts are generated in the retinal photoreceptors and to oxidative stress and patients with age-related macular degeneration (AMD) have circulating CEP autoantibodies. The goal of this work is to test the role of anti-CEP antibody production in the generation of immune mediated retinal degeneration.

Methods: C57BL/6 were immunized in the footpad with CEP-MA or non-adducted MSA emulsified in complete Freund’s adjuvant (CFA) followed with a subcutaneous challenge at day 10 with incomplete Freund’s adjuvant-CEP-MA in the skin of the neck, followed by a second subcutaneous challenge two months later with CFA-MA. Serum was collected at different time points from CEP-MA immunized and control groups by ELISA. Specificity of anti-CEP autoantibody to retinal tissue was assessed by performing western blot analysis on retinal extracts and immunohistochemical staining using sera from immunized mice. Anti-CEP sera from immunized groups were also tested on human eye tissue from AMD and control individuals.

Results: Anti-CEP-MA autoantibody could be detected in immunized mice by day 30 after immunization and persisted for 12 months. A single band of approximately 40 kD was detected by western blot analysis of retinal extracts probed with sera from immunized mice. Immunohistochemical analysis demonstrates that the anti-CEP sera antibodies localized to scattered areas of the retinal pigment epithelium and photoreceptors. Moreover, anti-CEP serum binds to areas of drusen formation in the retina of human patients with AMD.

Conclusions: Immune mediated retinal degeneration correlates with the production of autoantibodies against CEP modified proteins. These antibodies recognize specific areas of the retina where the antigen is present and have the potential of mediating disease.

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HLA Cw*0701 Genotype and Natural Killer Cell Receptor AA Haplotype Are
Associated With Age-Related Macular Degeneration
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Purpose: The role of inflammatory events in Age related macular degeneration (AMD) is now well established. HLA genotypes have previously been associated with AMD. However, the role of different HLA class I molecules on NK cell function, which are expressed predominantly by natural killer (NK) cells. NK cells are involved in early responses against infected or transformed cells by production of cytokines and direct cytotoxicity. We hypothesised that certain combinations of HLA Cw and KIR genes may influence susceptibility to AMD. To test this we evaluated the association of HLA Cw and it’s cognate KIR (killer-cell immunoglobulin-like receptors (KIRs)) ligands which are expressed predominantly by natural killer (NK) cells. NK cells are involved in early responses against infected or transformed cells by production of cytokines and direct cytotoxicity. We hypothesised that certain combinations of HLA Cw and KIR genes may influence susceptibility to AMD. To test this we evaluated the association of HLA Cw and its cognate KIR (killer-cell immunoglobulin-like receptor) ligands with AMD.

Methods: HLA C principal allele groups were genotyped in a cohort of 104 AMD cases and 150 age-matched controls from Southampton using PCR-SSP method. This cohort was then genotyped for 16 KIR genes by PCR-SSP. Frequencies of the tested HLA / KIR alleles were then compared between AMD patients and normal controls. HLA C1, Cw*07, Cw*0701 genotypes and their combinations with KIR genotypes / haplotypes were tested for association with AMD. P values were obtained using 2-tailed chi-squared test and bonferroni corrections applied for multiple testing (Pc).

Results: HLA Cw*0701 allele in combination with the inhibitory KIR AA haplotype was seen to strongly associate with AMD status following logistic regression analysis (P=0.006, P=0.036, OR= 4.35, 95% CI=1.41-13.44).

Conclusions: HLA Cw*0701 and KIR haplotypes AA are associated with AMD. This genotype combination could play an important role in AMD. Natural killer cells may therefore have a role in the pathogenesis of AMD.

CR: S.V. Goverdhan, None; S.I. Khakoo, None; H. Gaston, None; H. Griffiths, None; A.J. Lotery, None.
In Old Mice, the Retinal Pigment Epithelium/Choroid Becomes Immunologically Active

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**Purpose:** Recent studies suggest a role for the immune system in age-related macular degeneration (AMD). Our microarray analyses of retinal pigment epithelium (RPE)/choroid from normal, old mice showed intense immunological activity in old animals. To further characterize age-related changes in immune and inflammatory activities of normal, old mice, we studied expression of immunological molecules in the normal, aged RPE/choroid.

**Methods:** Comparing young (4 mos) and old (26 mos) mice, we determined the expression levels of genes that participate in immunological and inflammatory pathways using real-time RT-PCR, the distribution of the proteins in RPE/choroid by immunofluorescence staining and the protein levels by Western blot.

**Results:** Quantitative real-time RT-PCR showed that the expression levels of selected genes from the upregulated pathways, e.g. leukocyte extravasation signaling (ICAM1, ITGB2), complement cascade (C1, C3), natural killer cell signaling (FCER1G, SH2B2, SYK), IL-10 signaling (CCR1, IL1RN), and B cell receptor signaling (B2D1A1, CD45), were significantly increased in the normal RPE/choroid of old animals compared to young animals. Immunofluorescence labeling showed the presence of key proteins in immunological pathways in the normal RPE/choroid of old animals. In young animals, there was little to no labeling. Western blots showed the levels of these proteins were increased in the normal RPE/choroid of old animals compared to young animals.

**Conclusions:** The present study demonstrates upregulation of gene products that participate in immune responses and inflammatory activity in the RPE/choroid in normal, old animals. These marked changes suggest that the aged RPE/choroid had become immunologically active and might contain the underlying cellular and molecular changes that permit the development of AMD.

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Investigation of the Role of Cd1d-restricted Invariant NKT Cells During the Formation of Experimental Choroidal Neovascularization


**Purpose:** Choroidal neovascularization (CNV) is directly related to visual loss in age-related macular degeneration and other macular disorders. Several studies suggested the involvement of inflammation to form CNV. Natural Killer T (NKT) cells belong to a specialized population of lymphocytes that coexpress the T cell receptor and NK marker. In this study, we elucidated the role of NKT cells, which restricted CD1d molecule and participate in the innate immune response, in laser-induced experimental CNV.

**Methods:** We used female 8-10 weeks C57BL/6 mice, and two independent NKT cell-deficient mice; CD1d knockout (K0) mice and Jα18 KO mice. Experimental CNV was induced with rupturing in Bruch’s membrane by laser photocoagulation (PC). Seven days after PC, the eyes were enucleated and the areas of CNV were measured in the choroidal flat mounts.

**Results:** We detected the increase of NKT cell-related genes (Val14 and CXCL16) in the whole CNV-induced eye by quantitative real-time PCR, which indicated local accumulation of NKT cells. Both CD1d KO mice and Jα18 KO mice showed significant reduction of experimental CNV and decrease of VEGF in the concentration of ocular fluid at 24h after PC. In vitro co-culture of CNV-positive retinal pigment epithelial cells and purified splenic T cells, which partially resembled the interaction of resident ocular cells and NKT cells, showed the decrease of VEGF in the cultured supernatant in either NKT cell-deficiency or inhibiting NKT cell activation by the treatment of anti-CD1d antibody.

**Conclusions:** NKT cells play an important role to form CNV as one of the inducer of VEGF.

CR: K. Sonoda, None; K. Hijikja, None; C. Tsutsuami-Miyahara, None; T. Iwashita, None.

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Immunoglobulin Biosynthesis by Human Retinal Pigment Epithelium (RPE)

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Purpose: To characterize immunoglobulins (Igs) produced by human RPE and to determine the effect of age on Ig gene synthesis.

Methods: Human donor eyes (25-72 years old) were obtained from the eye bank within 24 hours of death. RPE proteins were extracted and focused using non-linear (pH 3.0-10) 7.7cm IEF strips. Proteins were further separated in the second dimension on 4-12% Bis-Tris gels. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to identify Ig components. Results were confirmed with Western blotting and RT-PCR. Presence of T-(CD3), B-lymphocytes (CD20), B-cell lambda/lambda (CD19) and IgM were also investigated with Western blotting, RT-PCR and immunohistochemistry to ascertain RPE as the source of Igs. The effect of age on Ig production in vivo was determined by plotting cytotoxic RPE-Ig against the donor's age. LPS (100 agon/ml) was used to induce Ig production of cultured human RPE and ARPE-19 cells in vitro.

Results: Human RPE proteome reveals several components of Igs including variable regions of heavy and light chains. RT-PCR and Western blot showed that Ig is the main Ig type within RPE cytosol. Absence of other lymphocyte markers (CD5 and CD20) indicated the source of Igs as RPE itself. Ig gene synthesis increases by age (r=0.73, p<0.05). Human RPE and ARPE-19 cells continue to produce Ig in vitro. Addition of LPS increased RPE-Ig production 2.3±0.4 times.

Conclusions: Human RPE produces Igs. These Igs may play a role in initiating or augmenting the cellular damage in several outer retinal pathologies including age-related macular degeneration.

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Generation and Characterization of Chimeric Complement Factor H Transgenic Mice With an “At-Risk” Mutation for Age-Related Macular Degeneration

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Purpose: Complement factor H (Cfh) is a key regulator of the alternative complement pathway. A single nucleotide polymorphism that leads to the absence of a tyrosine at amino acid position 402 of Cfh (Y402H) increases the risk for age-related macular degeneration (AMD) up to 7-fold. Our goal is to generate transgenic mice expressing either the tyrosine or the histidine variants of Cfh in order to: (a) identify the molecular basis for the susceptibility to AMD observed in human Cfh carriers, and (b) generate a relevant model of AMD that would allow the dissection of the different arms of the immune system in the disease.

Methods: Cfh consists of 20 short consensus repeats (SCR). Y402H localizes to SCR7. We have generated two transgenic constructs which consist of the human SCR6-8 sequence flanked by the mouse SCR1-5 and SCR9-20 sequences. One of the constructs codes for a tyrosine (Y-Cfh) at amino acid position 402 of Cfh (Y402H) increases the risk for age-related macular degeneration. We then used a western blot to determine the level of Cfh protein expression in the serum. The degeneration is complicated by anterior segment and vitreous inflammation, cytokoid macular edema (CME), retinal neovascularization, retinal detachment, and phthisis. Although AMD is a degenerative disease, we hypothesized that inflammation may play a primary role in AMD pathogenesis.

Methods: Two transgenic mice were examined with panels of standard histopathological and immunohistochemical markers (CD3, CD4, CD8, CD19, CD20, CD15, CD68, CD36, CD34, CD3, GFAP and s100B). Clinical phenotypes were classified with optical coherence tomography (OCT). A review of patients who had CME due to ADNIV and were treated with local immunosuppression was conducted. Then, using a phenotype-guided bioinformatics approach, candidate genes were identified within the ADNIV interval on chromosome 11q31.

Results: Immunohistopathology revealed a T-cell infiltrate in ADNIV eyes. OCT demonstrated early vision loss due to severe macular edema with leakage on fluorescein angiography. The CME and vision improved after either subtenon’s triamcinolone or flucinolone implants in ADNIV patients. Gene ontology terms corresponding to this clinical response and immuno-histopathology studies were identified and queried against over 100 candidate genes, and ranked candidates emerged for directed mutational analysis.

Conclusions: Ablation immune-cell mediated processes may underlie ADNIV. Clinical symptoms and vision loss can be mitigated by local corticosteroid therapy. Identification of the genetic cause of ADNIV will provide critical insight into the degenerative and inflammatory mechanisms of this disease.

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