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Ocular  
Inflammation  
**Animal Model**  
Workshop



ANIMAL MODELS OF OCULAR  
INFLAMMATION,  
NEO-VASCULARIZATION,  
AMD, DIABETES,  
INTRAOCULAR LYMPHOMA,  
GLAUCOMA AND OPTIC NEURITIS

RELEVANCE TO HUMAN  
OCULAR DISEASES

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# Animal Models of Ocular Inflammation, Neo-vascularization, AMD, Diabetes, Intraocular Lymphoma, Glaucoma and Optic Neuritis Relevance to Human Ocular Diseases

PROGRAM

Organizers: Bahram Bodaghi MD, PhD (France) and Phuc LeHoang MD, PhD (France)  
Scientific Committee: Bahram Bodaghi MD (France), PhD; Yvonne de Kozak MD, PhD (France), Narsing Rao MD (USA)

**Workshop Venue : Hotel Le Meridien Etoile**  
81, boulevard Gouvion Saint-Cyr - 75017 Paris - France

**Saturday, September 15**

**1 pm: Introduction** ..... **Bahram Bodaghi**

**1:05 – 3 pm: AMD**

**Moderators: Marc de Smet (Belgium) and Shigeaki Ohno (Japan)**

- Age-related-blindness in mice lacking av $\beta$ 5 integrin.....	Emeline Nandrot (USA)	p3
- Chemokines and AMD.....	Christophe Combadière (France)	p4
- AMD secondary to a CX3CR1-dependent subretinal microglia accumulation.....	Florian Sennlaub (France)	p4
- Cx3cr1/Ccl2 deficient mice: an animal model for AMD.....	Chi Chao Chan (USA)	p5

**3 - 3:30 pm: Discussion**

**Moderators: David BenEzra (Israel) and Yvonne de Kozak**, Marc Abitbol (France), Rachel Caspi (USA), Andrew Dick (UK), Marc de Smet (Belgium), Dale Gregerson (USA), Justine Smith (USA)

**4 – 6 pm: Uveitis I**

**Moderators: Chi Chao Chan (USA) and Andrew Dick (UK)**

- Tyrosinase-related proteins and VKH.....	Shigeaki Ohno (Japan)	p6
- Melanin-induced uveitis.....	Justine Smith (USA)	p7
- Rat models of autoimmune uveitis.....	Gerhild Wildner (Germany)	p7
- Ocular and systemic bio-distribution of rhodamin-conjugated liposomes injected into the vitreous: therapeutic potential of liposomes to deliver VIP during ocular inflammation.....	Serge Camelo and Yvonne de Kozak (France)	p8

**6:30 pm: Buffet Cocktail and Dinner**

**Sunday, September 16**

**8 – 10 am: Uveitis II**

**Moderators: John Forrester (UK) and Dale Gregerson (USA)**

- Uveitis in mice directed at retinal neoantigens.....	Céline Terrada, Benoit Salomon, Phuc LeHoang, Bahram Bodaghi	p9
- Horse model of uveitis.....	Cornelia Deeg (Germany)	p10
- Transgenic models of autoimmune uveitis.....	Dale Gregerson (USA)	p12
- Mitochondrial Oxidative Stress model.....	Narsing Rao (USA)	p12

**10:15 – 12:45 am: Uveitis III**

**Moderators: Rachel Caspi (USA) and Robert B Nussenblatt (USA)**

- Model of acute anterior uveitis.....	Jim Rosenbaum (USA)	p13
- Mouse models of autoimmune uveitis.....	Rachel Caspi (USA)	p14
- Uveitis and retinopathy in HLA-A29 transgenic mice.....	Yvonne de Kozak (France)	p15
- Primate model of uveitis.....	Phuc LeHoang, Margaret Sterkers (France)	p16
- Anterior uveitis accompanies joint disease in a murine model of proteoglycan induced arthritis.....	Holly Rosenzweig (USA)	p16
- "Spontaneous autoimmune ocular inflammation" induced by TLR ligands.....	Igal Gery (USA)	p17

**1:00 pm: Buffet Lunch**

**2:15 – 3:15 pm: Relevance of animal models to human uveitis-Discussion**

**Moderators: Narsing Rao (USA) and Manabu Mochizuki (Japan)**

Panel members: Andrew Dick (UK), John Forrester (UK), Robert B Nussenblatt (USA), Jim Rosenbaum (USA), Grace Shen (USA), Stephan Thurau (Germany)

**3:30 – 6 pm: Diabetes, Ocular lymphoma, Glaucoma and Optic neuritis**

**Moderators: Bahram Bodaghi and Uwe Pleyer (Germany)**

- PIGF-1 and epithelial hemato-retinal barrier breakdown: Potential implication in the pathogenesis of diabetic retinopathy.....	Francine Behar Cohen (France) Jean-Claude Jeanny (France) David Ben Ezra (Israel)	p18
- Murine model of ocular B-cell lymphoma.....	Valérie Toutou and Sylvain Fisson (France)	p19
- Immunopathogenesis of corneal graft rejection.....	Uwe Pleyer (Germany)	p19
- Glaucoma.....	Jun Song Mo (USA)	p20
- Optic neuritis.....	John Guy (USA)	p21

**Conclusion** ..... Phuc LeHoang

PROGRAM

## Age-related blindness in mice lacking $\alpha v\beta 5$ integrin

EMELINE NANDROT

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Daily phagocytosis of spent photoreceptor outer segment fragments by the retinal pigment epithelium (RPE) is critical for vision. In the retina, early morning circadian photoreceptor rod shedding precedes synchronized uptake of shed photoreceptor particles by RPE cells. *In vitro*, RPE cells employ the integrin receptor  $\alpha v\beta 5$  for particle binding. We analyzed RPE phagocytosis and retinal function in  $\alpha v\beta 5$  integrin-deficient mice, which specifically lack  $\alpha v\beta 5$  receptors.  $\alpha v\beta 5^{-/-}$  RPE cells in culture fail to take up isolated photoreceptor particles.  $\alpha v\beta 5^{-/-}$  RPE cells *in vivo* retain basal uptake levels but lack the burst of phagocytic activity that follows circadian photoreceptor shedding in wild-type.

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# CX3CR1-dependent subretinal microglia cell accumulation leads to cardinal features of age-related macular degeneration

CHRISTOPHE COMBADIÈRE, FLORIAN SENNLAUB

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Christophe Combadiere<sup>1,2,3\*</sup>, Charles Feumi<sup>4,5,6\*</sup>, William Raoul<sup>4,5,6\*</sup>, Nicole Keller<sup>4,5,6</sup>, Mathieu Rodéro<sup>1,2</sup>, Adeline Pezard<sup>1</sup>, Sophie Lavalette<sup>4,5,6</sup>, Marianne Houssier<sup>4,5,6</sup>, Laurent Jonet<sup>4,5,6</sup>, Emilie Picard<sup>4,5,6</sup>, Patrice Debré<sup>1,2,3</sup>, Mirna Sirinyan<sup>7</sup>, Philippe Deterre<sup>1,2</sup>, Tania Ferroukhi<sup>1</sup>, Salomon-Yves Cohen<sup>8</sup>, Dominique Chauvaud<sup>9</sup>, Jean-Claude Jeanny<sup>4,5,6</sup>, Sylvain Chemtob<sup>7</sup>, Francine Behar-Cohen<sup>4,5,6,9</sup> and Florian Sennlaub<sup>4,5,6</sup>

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The role of retinal microglial cells (MCs) in age-related macular degeneration (AMD) is unclear. Here we demonstrated that all retinal MCs express CX3C chemokine receptor 1 (CX3CR1) and that homozygosity for the CX3CR1 M280 allele, which is associated with impaired cell migration, increases the risk of AMD. In humans with AMD, MCs accumulated in the subretinal space at sites of retinal degeneration and choroidal neovascularization (CNV). In CX3CR1-deficient mice, MCs accumulated subretinally with age and albino background and after laser impact preceding retinal degeneration. Raising the albino mice in the dark prevented both events. The appearance of lipid-bloated subretinal MCs was drusen-like on funduscopy of senescent mice, and CX3CR1-dependent MC accumulation was associated with an exacerbation of experimental CNV. These results show that CX3CR1-dependent accumulation of subretinal MCs evokes cardinal features of AMD. These findings reveal what we believe to be a novel pathogenic process with important implications for the development of new therapies for AMD.

# Ccl2/Cx3cr1 deficient mice: an animal model for age-related macular degeneration

CHI-CHAO CHAN, M.D.

National Eye Institute, National Institutes of Health, USA

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**Background/Aims:** Senescent *Ccl2*<sup>-/-</sup> mice develop cardinal features of human age-related macular degeneration (AMD). Loss-of-function single nucleotide polymorphisms within *CX3CR1* are associated with AMD.

**Methods:** We generated *Ccl2*<sup>-/-</sup>/*Cx3cr1*<sup>-/-</sup> (DKO) mice and evaluated the eyes using routine histology, immunochemistry, biochemistry and proteomics.

**Results:** At 6-weeks old, all DKO mice developed AMD-like retinal lesions such as abnormal RPE cells, drusen, photoreceptor atrophy and choroidal neovascularization, which progressed with age and reversed with high omega-3 LCPUFA diet. A2E, a major lipofuscin fluorophore, illustrated by an emission peak at ~ 600nm, was significantly higher in DKO RPE. Decreased ERp29 was found in the retina of DKO mice.

**Conclusion:** A broad spectrum of AMD pathologies with early onset and high penetrance in these mice implicate certain chemokines, A2E, and endoplasmic reticulum proteins in AMD pathogenesis.

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## Contributors:

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## Animal models of VKH/SO disease

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### Introduction:

Vogt-Koyanagi-Harada(VKH) disease is an autoimmune disease against melanocytes. Many attempts have been done to make animal models for VKH disease using the uveal extracts. In spite of many works, most of them were not well reliable models. In melanocytes, there are many kinds of antigenic materials. Among these antigens, tissue differentiation antigens of melanocytes are melanin pigment associated proteins. However melanin pigments are highly insoluble particles and difficult to use as immunizing antigens and hard to process. For these reasons, it was difficult to establish a good VKH disease models (autoimmune disease against pigment cells). We used the peptides derived from melanocytes specific tyrosinase family proteins as immunogens and successfully established VKH disease models in various kinds of animals.

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### Materials and Methods:

Materials: For experimental animals, the pigmented rats of F2 (Lewis X F1 of Lewis and Brown Norway (BN)), Akita dogs, and rhesus monkeys were used. For antigens, the peptides derived from tyrosinase, tyrosinase related protein 1 (TRP1) and/or tyrosinase related protein 2 (TRP2) were used.

Methods: Mixture of peptides and complete Freund adjuvant was injected into hind foot pads with intravenous injection of inactivated Bordetella pertussis.

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### Results:

All of the Lewis rats developed experimental uveitis (EAU). F1 rats of Lewis X BN rat did not developed EAU. Fifty percent of F2 rats of Lewis X F1 developed EAU, highly resembling human VKH disease, by immunization of the peptides derived from tyrosinase, TRP1 and TRP2. Akita dogs also developed experimental dog VKH (EDVKH) by immunization with peptides derived from TRP1. Rhesus monkeys also developed experimental uveitis by immunization of TRP1.

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### Conclusions:

We have successfully developed EAU highly resembling to VKH disease and/or dog VKH disease. This model may serve as human VKH disease models.

## **Experimental Melanin-Protein-Induced Uveitis: an experimental model of human acute anterior uveitis**

JUSTINE R. SMITH,<sup>1</sup> JAMES T. ROSENBAUM,<sup>1</sup> KERYN A. WILLIAMS<sup>2+</sup>

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Experimental melanin-protein-induced uveitis (EMIU), which is also known as experimental autoimmune anterior uveitis (EAAU), was first described in 1993 by Broekhuysse and colleagues. This experimental uveitis may be induced in certain inbred and outbred rat strains by immunization with bovine ocular melanin. The inflammation has many clinical features in common with human acute anterior uveitis. Duration of the first episode is approximately one month.

Spontaneous recovery to a near normal clinical state is the rule, but multiple recurrences are common. Slit-lamp biomicroscopic examination reveals a florid anterior chamber reaction, with formation of a “hypopyon”, fibrin clots and posterior synechiae. At a microscopic level, leukocytic infiltration is first observed in the anterior uvea. Although this tissue remains the site of maximum inflammation throughout an attack, in severe cases limbitis, vitritis and choroiditis are also observed.

Abrogation of EMIU occurs after treatment with anti-CD4 antibody, indicating that the uveitis is controlled by CD4-positive T cells. Several research groups, as well as our own, have used EMIU to investigate various aspects of the pathogenesis of acute anterior uveal inflammation, including the participation of different leukocyte subsets; the expression of cell adhesion molecules, cytokines, chemokines and nitric oxide; the role of complement; and the impact of apoptosis. In addition, EMIU has also been used to evaluate various biologic interventions with potential implications for the treatment of human disease.

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## **Rat models of autoimmune uveitis**

GERHILD WILDNER, MARIA DIEDRICHS-MÖHRING, STEPHAN R. THURAU

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The major retinal autoantigens defined by their ability to induce experimental uveitis Lewis rats are retinal soluble antigen (S-Antigen, S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), both expressed by photoreceptor cells. The immunogenic and pathogenic epitopes are well characterized.

We postulate that the autoimmune response leading to uveitis in humans has to be initiated outside the eye, for the eye as an immune privileged organ can only be entered by already activated T cells. Considering the sequestered expression of retinal autoantigens the extraocular antigens eliciting the immune response must mimic the retinal epitopes. After invading the eye, crossreactive T cells will subsequently get re-activated by local retinal autoantigen presentation and induce uveitis by recruiting inflammatory cells.

We have described two environmental peptides mimicking a highly pathogenic epitope from retinal S-Antigen: one from rotavirus, a common pathogen causing gastroenteritis, the other from bovine milk as2casein, a frequent nutritional protein. In Lewis rats we can induce T cells crossreactive with mimotopes and S-Ag peptide as well as EAU by s.c. immunization with mimicry peptides and even casein protein. Moreover, also oral immunization with casein protein and native cholera toxin as adjuvant leads to the development of uveitis, imitating the natural immunity to gastrointestinal antigens. In contrast to retinal autoantigens, these mimicry antigens fail to induce oral tolerance for protection from uveitis. Patients with uveitis show enhanced and more frequent humoral and cellular immune responses to these antigens compared to healthy individuals, suggesting a potential role of these mimotopes in human disease.

We could furthermore describe a mimotope of retinal S-Ag peptide derived from the sequence of HLA-class I B antigens (HLA-B27, B51 and many others). T cells escaping thymic selection despite recognizing an equivalent of peptide B27PD might have a potential to become autoaggressive lymphocytes that cause uveitis by crossreacting with retinal autoantigen peptide. On the other hand, those T cells as well could become Tregs with crossreactivity to retinal autoantigen.

The fact that in rats the HLA peptide B27PD was at least as effective in inducing oral tolerance but much less uveitogenic than the retinal autoantigen itself rendered it attractive as a potential oral tolerogen for patients with autoimmune uveitis. A first therapeutic trial with uveitis patients and very promising results was conducted, which will be followed by a multicenter phase II trial soon.

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## Ocular and systemic bio-distribution of rhodamin-conjugated liposomes injected into the vitreous: therapeutic potential of liposomes to deliver VIP during ocular inflammation.

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**Purpose:** Treatment of ocular inflammation requires large quantities of therapeutic molecules to be injected systematically to penetrate the ocular tissues. However, systemic injections induce severe systemic side effects in patients. Local delivery of therapeutic molecules encapsulated within liposomes is a promising method to treat ocular diseases while limiting the systemic side effects. Moreover, liposome encapsulation increase the time of action of therapeutic molecules and avoid complications due to repeated intraocular injections. The purpose of the present study was to define the biodistribution of free liposomes loaded with VIP and of cells internalizing liposomes following their intravitreal (IVT) injection.

**Methods:** Twenty-four hours after IVT injection of Rhodamin-conjugated liposomes (Rh-Lip) into the eye of 7-8 week-old Lewis male rats, eyes, lymph node (LN) and spleen were collected. The phenotype and distribution of cells internalizing Rh-Lip in ocular tissues and lymphoid organs was determined by immuno-fluorescence and confocal microscopy. Interactions with T cells were assessed in

the cervical LN and the spleen. The effect of IVT injection of VIP-loaded Rh-Lip on local and systemic immune response during endotoxin induced uveitis (EIU) and experimental autoimmune uveitis (EAU) was assessed.

**Results:** Following IVT injections, Rh-Lip were detected mainly in the posterior segment of the eye (vitreous, retina) and in conjunctiva and episclera. In the retina, Rh-Lip were internalized by Muller cells, but not microglia. Following IVT injection Rh-Lip were found almost exclusively internalized by resident macrophages in the cervical LN, and very few in the spleen. Rh-Lip found in the secondary lymphoid organs were adjacent to T lymphocytes. Intraocular injection of VIP-Rh-Lip reduced ocular inflammation and systemic immune response during EIU and EAU.

**Conclusion:** Our data show that a local (IVT) injection of liposomes containing an immuno-regulatory molecule has systemic effects on the immune response. Whereas large amounts of IVT injected-Rh-Lip were internalized by subcapsular macrophages in the cervical lymph nodes up to 14 days following injection, only very limited amounts of liposomes reached the marginal zone of the spleen. This suggests that IVT injected liposomes loaded with VIP potentially modulate the loco regional immune microenvironment for long period of time.

## Uveitis in mice directed at a retinal neoantigen

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**Purpose:** We have described a murine model of experimental uveoretinitis. Regulatory T cells play a role in this model. In this study, we have compared the therapeutic effects of polyclonal versus antigen specific CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells after intravenous or intravitreal injection.

**Methods:** In mice expressing hemagglutinin (HA) in the retina after subretinal injection of rAAV, uveitis was induced by intravenous administration of  $2 \times 10^6$  activated HA-specific T cells. These cells were obtained from purified Thy-1.1 TCR-HA CD25<sup>-</sup> cells and stimulated by irradiated BALB/c splenocytes and HA peptide for 4 days. At the same time or 4 days later, HA-specific or polyclonal BALB/c CD4<sup>+</sup>CD25<sup>+</sup> T cells were injected intravenously or intravitreally. A challenge was performed by intravenous activated HA-specific effector T cells, 21 days after induction of uveitis. Intraocular inflammation was clinically and histologically studied in all animals.

**Results:** CD4<sup>+</sup>CD25<sup>+</sup> T cells controlled uveitis only if they were specific for the target antigen (HA). Compared to intravenous injection, the effect observed after intravitreal injection was obtained with low number of cells. Furthermore, protection against a challenge was achieved only after local administration of HA-specific regulatory T cells.

**Conclusions:** Regulation of experimental uveoretinitis may be obtained by using CD4+CD25+ T-cells. Specificity and activation status of these cells should be further analyzed in order to develop new in situ therapeutic strategies.

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## The horse model of uveitis

CORNELIA A. DEEG

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Equine recurrent uveitis (ERU) is an autoimmune disease with high prevalence (10%) in horses and the only spontaneous model for human autoimmune uveitis leading to blindness. (de Smet and Chan, 2001; Deeg et al., 2001). Lewis rat models helped to make progress in understanding etiology and pathogenesis of uveitis, since they enabled testing uveitogenicity of a considerable amount of proteins or peptides. However, uveitis in the Lewis rat is mainly monophasic and has therefore severe limitations for investigating the disease-characteristic recurrences. Since uveitis has to be induced by an artificial setting in these animals, insights into disease elicitors are almost impossible. Although equine recurrent uveitis also has disadvantages as a model, it also has major benefits in our opinion. As a spontaneously occurring disease with high prevalence it allows studies concerning the initiating events for recurrent uveitis. ERU shares many similarities with human autoimmune uveitis, including clinical characteristics such as the remitting-relapsing onset of disease. Further, large animal models are convenient for long-term studies due to their long life-span and enable access to repeated blood or cell samples for studying disease kinetics.

We could demonstrate in the equine model that epitope spreading also plays a role for autoimmune uveitis, since there were only very preliminary data published from human uveitis patients before (de Smet et al., 2001). The findings of epitope spreading confirm the relevance of the full detection and characterization of all participatory uveitis autoantigens. Therefore, we analyzed the immune response of the spontaneous diseased animals and identified two novel, potential autoantigens with 2DE Western blots using the retinal proteome as autoantigenic source. Characterization of cellular retinaldehyde-binding protein (CRALBP) revealed that this autoantigen meets Witebsky's postulates, and therefore proved to be a novel autoantigen in ERU, not described before (Deeg et al., 2006). We hypothesise a similar underlying pathogenesis for both, human and equine recurrent uveitis, underscoring the importance of the spontaneous horse model for human autoimmune uveitis. Therefore, we tested the immune response of human uveitis patients to the novel uveitis autoantigen CRALBP. Frequency of autoantibodies was 53% in tested uveitis patients compared to 17% of healthy negative controls.

Currently, we use proteome analysis as a comprehensive approach to disease pathogenesis and etiology, whereby protein expression patterns reflect the complexity of molecular processes and are potentially able to provide the molecular basis for specific diagnosis and understanding of disease pathogenesis (Jungblut et al., 1999). Since disease mechanisms are highly complex and involve many different proteins, a high resolution approach is therefore useful for detecting all molecules participating in the inflammatory process (Thiel and Thiesen, 2005). Our studies focussing on the target tissue proteomes of uveitis revealed novel candidates involved in disease pathogenesis (Deeg et al., 2007a; Hauck et al., 2007) and additionally unravelled unchanged expression of autoantigens despite complete destruction of their normal expression sites (Deeg et al., 2007b).

Studying the spontaneous uveitis model in horses has led to further knowledge of the events accompanying disease initiation and progression.

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## Peripheral induction of regulatory T cells specific for beta-galactosidase expressed in retinal photoreceptor cells

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Immunologic self-tolerance is provided by mechanisms of central and peripheral tolerance. Expression of tissue-specific antigens (Ags) in the thymus leads to both negative selection of T cells with specificity for self-Ags, and to the generation of Ag-specific regulatory T cells (Tregs) that bear the CD4<sup>+</sup>25<sup>+</sup>foxp3<sup>+</sup> phenotype. Experimental autoimmune uveoretinitis (EAU) is a retinal autoimmune disease mediated by T cells directed to retinal Ags including rhodopsin, arrestin, recoverin and phosducin. Using beta-galactosidase (b-gal) transgenic mice to achieve transgene expression in retinal photoreceptor cells, we previously found that b-gal expression based on the activity of the retina-specific arrestin promoter (hi-arr-b-gal mice) led to spontaneous immunoregulation that altered the immune response to b-gal elicited by immunization with b-gal in CFA.

Analysis of the hi-arr-b-gal Tg mice that express b-gal in the retina has not revealed detectable levels of b-gal in thymus, but very low levels in thymus could generation of Tregs. Since Tregs can be induced by extrathymic Ag exposure, the goal of this study was to determine whether or not Ag expression in the retina contributes to the generation of Tregs, regardless of thymic expression and its possible contribution. Using T cell receptor transgenic mice (3E9 mice) producing CD4 T cells specific for an epitope of B-gal, and Rag1-deficient mice, several strategies were used to determine if B-gal expressed in the retina led to spontaneous, thymus-independent Treg development. The results show that the intracellular expression of Ag in photoreceptor cells, and in the absence of exogenous Ag administration, leads to the generation of Tregs that exhibit phenotypic and functional properties similar to those of thymus-derived Tregs.

## Mitochondrial Oxidative Stress model

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Blood-borne, activated macrophages are generally believed to be the major effectors of tissue damage observed during uveitis. The infiltrating macrophages are known to damage the retina by releasing cytokines, proteolytic enzymes, and free radicals, including the highly cytotoxic peroxynitrite. We have recently shown that peroxynitrite-mediated nitration of retinal mitochondrial proteins; including cytochrome c occurred in early Experimental Autoimmune Uveitis (EAU) before the macrophages infiltrate the retina. These observations suggest that mitochondrial oxidative stress could be the initial pathologic event leading to retinal damage.

In early EAU retina, a several fold upregulation of TNF $\alpha$ , IL-1, IL-12, IL-15, IL-17, and IL-18 is detected by real time PCR. Such a response is seen in the presence of few infiltrating CD3- and IL-17-positive cells in the retina. These findings indicate that a few infiltrating auto reactive T cells in organ-specific autoimmunity control the subsequent recruitment of nonspecific T cells and other inflammatory cells, resulting in the amplified inflammatory insult. Although various retinal neuronal cells, endothelial cells, and retinal pigment epithelial cells can generate oxidants in EAU, only mitochondrial protein nitration was found in the early phase, and this was found to be exclusively localized in the photoreceptor inner segments. Such findings indicate that photoreceptors are the primary target in the peroxynitrite mediated mitochondrial oxidative stress in early EAU.

Select Th1 and Th17 cytokines, particularly TNF- $\alpha$ , are known to upregulate iNOS. Such upregulation of iNOS causes mitochondrial oxidative stress resulting in nitration of photoreceptor mitochondrial protein, cyto c. The nitrated cyto c is released into the cytosol leading to apoptosis of photoreceptors. Release of cyto c from mitochondria signifies an early event in apoptosis. In contrast iNOS KO mice with early EAU do not show the mitochondrial oxidative stress and photoreceptor apoptosis.

The fact that nitration of photoreceptor mitochondria commences long before the arrival of macrophages does not support the current belief that retinal tissue damage in uveitis is initiated only by the infiltrating cells. Moreover, the early EAU offers a unique model in addressing photoreceptor mitochondrial oxidative stress and subsequent apoptosis of these mitochondria rich retinal cells.

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## Uveitis secondary to bacterial products

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Bacteria are suspected contributors to several forms of immune-mediated, non-infectious forms of uveitis including that associated with ankylosing spondylitis, sarcoidosis, Behcet's disease, and inflammatory bowel disease. Endotoxin-induced uveitis has been a widely used model for more than two decades. Both rats and mice develop a transient, bilateral anterior uveitis after a systemic injection of endotoxin. Inflammation posterior to the lens is generally milder than anterior segment inflammation. The uveitis is more severe if the LPS is injected intra-ocularly. The model has been invaluable in helping to identify mediators induced in the inflamed eye and in testing pharmacologic approaches to reduce eye inflammation.

Muramyl dipeptide (MDP) is another bacterial cell component that can induce uveitis in laboratory animals. MDP is especially intriguing as a cause of uveitis because it activates the intracellular protein, Nod2/Nod2OD2, and mutations in the NOD2 gene are the cause of the autosomal dominant form of uveitis, that is one manifestation ofis characteristic of Blau Syndrome. Since a mutation in a gene that codes for a protein which senses a bacterial product consistently results in uveitis, it is critical to understand more fully the mechanisms by which bacterial products cause uveitis in laboratory animals.

## Mouse models of uveitis

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Experimental autoimmune uveitis in mice (EAU), induced by immunization with retinal Ag in complete Freund's adjuvant, has served as a useful model for studying basic mechanisms and therapeutic approaches applicable to human ocular autoimmune disease. Human uveitis patients who have immunological responses to retina usually recognize retinal arrestin (S-Ag). The reason for this preference is not known, but a similar preference, though to a different retinal Ag, is seen in mice. In spontaneous EAU-like uveitis in AIRE deficient mice and in athymic nude mice implanted with a neonatal rat thymus, the Ag recognized is interphotoreceptor retinoid binding protein (IRBP). IRBP is also the Ag of choice for induction of EAU in mice by immunization, whereas S-Ag elicits little or no disease. However, in HLA DR3 transgenic mice S-Ag is highly uveitogenic and peptide N, a promiscuous epitope recognized by patients, is a dominant uveitogenic epitope. This further validates the mouse EAU model as a relevant model for human uveitis.

Immunological mechanisms that drive uveitis are being studied in the EAU model. Inflammatory effector T cells can belong to one of three known lineages, Th1, Th2 and Th17. The IRBP/CFA induced EAU model, previously thought to be Th1 driven, was recently demonstrated to be very much Th17 dependent: neutralization of IFN- $\gamma$  (but not IFN- $\alpha$ ) prevented and reversed disease in the CFA induced model. However, if the disease is not induced in the context of CFA, IL-17 is not dominant and appears to be superfluous or insufficient. One example is EAU induced by adoptive transfer of a Th1 cell line, where neutralization of host IL-17 fails to inhibit disease. Another example is a new model of EAU induced by injection of dendritic cells that have been *in vitro* matured with anti-CD40 and LPS and pulsed with IRBP peptide. DC-EAU is a new and distinct model of EAU that is IFN- $\gamma$ -dependent, as IFN- $\gamma$  deficient mice fail to develop disease after infusion of uveitogenic DC. Importantly, in addition to having a different immunological profile than CFA-EAU it also differs in severity, duration, fundus appearance and composition of the inflammatory infiltrate.

In the aggregate, the data suggest that autoimmune uveitis can be either Th17 or Th1 driven, and demonstrate that Distinct clinical types of EAU in terms of immune response profile, disease course, clinical signs and histological appearance can be induced on the same genetic background and with the same antigen, if it is initially recognized by the immune system in a different context of innate stimulation. These findings may help to explain the heterogeneous nature of human uveitis in the face of responses to the same retinal Ag.

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# Pathological aspects of experimental uveitis and retinopathy in HLA-A29 transgenic mice

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## Introduction and objectives:

The association between birdshot retinochoroidopathy and HLA-A29 antigen is the strongest disease association with an HLA-A locus. Experimental autoimmune uveoretinitis (EAU) serves as a model for human autoimmune uveitis associated with major histocompatibility complex (HLA) genes and leading to blindness. In order to develop an animal model of HLA-A29-associated disease, a transgenic mouse expressing HLA-A29 molecules was developed and the spontaneous eye disorders arising in these mice was compared with eye pathology occurring in HLA-A29-associated birdshot retinochoroidopathy and in retinal-antigen-induced EAU.

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**Methods:** HLA-A2902 cDNA (A29c) was obtained from a patient suffering from birdshot retinochoroidopathy and used for transgene construct to generate HLA-A29 transgenic mice (Yann Szpak et al., PNAS, 2001). Transgenic mice were observed at 6 and 12 months. C57Bl/6 mice 8-wk-old were immunized with IRBP or IRBP residues 1-16 in CFA and pertussis toxin injected intraperitoneally. Histopathology was evaluated on paraffin sections after haematoxylin-eosin-safran staining.

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**Results:** No pathological changes were found in the eyes of A29-negative mice aged 12 months or more or in eyes from  $\leq 6$  month-old A29-positive mice. In contrast, spontaneous pathological changes were detected in aged ( $\geq 12$  months) A-29-positive mice: 80% of A29-positive mice presented pathological changes in the retina, most often bilateral: retinal vasculitis, retinal folds with clusters of cells containing pigment and retinal pigment epithelial thickening. Retinal detachment and inflammatory cells were detected in the vitreous, the retina, the choroid and the optic disk with the photoreceptor cells alterations. This spontaneous eye disease showed striking histological similarities to the HLA-A29-associated disease in humans and also to murine EAU.

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## Primate Model of Uveoretinitis and Vasculitis / Experimental Autoimmune Uveoretinitis (EAU) induced in monkeys by retinal S Antigen : Clinical, Angiographic and Histological features.

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Purpose : To describe the clinical, angiographic and histological changes in an experimental model of autoimmune uveoretinitis and vasculitis in primates.

Methods : Seven Cynomolgus monkeys received a single subcutaneous immunization in the interscapular area with either 50 or 100 micrograms of human (5 animals) or 100 micrograms of bovine S antigen (2 animals) with complete Freund's adjuvant. Biweekly fluorescein angiograms were performed during a one year follow up period.

Results : All the animals had bilateral intraocular inflammation. Clinically, the intensity of the disease distinguishes two forms : a severe and a mild form. Common characteristics are shared by both forms : it is a long term disease with a sudden, often asymmetrical onset, occurring usually in one eye approximately four weeks after immunization, the second eye being involved three weeks later. A cyclic course of the disease is observed in both forms. This recurrent pattern could be accurately shown with the help of repeated fundus fluorescein angiograms. The initial and principal manifestation consisted of retinal vascular sheathing affecting veins and venules often accompanied with papillary edema. The more severe forms showed areas of posterior uveoretinitis, dense vitritis and anterior uveitis. Histological studies showed that EAU in monkeys is a composite of retinal vasculitis, choroiditis and anterior uveitis. At the early stages, vascular parietal alterations led to intraretinal hemorrhages. Later, a granulomatous infiltration occurs as in sympathetic ophthalmia. At the end stages, after several months of recurrent disease, atrophy of the retina was associated with pigment migration and minimal inflammation

Conclusion : A single systemic injection of pure human or bovine retinal S antigen induced a long term ocular disease consisting primarily of retinal periphlebitis that can be associated with retinitis, vitritis and occasionally anterior uveitis. The clinical and angiographic features of experimental uveoretinitis in monkeys are very similar to human retinal vasculitis.

## Anterior Uveitis Accompanies Joint Disease in a Murine Model Resembling Ankylosing Spondylitis

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Uveitis is often associated with a systemic inflammatory disease such as ankylosing spondylitis, sarcoidosis, juvenile idiopathic arthritis, Behcet's disease or inflammatory bowel disease. Our understanding of the eye's susceptibility to immune-mediated uveitis as occurs in the apparent absence of infection has been limited by a relative lack of experimental models. Here we sought to assess whether ocular inflammation occurs in a previously described murine model of proteoglycan-induced arthritis (PGIA).

In this model, mice immunized with purified human proteoglycan (PG), an antigen found in both the eye and joint, develop progressive polyarthritis and sacroiliitis—features common to the clinical presentations of ankylosing spondylitis. Using intravital microscopy we examined the ocular inflammatory response after the onset of arthritis in both BALB/c wild-type mice and mice that over expressed the T cell receptor specific for a dominant arthritogenic epitope of cartilage PG (TCR-Tg mice). Due to the increased CD4+ T cell population reactive to PG, the TCR-Tg mice exhibit increased incidence and severity to PGIA compared to wild-type BALB/c mice. While the BALB/c mice showed minimal increase in the intravascular inflammatory response, the TCR-Tg mice showed a significant increase in the number of rolling and adhering cells within the iris vasculature compared to adjuvant-control mice.

Cellular infiltration within the iris tissue, as assessed by intravital microscopy was also increased. We have begun to characterize the temporal ocular inflammatory response as occurs in the initiation of PGIA, and find that the TCR-Tg mice immunized with PG show significant increased numbers of rolling and adhering cells within the iris vasculature by 14 days post immunization, a time at which minimal systemic arthritis has developed. Interestingly, this response diminishes at 34 days post immunization, which suggests that recurrent episodes of ocular inflammation may occur during PGIA. These preliminary studies indicate that TCR-Tg mice show an increased ocular inflammatory response that coincides with systemic inflammation. Although these data are preliminary, this model has the potential to clarify the mechanisms accounting for the co-existence of eye and sacroiliac inflammation as occurs in patients with ankylosing spondylitis.

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## Spontaneous autoimmune ocular inflammation induced by TLR ligands

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The notion that microbial infection is responsible for a major portion of pathogenic autoimmunity, proposed several decades ago, has recently received support from two discoveries; (i) T-lymphocytes specific against self antigens escape deletion, but remain harmless in a naive state in normal situations and (ii) the Toll-like receptor (TLR) system in which microbial products interact with TLR on APC and enable them to activate naïve T-cells. Activation of self-specific cells could trigger pathogenic autoimmunity. We developed a system that imitates this hypothetical pathogenic process. The system consists of two lines of transgenic (Tg) mice, with mice of one line ("HEL-Tg") expressing hen egg lysozyme (HEL) in their eyes and mice of the other line ("3A9") expressing HEL-specific TCR on their T-cells. Transferring naïve T-cells from 3A9 mice into HEL-Tg recipients caused no ocular changes in HEL-Tg mice. On the other hand, severe ocular changes developed in recipient eyes when the transferred 3A9 T-cells were previously activated in vitro. This system thus allowed us to test the capacity of TLR ligands to trigger ocular inflammation in recipients of naïve 3A9 cells. Treatment with any one of seven tested TLR ligands induced inflammation in recipient eyes, thus providing direct evidence to the notion that microbial products are capable of triggering pathogenic ocular autoimmunity. Different levels of ocular severity were induced by the tested TLR ligands, with the changes induced by pertussis toxin (PTX) being the most severe by far than any of the other ligands. Analysis of the PTX superior activity revealed it could be attributed to three effects: (i) highest rate of

proliferation of the donor cells; (ii) most efficient expression of surface molecules on donor cells, that facilitate their tissue invasion and (iii) highest expression of pro-inflammatory cytokines.

The experimental system also made it possible to analyze the involvement of Th1 and Th17 cells in the pathogenic process induced by different TLR ligands. Findings of interest: (i) both Th1 and Th17 cells were involved in disease induction by different TLR ligands; (ii) different ratios were found, however, between Th1 and Th17 in inflammation induced by different ligands: the ratio between Th17 and Th1 was 5:1 for PTX, 2:1 for LPS, 1:1 for poly (I:C) and 1:2 for CpG; (iii) transcript levels for Th17 cytokines, IL-17 and IL-22, were higher in PTX-induced inflamed eyes than in eyes from mice treated with other TLR ligands. Thus, selective involvement of Th17 cells could be partly responsible for the excessive severity of ocular inflammation in PTX-treated mice.

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## PIGF-1 and epithelial hemato-retinal barrier breakdown: Potential implication in the pathogenesis of diabetic retinopathy

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**Aims/hypothesis:** Disruption of the retinal pigment epithelial (RPE) barrier contributes to sub-retinal fluid and retinal oedema as observed in diabetic retinopathy. High placental growth factor (PIGF) vitreous levels were found in diabetic patients. The purpose of this work is to elucidate the influence of PIGF-1 on human RPE (ARPE-19) barrier *in vitro* and on normal rat eyes *in vivo*.

**Methods:** ARPE-19 permeability was measured using transepithelial resistance (TER) and inulin flux under PIGF-1, VEGF-E, and VEGF 165 stimulation. The effect of hypoxic conditions or insulin was evaluated on TER and on PIGF-1 and VEGF receptors using RT-PCR. The involvement of MEK/ERK signaling pathways under PIGF-1 stimulation was evaluated using western blot analysis and specific inhibitors. The effect of PIGF-1 on the external hemato-retinal barrier was evaluated after its intravitreous injection in the rat eye, using semi-thin analysis and ZO-1 immunolocalisation on flat-mounted RPE.

**Results:** *In vitro*, PIGF-1 induces a reversible TER decrease and enhances tritiated inulin flux. These effects are specifically abolished by an antisense oligonucleotide directed at VEGFR-1. Exposure of ARPE-19 cells to hypoxic conditions or to insulin induces an up-regulation of PIGF-1 expression along with an increased trans-cellular permeability. The PIGF-1-induced RPE cell permeability involves the MEK/ERK signaling pathway. Injection of PIGF-1 in the rat eye vitreous induces an opening of the RPE tight junctions with subsequent sub retinal fluid accumulation, retinal oedema and cytoplasm translocation of junction proteins.

**Conclusion:** Our results indicate that PIGF-1 may be a potential regulation target for the control of diabetic retinal and macular oedema.

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## Animal models of primary intraocular lymphomas

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Primary intraocular lymphoma (PIOL) is a high grade non-Hodgkin lymphoma which pathogenesis is still unclear. Few animal models exist in order to study this condition.

Although intraocular lymphomas in human are usually B-cell lymphomas, most of these models are T-cell lymphomas. Recently, a major step forward has been realized with the development of new models of intraocular B-cell lymphoma. New therapeutic tools are being evaluated in these models of B-cell lymphoma, such as the use of lymphotoxin, or the manipulation of the immune microenvironment in order to control the tumour growth (monoclonal antibodies, cellular and molecular immunotherapy). We evaluate the contribution of the different animal models available in the study of intraocular lymphoma, and discuss the new therapeutic strategies and their various targets in the tumour as well as in the environment, which are currently investigated through the development of these models.

## The taming of the shrew - or - what have we learned from experimental keratoplasty ?

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The presence of an immunologically privileged organ makes it more difficult to study the underlying immunobiological mechanisms after keratoplasty: it places limits on theoretical studies of graft reaction, its immunobiology and reduces the means of testing new therapeutic options. Therefore in the past decades rabbits, rats and mice but also sheep and pigs have been used extensively to study the process of immunologically mediated corneal allograft rejection. Basic clues to graft rejection were initially found in rabbit eyes, which are comparable in size to the human eye and offer relatively easy operative access with conventional surgical instruments. However, more detailed studies were hampered by the inadequate knowledge of rabbits immune system. As a logical next step, Williams and Coster presented a rat eye model of penetrating keratoplasty which avoids these primary disadvantages. Using this model and subsequent transfer to the mouse, several researchers including our group have contributed further details on the immune reaction as well as new preventive measures. The major findings regarding corneal allograft immune pathophysiology and its therapeutic modulation in experimental animals will be reviewed.

Supported in part: (DFG PI 150/14-2)

  
 **Glaucoma**

*JUN SONG MO*

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## Animal models of optic neuritis

JOHN GUY

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Animal models of optic neuritis include

1) *experimental allergic encephalomyelitis (EAE), an autoimmune disorder induced by sensitization of animals to CNS myelin antigens, myelin basic protein or proteolipid protein,* 2) *viral-induced demyelination and* 3) *antibody-induced demyelination.* The characteristic relapsing neurological signs of paralysis, ataxia and visual loss in the chronic EAE model are similar to those of patients with MS. Histopathology of inactive EAE lesions shows foci of gliosis without active inflammation that are also seen in MS. Active optic nerve lesions in EAE reveal demyelization, mononuclear cell infiltration and phagocytosis of axons and myelin by effector macrophages. These findings are also seen in MS. In EAE there is also an immunogenetically restricted recognition system that involves the major histocompatibility antigens. Helper CD4 lymphocytes first adhere to endothelial cells, and then they infiltrate the CNS. The inflammatory response is amplified by recruitment of inflammatory cells and release of mediators, such as cytokines, antibodies and reactive oxygen species (ROS). It is presumed that similar mechanisms may contribute to the pathogenesis of MS, but this is not known because in most patients the disease is already well established at clinical presentation.

The EAE animal model has impacted the design and direction of both basic and clinical research to understand the pathogenesis and treatment of MS. Immunomodulatory cyclophosphamide, cyclosporine A, copolymer 1, antibodies to specific lymphocyte subsets, and immunization with T cell receptor peptides initially evaluated in EAE have been or are being applied to MS. The EAE model has an additional important advantage over other animal models. The alterations in the permeability of the blood-brain barrier (BBB) play a major role in the pathogenesis of EAE induced demyelination. Comparable disruption of this barrier occurs in immune-mediated disorders such as optic neuritis and MS. In fact, optic neuritis and MS are believed to be disorders of the BBB through which inflammatory cells and humoral factors producing demyelination gain access to the CNS. We have proven that MRIs of the optic nerve showing contrast enhancement and demyelination are similar in EAE and human disease. Moreover, the histopathologic findings of biopsy specimens of patients with optic neuritis exhibit inflammation and demyelination that is also seen in the EAE optic nerve. The MRI, histopathologic and ROS similarities of the EAE animal model to human optic neuritis suggest that EAE is the ideal model system to test potential treatment agents.

The traditional view of optic neuritis and MS emphasizes demyelination as the primary event in the disease process. The targeted cells appear to be the oligodendrocytes that are responsible for producing axon's myelin. In fact, apoptosis of oligodendrocytes has been described as the earliest event in the early lesions of MS. Recently this focus has changed. Axonal and neuronal loss are increasingly recognized as the primary factors contributing to persistent deficits and disability in MS and optic neuritis, as also revealed by optical coherence tomography (OCT). Axonal loss is seen in acute EAE. Loss of RGCs is also common to chronic EAE as well as to relapsing/remitting EAE. The incidence of optic neuritis is very high in both model systems with one significant difference. In chronic EAE, RGC loss occurs prior to the infiltration of inflammatory cells, but not in relapsing and remitting EAE or in MOG-specific TCR transgenic mice that develop isolated optic neuritis usually without any other characteristic lesions of EAE in the brain or in the spinal cord. Moreover, these transgenic mice need no sensitizing injection for the development of isolated optic neuritis, though the incidence of optic neuritis can be doubled by intraperitoneal injection of small amounts of pertussis toxin. Since the mechanisms of axonal and neuronal degeneration may be different in the EAE animal model relative to the transgenic mouse as is also the likelihood in patients with optic neuritis or MS, we believe that it is prudent to study both model systems.

One in which inflammatory cells may play a major role in neurodegeneration, and the other in which it may not.





