Aus dem Institut für Hygiene und Mikrobiologie

der Universität Würzburg

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The Role of Endosymbiotic *Wolbachia Bacteria* in the Pathogenesis or River Blindness

Inaugural – Dissertation

zur Erlangung der Doktorwürde der

Medizinischen Fakultät

der

Julius-Maximilians-Universität zu Würzburg

vorgelegt von

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Würzburg, November 2007

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Tag der mündlichen Prüfung: 02.12.2008

Die Promovendin ist Ärztin

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INTRODUCTION

1 Onchocerciasis

Onchocerciasis, or river blindness, is a parasitic worm disease caused by the filarial nematode *Onchocerca volvulus*. It affects 17.7 million people in Africa, Latin America, and Yemen and is the second most common cause of preventable blindness in sub-Saharan Africa [1]. Ocular disease is thought to result from a host–derived inflammatory reaction which occurs in response to the death of parasite microfilaria in the eye. Using a mouse model (Figure 1) for river blindness in which soluble extracts of filarial nematodes are injected into the corneal stroma,

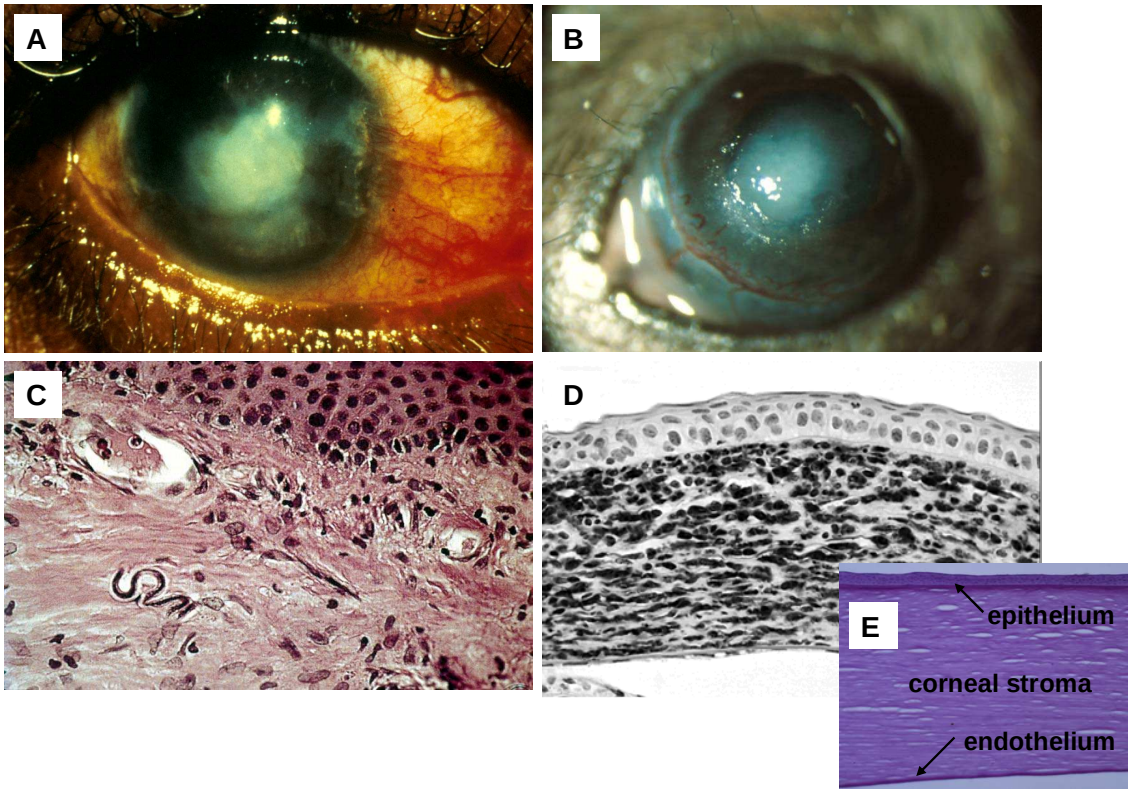

Figure 1 Mouse model of *O. volvulus* keratitis. Injection of *O. volvulus* antigens into the murine corneal stroma results in progressive corneal opacification and neovascularization (B), similar to human Onchocerca keratitis (A) (Photo by Dr. H. Taylor (University of Melbourne, Australia)) and (C) (Photo by D.H. Connor (University of Wisconsin, USA)) with stromal thickening and migration of inflammatory cells to the cornea. (D) demonstrates infiltration of neutrophils to the corneal stroma of a C57BL/6 mouse 24 hours after intracorneal injection of *Wolbachia* containing *O. volvulus* extract. The cornea was immunostained with anti-neutrophil antibody and neutrophils visualized by bright field microscopy (original magnification is x 200). (E) shows a normal murine cornea without inflammation. Images (D) and (E) by A. v. Saint André.

this study investigates the role of the parasite's endosymbiotic *Wolbachia* bacteria in the pathogenesis of ocular onchocerciasis.

Onchocerciasis is transmitted by *Simulium* spp. blackflies, which transfer infective L3 stage larvae during a blood meal (Figure 2). These larvae mature to female and male worms within a year and are located in subcutaneous nodules (onchocercomas) within the human host. The numbers of female worms in infected people may range from 1 to 60 or more [2], each of them producing millions of microfilariae during their lifetime [3] which lasts 10 to 14 years. It is this microfilarial stage that is not only critical in the maintenance of the parasite's life cycle, but is the actual pathological agent of onchocerciasis – causing ocular and various forms of skin disease. Blindness – the most devastating disease manifestation – is caused by inflammation in the eye as a result of microfilariae migrating through the skin and conjunctiva into the cornea. Microfilariae dying and degenerating in the cornea lead to loss of corneal clarity and thereby visual im-

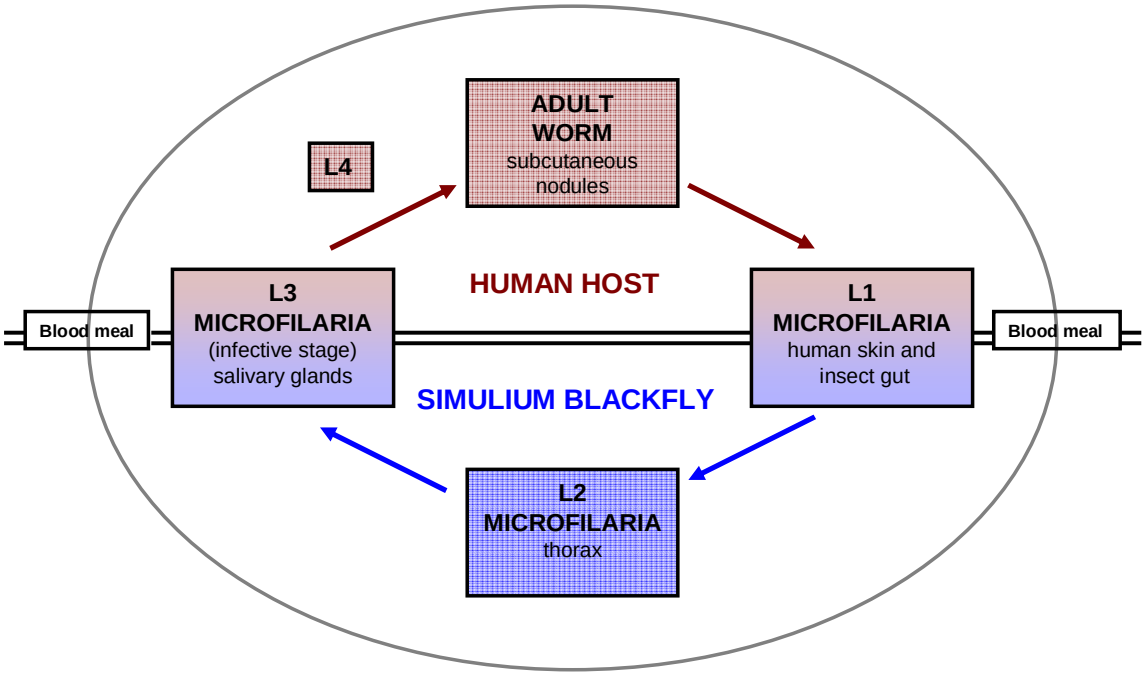

Figure 2 Transmission and Life Cycle of *O.volvulus.* The microfilaria is the most abundant stage, and it is critical in the maintenance of the parasite's life cycle, as well as being the pathological agent. Fertilized females release cyclically 1000-3000 microfilariae per day for the lifetime of the female. Microfilariae migrate through the human skin, and the parasite life cycle is continued after ingestion of microfilariae during the bloodmeal of a *Simulium* blackfly. Microfilariae undergo two molts in the blackfly, migrating through the insect gut, the thorax and into the salivary gland. On a subsequent blood meal, infective third stage larvae are transmitted to the next human host where they undergo a further two molts to become adult males and females. Image by A. von Saint André.

pairment. This sclerosing keratitis may be followed by posterior chamber involvement including chorioretinitis and optic atrophy [4].

Major steps have been taken towards control of onchocerciasis, due to the successful joint efforts and support from WHO and other UN agencies, the World Bank, and a coalition of non-governmental and development organizations. These onchocerciasis control programs utilize a twofold strategy. Mass distribution of the microfilaricidal drug ivermectin is used as the principal intervention [5], and application of larvicides to the breeding sites of the mosquito vector has been used to interrupt transmission. The Onchocerciasis Control Program in West Africa (OCP) initially targeted the *Simulium* vector, later complemented by the distribution of ivermectin. This successful program ended in 2002. Two further programs, the Onchocerciasis Elimination Program of the Amerocas (OEPA) and the African Program of Onchocerciasis Control (APOC) have been implemented based on mass distribution of the macrofilaricidal drug ivermectin, either semiannual (OEPA) or annually (APOC). Recent endemicity studies, however, suggest that, at least in Africa, this approach will not stop transmission of disease and in areas where interruption of transmission has been achieved by vector control, the infection will be reimported by (re)migration of people who still carry the infection [6, 7]. Even after the end of transmission, mass treatment has to continue at least as long as adult worms survive in humans (up to 14 years) [8]. Therefore, the *Conference on Eradicability of Onchocerciasis* concluded that eradication was not feasible given the need of sustainable treatment at a coverage of 65% for 35 or more years [7]. Furthermore, should resistance to ivermectin – the only currently available drug - for human onchocerciasis arise, as an *O. volvulus* phenotype in Ghana with suboptimal drug response after repeated ivermectin treatments recently suggested [9, 10], control activities would be severely compromised. Thus new chemotherapeutic strategies need to be developed. However, long-standing research to develop a macrofilaricide – a drug that could kill or permanently sterilize the adult *O. volvulus* parasite - with qualities compatible with public health use, has to date, failed.

2 *Wolbachia* endosymbiotic bacteria

It has been known since the mid-1970s that most filarial nematodes are infected with intracellular bacteria [11-13]. But it is only recently that the importance in the biology of these helminthes has been realized. *Wolbachia* are *Rickettsia*-like, matrilineally inherited [14], obligate intracellular bacteria that infect many species of invertebrates [15] and most filarial parasites of importance for human health, including *O. volvulus*, *B. malayi*, *W. bancrofti* and some *Mansonella* species. Three filarial parasite species, *Acanthocheilonema viteae* (infects rodents), *Onchocerca flexuosa* (infects deer) and *Loa Loa* [16, 17] do not naturally contain *Wolbachia* and are often used as experimental controls [18, 19] as in this study.

Phylogenetic classification of *Wolbachia* is within the order of Rickettsiales, family Anaplasmataceae, which also contains the bacteria *Ehrlichia*, *Anaplasma* and *Neorickettsia* [20]. Presently there is a single valid species in the genus *Wolbachia* for both filariae and arthopods, which is *Wolbachia pipientis* [18, 21-23]. Six major clades (A–F) of *Wolbachia* have been identified to date [23, 24]: A, B, E, and F have been reported from insects, arachnids, and crustaceans; C and D from filarial nematodes. The bacteria are found in all the stages of the nematodes' life cycle although they occur in varying proportions between individual worms and developmental stages [12, 25]. In adult nematodes, *Wolbachia* is concentrated in intracytoplasmatic vacuoles within the hypodermal lateral cords. In female worms, the bacteria are also present in the reproductive organs, including oocytes and developing embryonic stages in the uteri, whereas they have not been demonstrated in the male reproductive system [26-29] (see Figure 3).

Like other Rickettsia bacteria, *Wolbachia* are sensitive to the tetracycline class of antibiotics (tetracycline, doxycycline), and also to rifampicin and azithromycin [30-37]. There are currently no technologies that enable long or short term culture of these endosymbionts in cell-free medium, i.e. outside the cytoplasm of invertebrate cell lines or intact filarial worms. *Wolbachia* from nematodes do not appear to infect host tissues, although bacterial DNA has been detected in filarial-infected individuals after diethylcarbamazine (DEC) treatment [38].

In arthropods *Wolbachia* have mostly parasitic habits manipulating arthropods' reproduction. In nematodes, in contrast, the endobacteria live as symbionts, in-

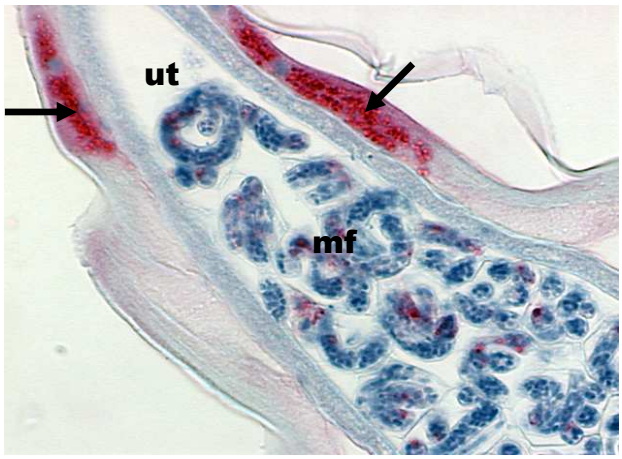

Figure 3 *Wolbachia* bacteria in section of female Brugia worm. *Wolbachia* bacteria (red) are concentrated in the hypodermal lateral cords (arrows) and around the uterus (ut) as well as within the microfilaria (mf) (original magnification X 200). Adult worms were fixed in 4% formaldehyde, embedded in paraffin and sliced into 5 µM sections followed by immunohistochemical staining using rabbit anti-recombinant wsp antisera and Vector red substrate. Image by Amy Hise (Case Western Reserve University, Cleveland, OH, USA).

creasing their own survival by increasing the fitness of the host. The long co-evolution of the bacteria and the worm [18, 22] is expected to result in co-adaptation and reciprocal dependence. Gene sequence analysis has provided a further indication that the association between *Wolbachia* and filarial nematodes is not parasitic [40, 41]. Although little is known about the specific molecular interaction between the symbiont and the host, *Wolbachia* appears to be essential for embryogenesis and larval development of the nematode and is, therefore, designated as obligatory symbiotic [19, 31, 32, 42]. As obligatory symbionts, *Wolbachia* can be used as a target for therapy.

3 Pathogenesis and host immune responses in *onchocerca* keratitis

The transparent nature of the mammalian cornea is due to the highly organized arrangement of collagen fibrils and a tightly regulated level of hydration, which is maintained by the resident cells. These include epithelial cells, which are the external barrier; stromal fibroblasts (keratocytes), which produce the collagen and proteoglycans that form the matrix of the stroma; and corneal endothelial cells, which maintain the hydration level of the cornea by pumping H_20 from the stroma to the anterior chamber [43]. The cornea is a unique tissue for studying immunologically regulated inflammatory processes, since it is considered to be immune privileged: due to the lack of blood and lymphatic vessels, i.e. structures that provide the conduit for transportation of immunologic components into and

out of the tissue, the cornea lacks direct access to the immune system. The normal cornea also lacks professional antigen presenting cells such as dendritic cells and macrophages that are resident in most other tissues. However, all of this immune apparatus is present in the limbus (Figure 4).

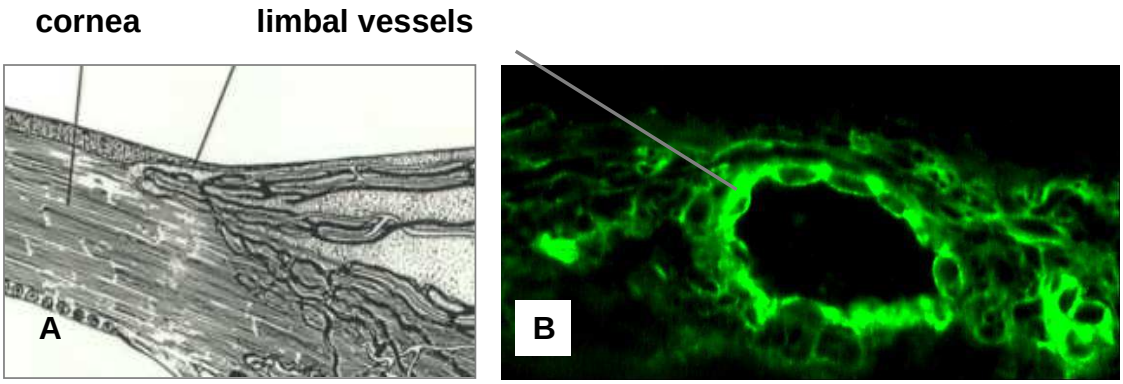

Figure 4 Recruitment of inflammatory cells from limbal vessels to the cornea. Since the normal cornea is avascular, leukocytes migrate from limbal vessels to the cornea. (A) Normal anatomy of the eye showing cornea and limbus (from "General Ophthalmology" 15th edition by D. Vaughan, T. Asbury, P. Riordan-Eva, Appleton & Lange; 1999). (B) Image of limbal vessel stained for PECAM-1 (image by A. v. Saint André). Eyes of C57BL/6 mice were enucleated 24 hours after intrastromal injection of *Wolbachia*-containing *O. volvulus* antigen, showing upregulation of PECAM-1 which promotes neutrophil recruitment to the cornea. Original magnification x 400.

During the course of inflammation, i.e. when microfilaria migrate to and degenerate in the cornea, leukocytes extravasate from blood vessels in the limbus, migrate to the center of the cornea and eventually this tissue becomes vascularized. Although inflammatory cells are important in host defense, they can also have cytotoxic effects on resident cells in the cornea, resulting in loss of corneal transparency, visual impairment, and blindness. Because the normal cornea is avascular and transparent, one can qualitatively and quantitatively observe the development of the inflammatory processes. The accessibility of the cornea and its relative separation from the immune system also facilitates local immunological manipulations without confounding system effects.

Previous studies in onchocerca keratitis focused on adaptive immune responses for at least two reasons: firstly, as a model for chronically infected individuals who are presumably sensitized prior to ocular involvement, there would be no example for innate immunity in the eye in the absence of an adaptive immune response, and secondly, experimental models showed no detectable corneal opacification or

neovascularization with methods available at the time, unless animals were first immunized [44-46]. The understanding of the pathogenesis of *onchocerca* keratitis in a mouse model up to the point when this study was performed is summarized here (see Figure 5): the intrastromal injection of *O. volvulus* antigen is associated with a predominant CD4+, Th2 response both systemically and in the cornea, with IgE and parasite specific IgG1 being the predominant isotypes produced, and with the predominant early cellular infiltrate being neutrophils followed by a long-time mobilization of eosinophils 3 to14 days after exposure [44, 47-49]. The use of B cell-deficient μMT mice and FcγR-/- mice revealed that Fc receptors on neutrophils and eosinophils facilitate degranulation of these cells and disruption of corneal clarity [50, 51]. Activation of resident corneal cells by breakdown products of microfilariae leads to secretion of proinflammatory cytokines, which stimulate synthesis of chemokines by keratocytes and induce the expression of adhesion molecules on vascular epithelial cells followed by recruitment of neutrophils and eosinophils: neutrophil recruitment is mediated by PECAM-1 and chemokine receptor CXCR2, whereas eosinophil recruitment is dependent on eotaxin, P-selectin and ICAM-1 [51-54]. In humans, CXCR2 is the receptor for IL-8 which is an important neutrophil attractant and activator during acute inflammation [55-57]. Mice, however, do not produce this chemokine but have two functional homologues, KC and MIP-2 that can bind to the murine CXCR2 receptor and are important mediators of neutrophil extravasation and recruitment to extravascular sites [58]. Both KC and MIP-2 are produced in murine models of *O. volvulus* keratitis [53, 59].

The high turnover rates of microfilariae – 20,000 to 300,000 or more per day in hyperendemic areas [3] - cause a persistent release of *O. volvulus* and *Wolbachia* products. The exposure of these antigens to the host defense system can induce immune responses to filarial as well as endobacterial molecules.

While it is well documented that helminth infections are principally characterized by Th2-like immune responses, increasing evidence at the time of this study [60-63] strengthened the notion that the innate immune system, neutrophil and Th1-type responses – observed in filarial infections preferentially after microfilaricidal treatment – may depend on stimuli derived from *Wolbachia* endobacteria.

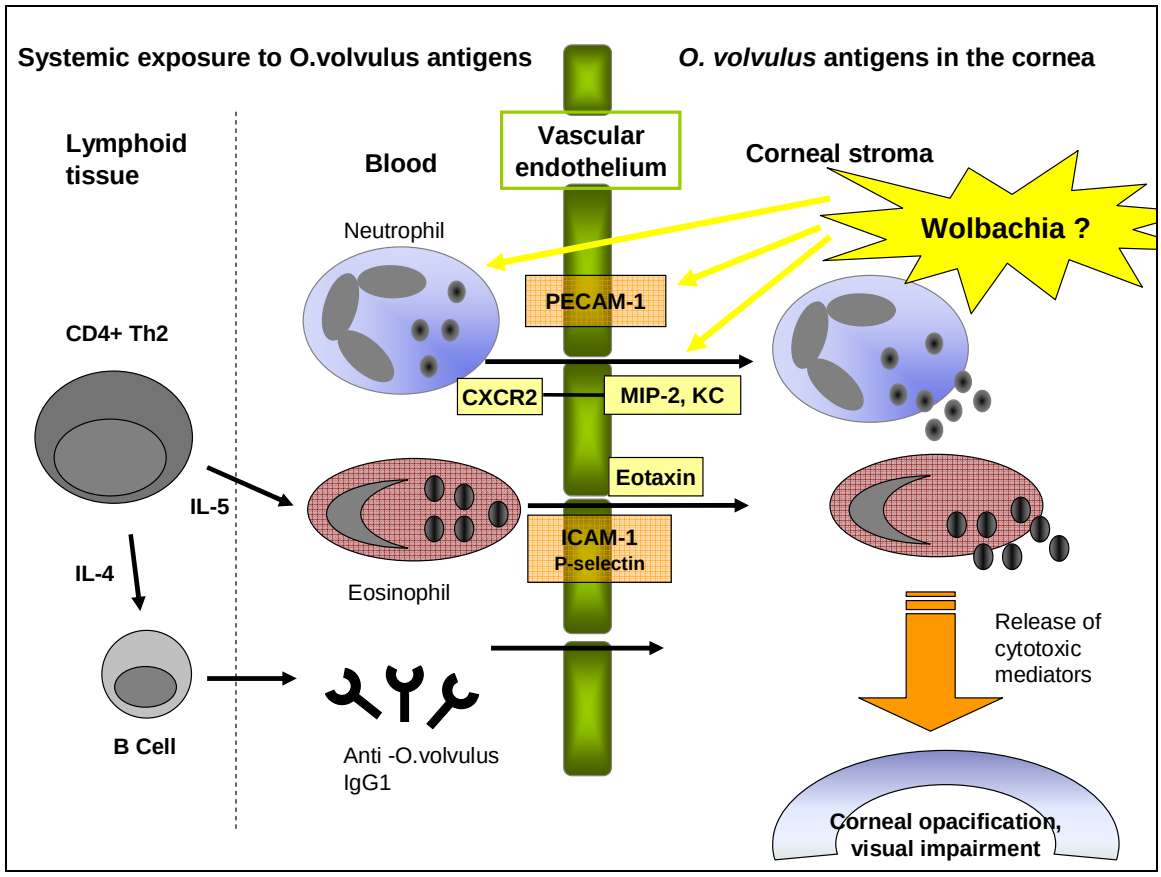

Figure 5 Proposed sequence of events in adaptive immune response underlying *Onchocerca* keratitis and possible role of *Wolbachia* in the pathogenesis of river blindness. Immunization or chronic infection induces a predominant Th2 response, with IL-4 leading to isotype switching to IgE and IgG1, and IL-5 inducing eosinophil differentiation; parasite antigens in the corneal stroma lead to activation of resident cells in the cornea, production of CXC and CC chemokines, elevated expression of adhesion molecules on vascular endothelial cells in the limbus, and biphasic infiltration of neutrophils and eosinophils to the corneal stroma. Immune complex-mediated cross linking of Fc receptors on neutrophils and eosinophils results in degranulation and release of cationic proteins and other cytotoxic mediators that disrupt normal corneal clarity, eventually leading to sclerosis and blindness. Image by A. v. Saint André, adapted from Kaifi et al.

The innate immune system in mammals senses the invasion of microorganisms using the family of Toll-like Receptors (TLRs), stimulation of which initiates a range of host defense mechanisms. TLR expression in the corneal epithelium has been confirmed. Initially and at time of these experiments, only TLR4 expression was reported [66], but recently other TLR including TLR2 have been found in the cornea [67-71] . Hence, this study investigates *Wolbachia*'s role in activating the innate immune system by looking at neutrophil recruitment, TLR4, PECAM-1 and CXC chemokine dependent mechanisms in the development of *onchocerca* keratitis.

4 Aim of this Study

This study aims to determine the role of *Wolbachia* endobacteria in the pathogenesis of *O. volvulus* keratitis. Using a murine model of corneal inflammation in which various parasite extracts are injected directly into the corneal stroma, corneas were examined by scanning confocal microscopy [72] and immunohistochemistry. Confocal microscopy enables *in vivo* measurement of stromal thickness and stromal haze, which are indicators of corneal edema and opacity, respectively, and can measure corneal abnormality responses that cannot be detected by slit lamp microscopy. The approach of characterizing *Wolbachia*'s role in river blindness is threefold:

Firstly, soluble extracts of *O. volvulus* worms recovered from doxycycline treated individuals are compared with worms from untreated individuals and the corneal inflammatory responses are evaluated by confocal microscopy and immunohistochemical staining for neutrophils and eosinophils. According to immunohistochemistry and DNA analyses, *O. volvulus* worms from individuals treated with doxycycline have no detectable *Wolbachia* – as opposed to worms recovered from untreated individuals [31].

Secondly, the corneal effects to extracts from the rodent filaria *Acanthocheilonema viteae*, which naturally does not harbor *Wolbachia* are compared to extracts from endosymbiont containing *Brugia malayi*, a related filarial worm that causes lymphatic filariasis.

The third step determines if *Wolbachia* bacteria mediate corneal pathology by activating Toll-like Receptor (TLR) 4. TLR4 is one of the best characterized and one of the most important TLRs that mediates signals for a broad spectrum of ligands including heat shock proteins, fatty acids, LPS and oligosaccharides [73]. C3H/HeJ mice contain a genetic mutation that results in a truncated and inactive TLR4 protein [74]. Following injection of corneas with *Wolbachia* harboring *O. volvulus* antigen, corneal pathology in TLR4-mutant C3H/HeJ mice is compared to the wild-type C3H/HeN strain.

An additional step investigates further mechanisms of inflammatory infiltration to the cornea. C3H/HeJ and C3H/HeN mice are utilized to evaluate TLR4 dependent expression of the adhesion molecule PECAM-1, and production of the CXC cytokines MIP-2 and KC, all of which are essential for neutrophil recruitment to the

cornea. Accordingly, PECAM-1 and KC expression is also investigated in C57BL/6 mice using *O. volvulus* antigen from doxycycline treated and untreated individuals to look at *Wolbachia* - dependent expression of these neutrophil attractant markers.

The aim of this study was to determine the role of *Wolbachia* in the pathogenesis of river blindness and thereby re-thinking our understanding of the immunopathology of filariasis, the adverse reactions to microfilaricidal drugs and new treatment options against filarial nematodes, which cause some of the most debilitating diseases in more than 150 million people [75-77] throughout the world's poorest communities.

METHODS

1 Antigen preparation

Doxycycline treated and non-treated *O. volvulus* antigen

O. volvulus worms containing *Wolbachia* were recovered from infected individuals in Ghana who were either untreated (OvAg) or had been treated with doxycycline (OvAg/doxy) [31]. Phosphate-buffered saline (PBS) soluble parasite extracts were prepared by grinding, ultrasonication and centrifugation at 10,000 X g for 30 minutes. The total concentration was determined by standard Bradford assay [78] (Bio-Rad, Richmond, CA, USA). All materials used for the production of parasite products were sterilized and procedures were performed with strict aseptic techniques. Endotoxin levels in parasite extracts were detected by a sensitive chromogenic Limulus amebocyte lysate (LAL) test (BioWhittaker, Walkersville, MD, USA) with a quantifying LAL-activity between 0.1 to 1.0 endotoxin per ml using several dilutions of the extracts [61].

The effect of doxycycline on reducing *Wolbachia* numbers in the worms was demonstrated by immunohistochemistry using antibodies to bacterial heat shock protein 60 (hsp60) [31]. Soluble extracts were frozen at -80°C until required. These parasite extracts were kindly provided by Achim Hoerauf and Norbert W. Brattig (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany).

O. volvulus antigen

O. volvulus worms were recovered from subcutaneous nodules that had been surgically removed from infected patients in Cameroon, and kindly sent to us by Dr. Janet Bradley (University of Nottingham, Nottingham, U.K.). Parasites were recovered after digestion with collagenase (Sigma, St. Louis, MO, USA) as described elsewhere [44], and homogenized in Hanks' Balanced Salt Solution (HBSS) using a mortar and pestle. Insoluble material was removed by centrifugation, and the concentration of protein was determined by a commercial adaptation of the Bradford [78] method (Bio-Rad). All procedures were performed with strict aseptic techniques and endotoxin-free materials. Endotoxin levels in

parasites were detected by quantitative LAL testing (BioWhittaker) using several dilutions of the extracts and quantifying LAL-activity between 0.1-1.0 endotoxin per ml.

B. malayi antigen

Adult worms of the filarial parasite *B. malayi*, obtained live from the NIH filaria repository and shipped in sterile RPMI-1640 were processed by washing extensively with sterile PBS, then culturing for 7 days in RPMI, 5% low endotoxin FCS and 1% penicillin/ streptomycin at 37°C in 5% CO_2. Daily media changes were performed to ensure that the final filarial extracts were free from contaminating bacterial products or endotoxin. After rinsing six times with cold sterile PBS, the worms were processed in cold RPMI by homogenization using endotoxin-free coarse glass tissue grinders followed by external sonication on ice. The filarial extracts were then centrifuged at 1500 rpm for 15 minutes at 4°C and the supernatant was collected and frozen at -20°C. All procedures were performed using strict aseptic techniques and endotoxin-free materials. Quantitative LAL testing (BioWhittaker) was used to monitor culture media during the processing. Protein concentrations were determined using a commercial adaptation of the Bradford [78] method (Bio-Rad).

B. malayi extracts were kindly provided by Amy G. Hise (Case Western Reserve University, Cleveland, OH, USA).

A. viteae antigen

Adult *A. viteae* worms which naturally do not harbor *Wolbachia* were obtained from subcutaneous tissues of Mongolian gerbils. Adult male and female worms were collected under aseptic conditions and washed four times in RPMI-1640 containing 5% FCS and 1% penicillin/ streptomycin (Gibco Brl, Gaithersburg, MD, USA). Parasites were cultured at 37°C in 5% CO_2 for 5 days to ensure the absence of contaminating microorganisms and to select viable motile parasites. Adult parasites were separated into male and female worms under sterile conditions and washed four times in sterile PBS. Extracts were prepared by finely

mincing the worms followed by homogenization on ice. All procedures were carried out under stringent sterile conditions with endotoxin-free materials. Extracts were centrifuged at 20,000 g for 30 minutes, and the supernatant was collected and stored at -20°C until required. Protein concentration of parasite extracts was determined by the Coomassie protein assay (Pierce Chemical Co., Rockford IL, USA) [60].

A. viteae extracts were kindly supplied by Mark Taylor (Liverpool School of Tropical Medicine, Liverpool, U.K.).

2 Animals

6 to 8 week-old female C57BL/6 and C3H/HeN mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and 6 to 8 week-old female C3H/HeJ mice from Harlan Sprague (Indianapolis, IN, USA). All mice were treated in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the “Use of Animals in Ophthalmic and Vision Research”.

Injections into the corneal stroma

Mice were anaesthetized with an intraperitoneal injection of 200 µl of a 1.2% solution of 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI, USA) containing 2.5% 2-methyl-2-butanol (tertiary amyl alcohol) (Aldrich) dissolved in distilled water. Using a sterile 30-gauge needle, the corneal epithelial layer was abraded, and 1 µg of extract in 5 µl of saline was directly injected into the corneal stroma using a 33-gauge needle attached to a Hamilton syringe (Hamilton, Reno, NV, USA).

In previous experiments the appropriate dosage for intracorneal injections had been made dependent on the extract’s protein concentration. Comparing the inflammatory responses in murine corneas 24 hours after injection of 2 mg/ml versus 1 mg/ml of the different extracts, two out of four corneas which had received the higher dose of each antigen turned completely opaque and impossible to examine by confocal microscopy and one animal died (unpublished

data). All the mice injected with the lower dose of extract survived and corneas were easily evaluated. All experiments were therefore performed using parasite extracts of 1 mg/ml of protein concentration.

Inflammatory responses to different parasite antigens were compared in pairs by injecting one of the matched up extracts into the left and one in the right eye of the same animal (see Figure 6). This set up of experiments excludes variation of host reactivity in different mice as a cause for altered reactions to the various parasite extracts.

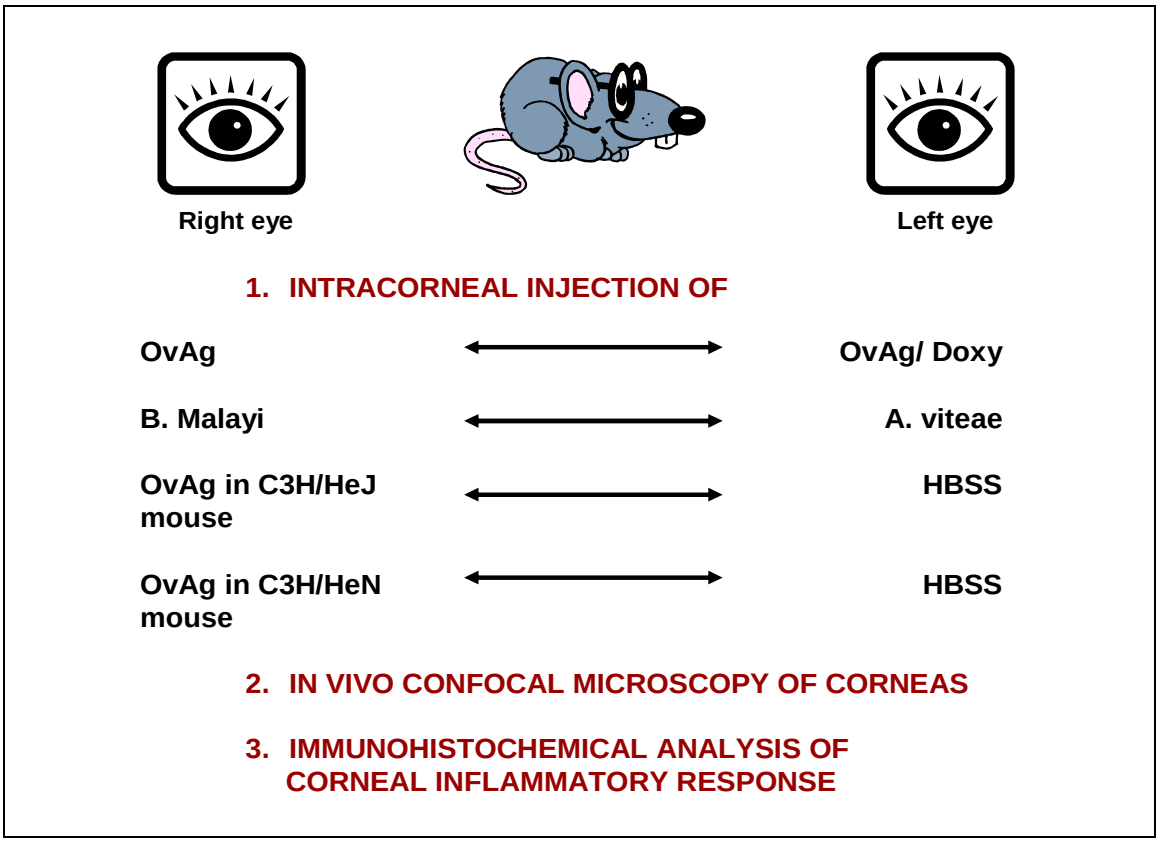

Figure 6 Basic set up and steps of experiments. Inflammatory reactions to different antigens were compared in the same animal by injecting one extract in the right, one in the left eye. In a second step corneas were examined by confocal microscopy, then mice were sacrificed and corneas analyzed immunohistochemically.

Immunizations

For the first set of experiment animals were given three weekly subcutaneous immunizations with 10 µl of *O. volvulus* antigens in a 1:1 ratio with adjuvant containing 10% squalene (Aldrich), 0.4% Tween Fisher (Fisher, Fair Lawn, NJ, USA), and 1% pluronic acid (BASF Bioresearch, Parsippany, NJ, USA).

The mouse model of *O. volvulus* keratitis had been established with a focus on a Th2 type response. Therefore weekly immunizations of mice had been performed in previous projects in order to stimulate the adaptive immune system. Since this study concentrated on the innate immune response, mice in all but the first experiment received intracorneal injections only without prior immunizations.

3 Confocal microscopy

In vivo examinations were performed using a scanning confocal microscope (Tandem scanning, Reston, VA, USA). The technique used was Confocal Microscopy through Focusing (CMTF). For this the mice were anesthetized as described previously. A drop of 2.5% hydroxypropyl methylcellulose was then

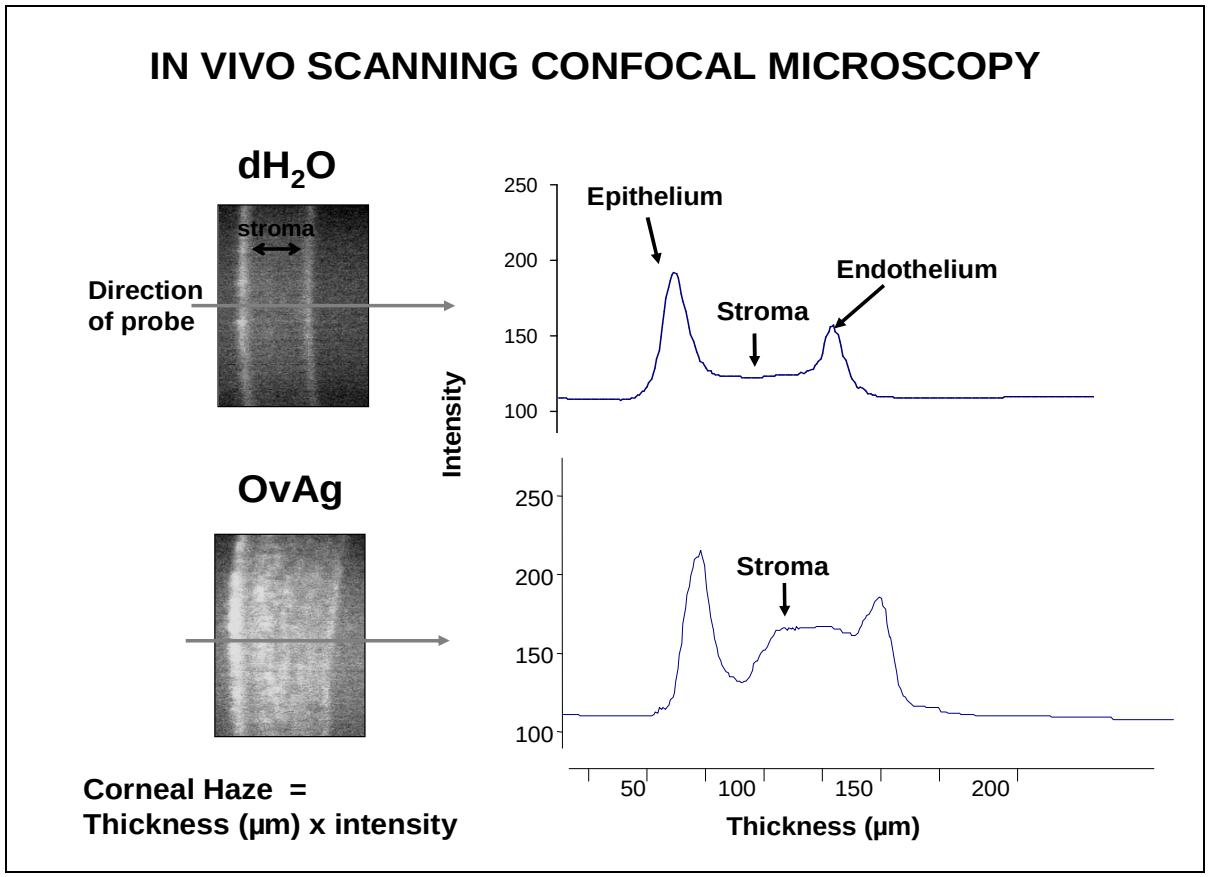

Figure 7 Confocal microscopy through focusing to measure stromal thickness and stromal haze of murine corneas. Injection of *O. volvulus* extract (OvAg) into the cornea (lower part) of C57BL/6 mice results in increased stromal thickness on both the CMTF derived image (left side) and intensity profile (right side), compared to injection of sterile water (upper part). Image by A. von Saint André.

applied to the objective tip to eliminate the bright field reflections and the objective was brought in contact with the mouse eye. Keeping the gain constant in all the readings and using double frame rate (60 frames/ sec) and lens speed of 160 µm/sec, the images and intensity profiles were obtained. From these profiles, the stromal thickness as well as the stromal haze were calculated [72] (Figure 7). CMTF was used to evaluate murine corneas 24 hours after intrastromal injection of parasite antigen.

4 Immunohistochemical analysis of corneal inflammation

Detection of Neutrophils and Eosinophils

For immunohistochemical staining, enucleated eyes were snap frozen in liquid nitrogen, stored at -70°C, and 5 µm sections were fixed in 4% formaldehyde for 25 min, then rinsed in PBS (Sigma) and covered with proteinase K (Dako, Carpinteria, CA, USA) for 8 minutes to improve accessibility of antibodies to target sites within the tissue, since proteolytic digestion by proteinase K exposes certain epitopes which may have been masked during formalin fixation. Neutrophils were detected by incubating slides for two hours with the rat anti-mouse antibody NIMP-R/14 (kindly supplied by Achim Hoerauf, Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany), diluted 1:100, followed by fluorescein isothiocyante (FITC)-conjugated rabbit anti-rat IgG (Caltag Laboratories, Burlingame, CA, USA) diluted 1:200 as secondary antibody for 45 minutes. Eosinophils were immunostained with rabbit antisera to eosinophil major basic protein (MBP, kindly provided by Jamie Lee, Mayo Clinic, Scottsdale, AZ, USA), diluted 1:5000. FITC-labeled goat anti-rabbit IgG (Caltag Laboratories), diluted 1:200, was used as a secondary antibody and incubated for 45 minutes. Stained sections were washed in PBS and coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) to inhibit quenching. Positively stained cells were examined by fluorescence microscopy. Cells were counted either throughout the corneal section or in representative fields at 600 x magnification.

Detection of PECAM-1

Eyes were snap-frozen in liquid nitrogen, stored at -70°C, and 5 μm sections were fixed for 10 minutes in –20°C acetone. Slides were air-dried, then rehydrated in PBS (pH 7.4) and stained with rat anti-mouse IgG against PECAM-1 (MEC 13.3, PharMingen, San Diego, CA, USA). Primary antibodies were diluted 1:100 in PBS containing 1% fetal calf serum and incubated for two hours at room temperature. FITC-labeled rabbit anti-rat IgG (H+L) (Caltag Laboratories) diluted 1:200 was used as a secondary antibody and incubated for 45 minutes. Stained sections were washed in PBS, coverslipped with Vectashield Mounting Medium (Vector Laboratories) and examined by fluorescence microscopy.

Evaluation of Staining Intensity

Evaluation of expression of FITC-stained adhesion molecules on limbal vessels was based on the method described by Tang and Hendricks [79] for detection of PECAM expression. After immunostaining for PECAM-1, images of limbal vessels were captured using a digital camera model DC330 (PAGE-MTI Inc., Michigan City, IN, USA) and Scion Image Software (Version 1.62c; National Institutes of Health, Bethesda, MD, USA, modified by Scion Corporation). To evaluate the relative fluorescence intensity, the mean brightness value of the green channel of the three most intensely stained vessel areas was determined using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA, USA) with a set 400 pixel square area. Four vessels from each eye were analyzed, the background reading in unstained areas of the cornea was subtracted from these values, and the mean plus/ minus standard error of the fluorescence intensity for each vessel was estimated. Data was then presented as the percent maximum value for the antibody.

5 Detection of chemokines in the cornea

To determine the concentration of the chemokines MIP-2 and KC in murine corneas, animals were sacrificed and corneas were carefully dissected to avoid

removing surrounding conjunctival tissue and underlying iris. The corneas were suspended in 400 ml RPMI-1640 medium, and sonicated for 90 seconds at 50 cycles per second (Sonics VibraCell, Danbury, CT, USA). After centrifugation, the concentrations of MIP-2 and KC were detected in supernatants by two-site ELISA following the manufacturer's directions (R&D Systems, Minneapolis, MN, USA). The limit of detection for MIP-2 was 1.5 pg/ml, and less than 2.0 pg/ml for KC.

6 Statistics

Statistical significance was determined using an unpaired Student's *t* test (Prism Graph Pad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered to be significant.

RESULTS

1 Doxycycline treated *O. volvulus* antigen has an impaired capacity to induce corneal inflammation

This first set of experiments compares the inflammatory infiltrate after exposure of C57BL/6 mice to *O. volvulus* extracts originating from onchocerciasis patients either with (OvAg/doxy) or without (OvAg) prior doxycycline treatment. As previous studies had shown, inflammatory cells infiltrate the cornea in a biphasic manner, with neutrophils predominant in the first 24 hours after intrastromal injection and their replacement by eosinophils after 72 hours [51, 53, 64]. Consistent with this observation, Figure 8 shows neutrophil recruitment peaking at 24 hours in eyes injected with OvAg. However, the number of these cells was significantly reduced in corneas treated with OvAg/doxy (p = 0.018), i.e. without *Wolbachia*. Eosinophil mobilization to the cornea increased three days after exposure to parasite antigens, however their number was the same for the two different extracts. Neutrophil, but not eosinophil infiltration therefore seems to be dependent on the presence of *Wolbachia* endobacteria.

LAL testing revealed an endotoxin level of 3.83 EU/ml for untreated OvAg and 1.98 EU/ml for OvAg/doxy extracts of the same protein concentration, therefore

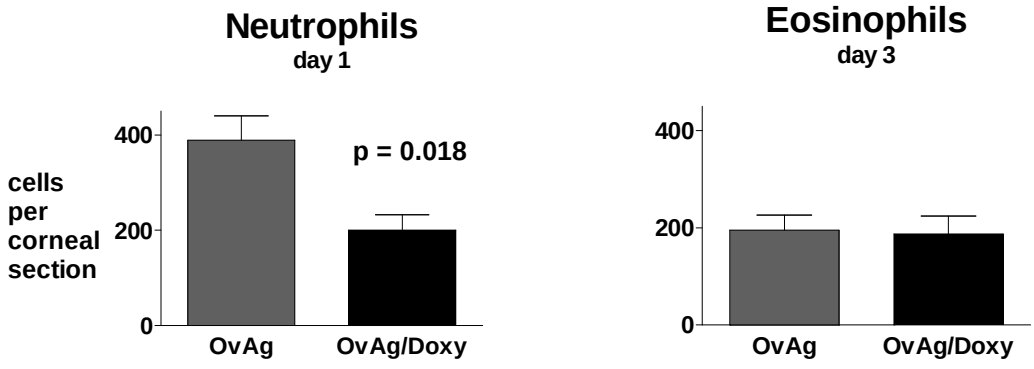

Figure 8 Diminished neutrophil recruitment to the cornea 24 hours after injection of *O. volvulus* worms derived from doxycycline treated individuals. Untreated *Wolbachia*-containing OvAg revealed a significantly higher neutrophil infiltration (p = 0.018), indicating that *Wolbachia* bacteria may contribute to the pathogenesis of river-blindness. Eosinophil numbers remained the same for both treated and untreated *O. volvulus* extracts 72 hours post intrastromal injection.

suggesting a small amount of endotoxin contamination of both extracts. The increased endotoxin level of untreated OvAg was at the time when this experiment was done interpreted as inherent *Wolbachia* – LPS. Recent genome sequencing of *Wolbachia,* however, has shown that the endosymbiont lacks the genes required for LPS biosynthesis [40, 80]. Although endotoxin levels in these two extracts are low and not of major difference, some degree of endotoxin related effect on neutrophil recruitment cannot be excluded in this experiment.

O. volvulus extracts from doxycycline treated worms (i.e. not containing

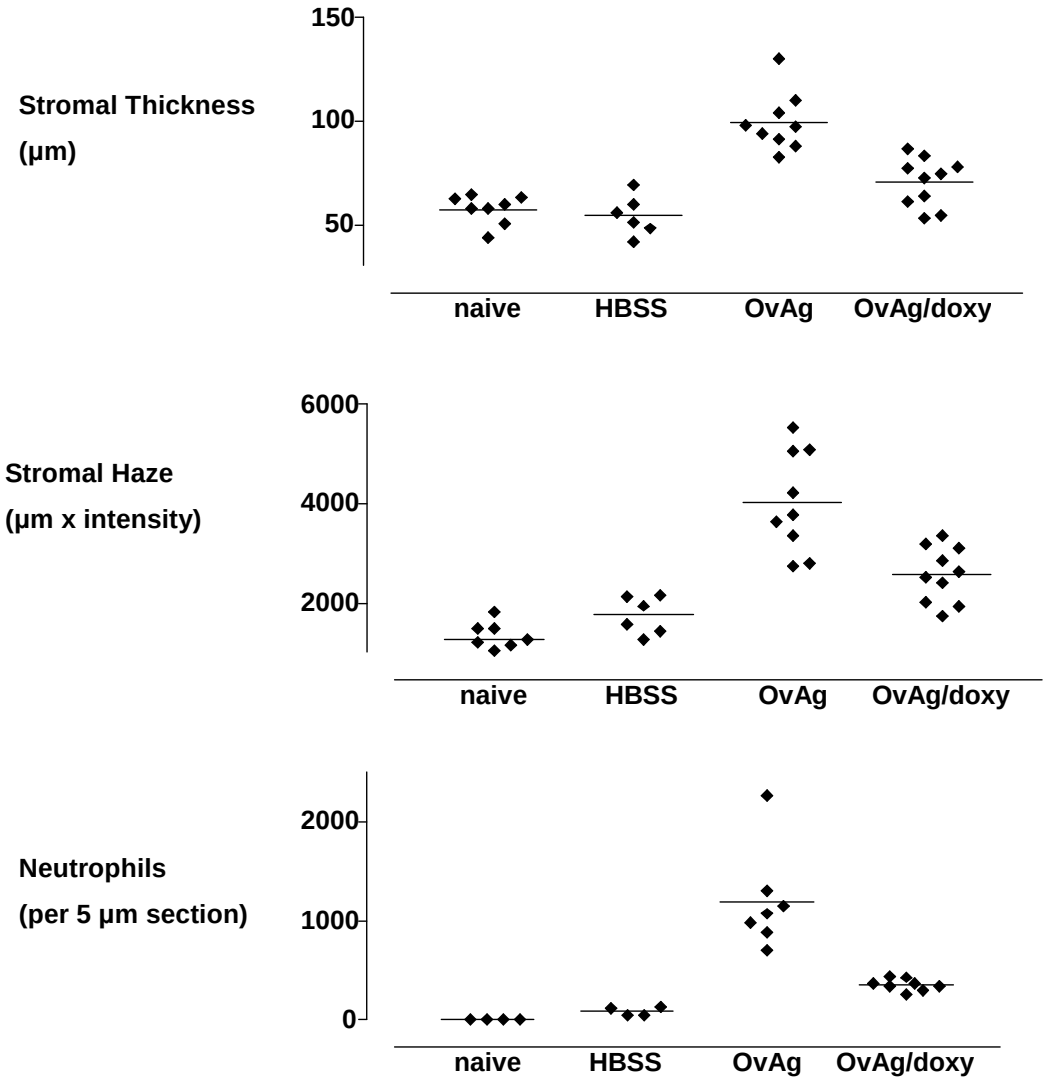

Figure 9 Diminished stromal thickness, stromal haze and neutrophil recruitment in corneas injected with doxycycline-treated and therefore *Wolbachia*-free *O. volvulus* extracts (OvAg/doxy). Untreated OvAg extracts led to a significantly higher inflammatory response. Data points represent individual corneas from a single experiment.

Wolbachia) induced significantly lower stromal thickness ($p = 0.0001$), stromal haze ($p = 0.0012$) and neutrophil infiltration ($p = 0.0005$) than untreated worms (i.e. containing *Wolbachia* bacteria) 24 hours after intracorneal injection (Figure 9). Although minor compared to untreated worms, extracts from treated worms also induced responses that were higher than naïve corneas or corneas injected with saline ($p < 0.0001$ for neutrophil infiltration, $p = 0.0129$ for stromal thickness and $p = 0.0071$ for stromal haze), indicating that not only *Wolbachia*, but also filarial antigens contribute to the inflammatory reaction. Again, the small endotoxin content of OvAg and OvAg/Doxy may also contribute to this effect. Stromal thickness and stromal haze values for saline-injected mice were similar to naïve mice, meaning that the trauma of the injection has no or little effect on these parameters. This experiment was repeated four times showing similar results with both immunized and unimmunized mice. Figure 10 shows the typical confocal microscopy images obtained in these experiments.

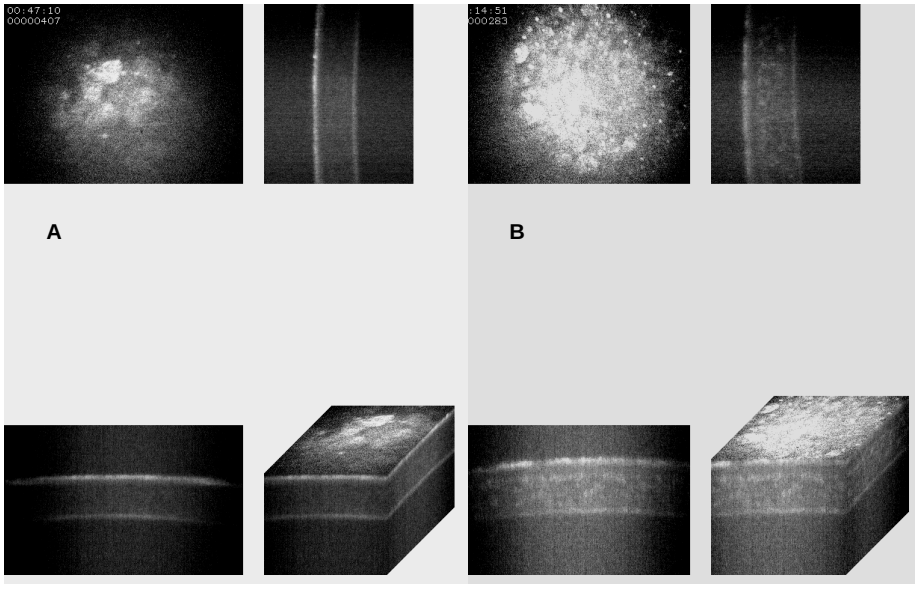

Figure 10 Confocal microscopy images of B57BL/6 mice 24 hours after injection of either saline (A) or untreated *Wolbachia*-containing *O. volvulus* extract (B). Stromal thickness (top right and bottom left of both A and B) and cell infiltration are increased in corneas exposed to parasite extract. Greater cell infiltrate can be seen especially in the corneal epithelial layer of OvAg-injected corneas (top left corner of B) compared to saline-treated eyes (top left corner of A). Images by A. von Saint André.

2 Filaria – induced inflammatory response is dependent on the presence of endosymbiotic *Wolbachia* bacteria

The inflammatory response to extracts of *Brugia malayi*, a filarial worm that contains *Wolbachia*, was compared to extracts from the rodent filaria *Acanthocheilonema viteae*, which naturally does not harbor the endosymbiotic bacteria. Corneas of B57BL/6 mice were injected with these extracts, and the inflammatory response was measured as described above. Figure 11 shows that injection of *B. malayi* extract stimulates a pronounced neutrophil infiltration, with elevated stromal haze and stromal thickness, similar to the untreated OvAg

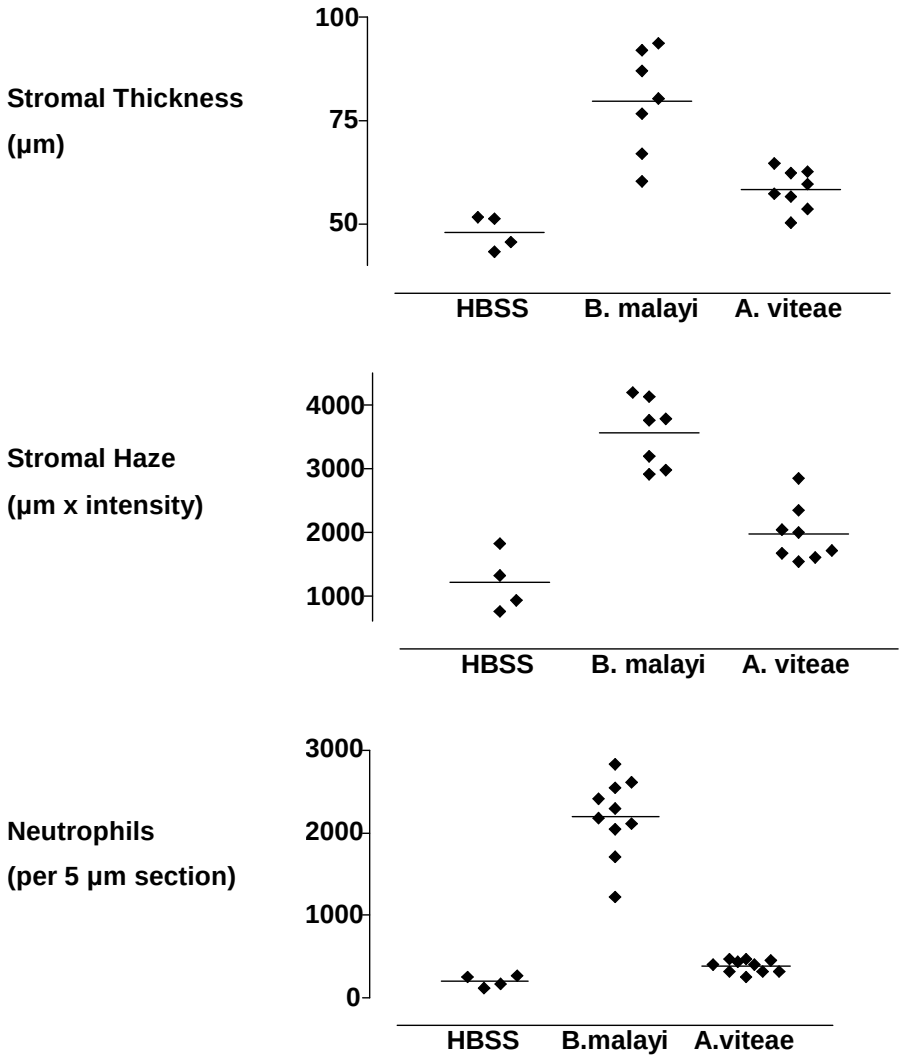

Figure 11 Significantly increased stromal thickness, stromal haze and neutrophil recruitment for *Wolbachia* containing *B. malayi* extracts compared to endosymbiont-free *A. viteae* extracts. Data points represent individual corneas from a single experiment.

response. In contrast, the inflammatory response to *A. viteae* extract was significantly lower compared to the reaction to *B. malayi* extracts (Figure 4) with p = 0.0007 for stromal thickness, p < 0.0001 for stromal haze and p < 0.0001 for neutrophil infiltration. Similar results were obtained by a repeat experiment. The immunoflorescence stain for neutrophils in Figure 12 demonstrates the marked difference in numbers of infiltrating cells between the two extracts.

Interestingly, LAL testing of *B. malayi* and *A. viteae* extracts revealed 68.2 EU/ml and 3.79 EU/ml respectively. Unfortunately, a repeat LAL test was not possible to perform due to shortage of extract. Again, with the recent insight into *Wolbachia*'s genome rendering the existence of *Wolbachia*-LPS unlikely [40, 80], the above results have to be interpreted with some precaution as discussed in detail later. Briefly, the higher endotoxin level may explain the increased number of neutrophils seen with the *B. malayi* extract compared to the untreated OvAg extract in the previous experiment, and it could also account for the difference in inflammatory response evocated by the *B. malayi* and *A. viteae* extracts. The fact that speaks for *Wolbachia* rather than endotoxin as the important piece in this pathogenesis puzzle is that the *Wolbachia*-free *A. viteae* reagent overall produces only a minor inflammatory response despite its endotoxin level being similar to the untreated OvAg extract which caused significant pathology. On the other hand, differences in filarial antigens with different potencies to cause disease can also be confounding factors in interpretation of these results.

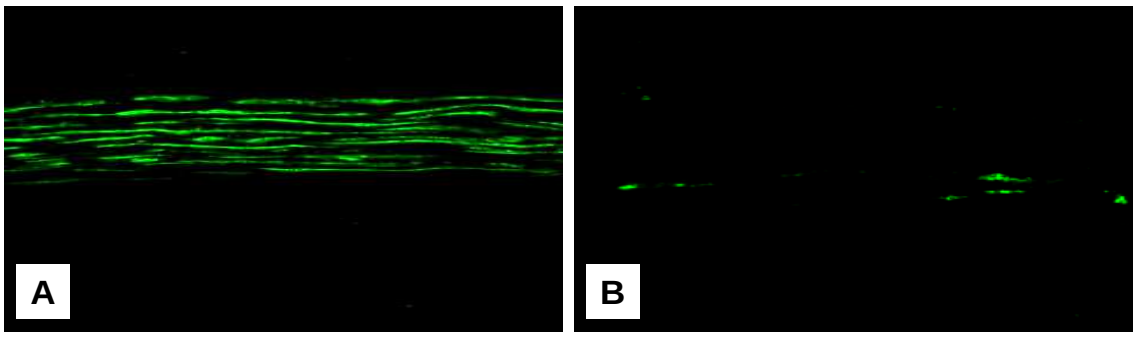

Figure 12 Increased neutrophil recruitment to the cornea with *Wolbachia* containing *B. malayi* extract (A) compared to endosymbiont free *A. viteae* antigen (B). Neutrophils stained with anti-neutrophil antibody NIMP-R/14 and visualized by fluorescence microscopy (original magnification is x 600). Images by A. von Saint André.

3 Toll - like receptor 4 dependence of *O. volvulus* keratitis

To determine if *Wolbachia* mediate corneal pathology by activating Toll-like receptor (TLR) 4, untreated *O. volvulus* extracts (i.e. containing *Wolbachia*) were injected into corneas of C3H/HeJ mice, which do not harbor an intact TLR4 and are hyporesponsive to LPS due to a single missense mutation within the TLR4 coding sequence [74]. The inflammatory response was compared with congenic, TLR4-mutant C3H/HeN mice. Figure 13 shows that in C3H/HeJ mice stromal

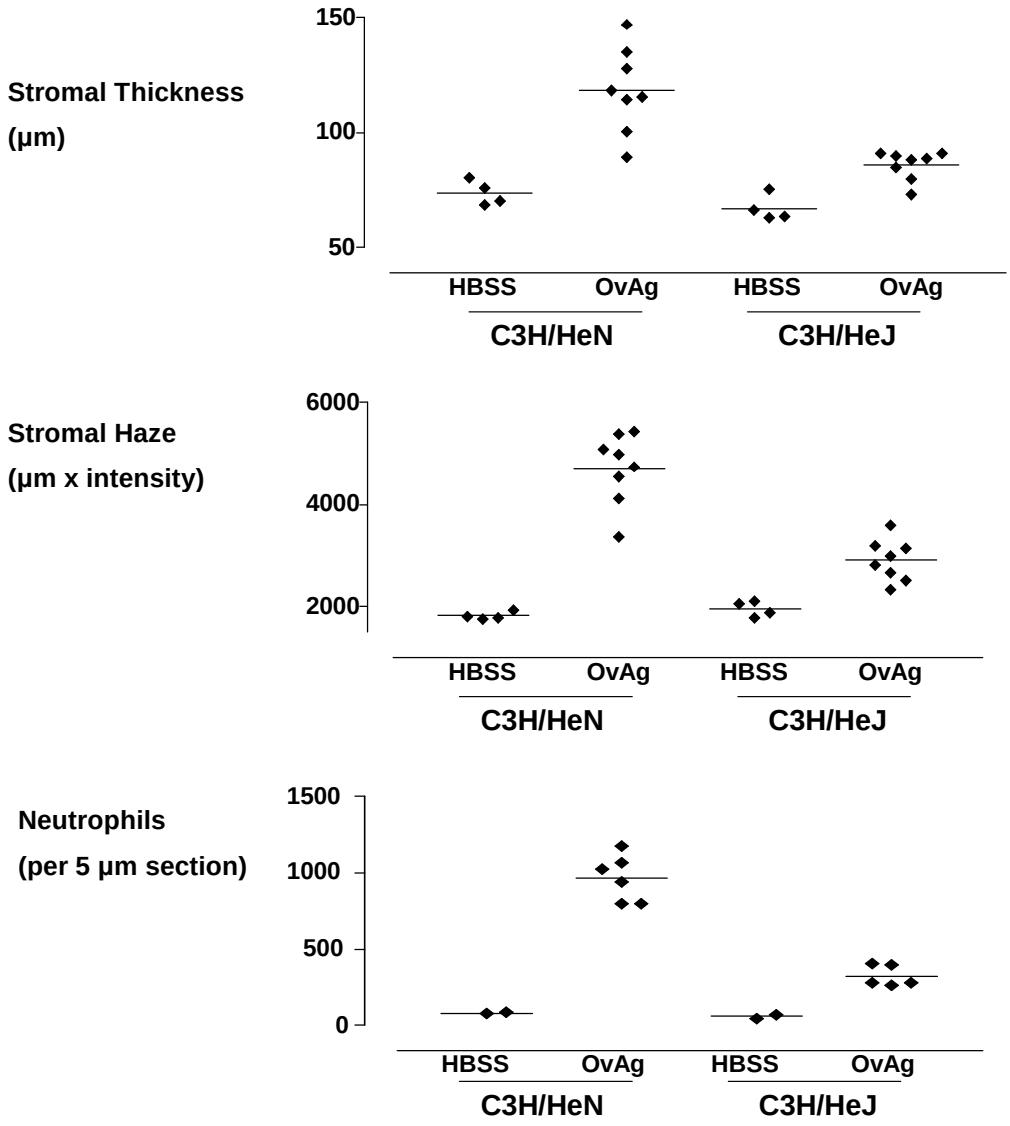

Figure 13 Decreased stromal thickness, stromal haze and neutrophil recruitment are found in TLR4-mutant C3H/HeJ mice challenged with *Wolbachia*-containing *O. volvulus* antigen. In contrary, TLR4-intact C3H/HeN mice show a significantly higher inflammatory response. Data points represent individual corneas from a single experiment.

thickness, stromal haze and neutrophil recruitment were significantly diminished compared with wild-type mice ($p = 0.0003$ for stromal thickness; $p < 0.0001$ for stromal haze; $p < 0.0001$ for neutrophil recruitment), indicating that TLR4 participates in regulating the development of *O. volvulus* keratitis. Two repeat experiments led to comparable results. This finding proposes an important role of *Wolbachia* bacteria or bacterial products in the pathogenic process of river blindness. LAL testing of this OvAg extract was 3.83 EU/ml, again raising the question of endotoxin contamination explaining part of these results.

4 TLR4 and *Wolbachia* dependent expression of PECAM-1 and of neutrophil chemokines MIP-2 and KC

Since the cornea is an avascular tissue, cellular infiltration originates from limbal vessels in the peripheral region of the cornea, and progresses toward the central cornea where the initial stimulus (injection of parasite antigens) is induced. This experiment identifies whether essential mediators of neutrophil recruitment are dependent on the presence of *Wolbachia*. Expression of Platelet Endothelial Cell Adhesion molecule (PECAM) –1, Macrophage Inflammatory Protein (MIP)-2 and KC are all essential for neutrophil recruitment to the cornea [52, 53, 58]. As Figure 14 demonstrates, their expression is significantly diminished in TLR4-mutant C3H/HeJ mice compared with wild-type mice ($p = 0.0054$ for PECAM–1; $p = 0.0007$ for MIP-2; $p < 0.0001$ for KC). Both mouse strains were challenged with intrastromal injections of untreated and therefore endosymbiont-containing *O. volvulus* antigen 24 hours prior to sacrificing the animals. Figure 15 shows the typical appearance of limbal vessels stained immunohistochemically for PECAM-1, with an obviously elevated expression of this adhesion molecule in wild-type mice. These findings which were confirmed by two repeat experiments indicate a regulatory role of TLR4 in *O. volvulus* keratitis by modulating the expression of PECAM-1, MIP-2 and KC in the cornea. Since the OvAg extract used here had an LAL level of 3.83 EU/ml, the same considerations of an endotoxin related inflammatory effect apply in interpreting the above results.

Interestingly, TNF-α which is also essential for leukocyte recruitment, induces KC and MIP-2 expression [58], and had been described as a key proinflammatory

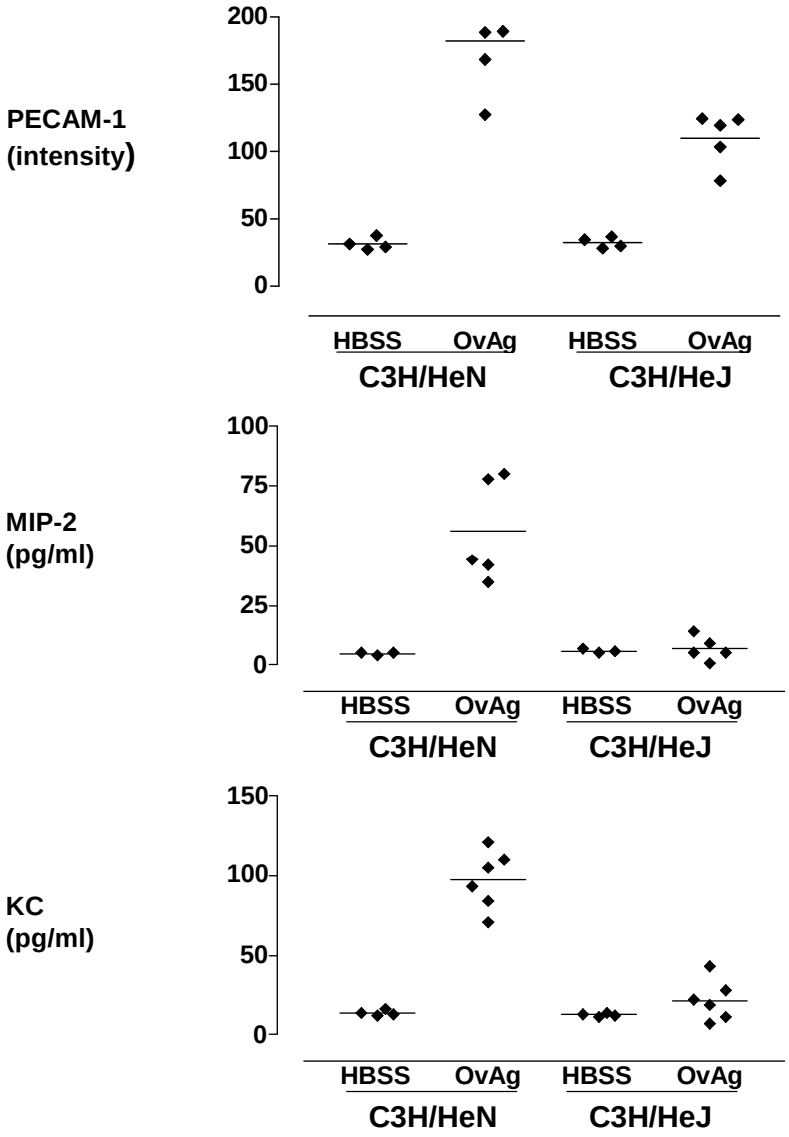

Figure 14 Decreased PECAM-1 expression and decreased MIP-2 and KC production in TLR4-mutant C3H/HeJ mice challenged with *Wolbachia*-containing *O. volvulus* antigen. In contrast, wild-type C3H/HeN mice show upregulation of these proinflammatory parameters in response to the same extract. Data points represent chemokine levels from individual corneas from a single experiment.

cytokine in filarial disease [60, 81] was undetectable in the corneal stroma of both C3H/HeN and C3H/HeJ mice (data not shown). This may have been caused by technical errors. However, this finding may also be due to a non-representative time point chosen for the detection of TNF-α in this experiment, since all cytokines were measured at 24 hours after intracorneal injection of parasite extract. Brattig et al found significant TNF-α production after 4 hours of stimulating human monocyte cultures with OvAg of untreated patients and decreasing levels after 24 hours [61]. Gillette-Ferguson et al [63] on the other hand were able to measure

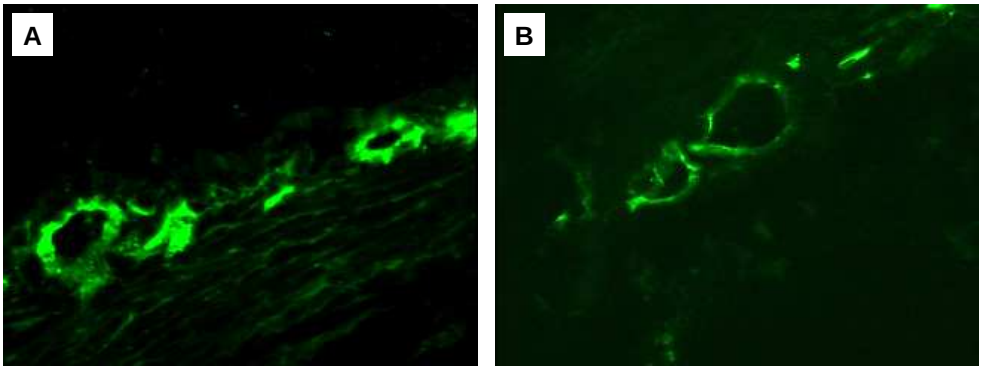

Figure 15 Increased staining intensity for PECAM-1 in TLR4-responsive C3H/HeN mice (A) compared to TLR4-mutant C3H/HeJ mice (B). Mice were sacrificed 24 hours after intracorneal injection of untreated *O. volvulus* antigen; 5 µm sections of murine eyes were immunostained with mouse antibody against PECAM-1 and visualized using FITC anti-rat IgG. Original magnification x 400. Images by A. von Saint André.

TNF-α levels in a dose dependent manner after 18 hours of incubation of murine neutrophil populations stimulated with *Wolbachia*-containing parasite extract.

In accordance to the TLR4 dependence of PECAM-1 and KC upregulation, their expression was diminished in corneas of C57BL/6 mice injected with OvAg/doxy (not containing *Wolbachia*) compared to those challenged with untreated *Wolbachia*-containing extract ($p < 0.0001$ for PECAM-1; $p = 0.0313$ for KC) (Figure 16). These results which were reproduced twice, point again to the role of *Wolbachia* in recruitment of neutrophils to the cornea and thereby developing keratitis.

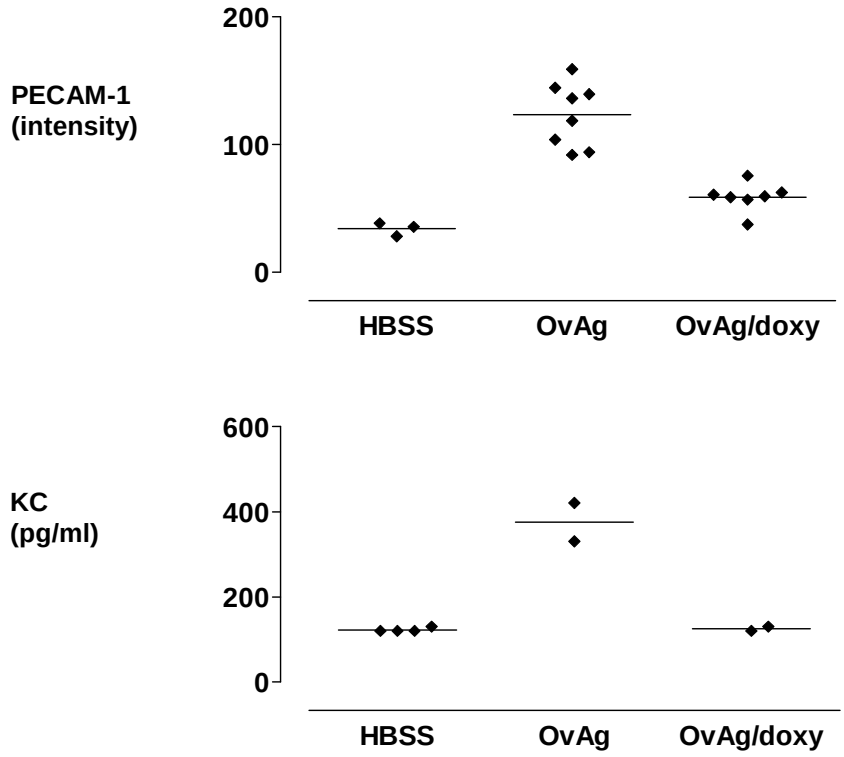

Figure 16 Reduced PECAM-1 expression and KC production with doxycycline treated *O. volvulus* extract in C57BL/6 mice. In contrast, untreated *Wolbachia*-containing extract revealed marked upregulation of the adhesion molecule PECAM-1 and the neutrophil chemokine KC. Data points represent chemokine levels from individual corneas from a single experiment.

DISCUSSION

1 *Wolbachia* as inducer of pathology

This study demonstrates in a mouse model of *onchocerca* keratitis that development of corneal opacification is associated with the presence of *Wolbachia* endobacteria, neutrophils and the innate immune system. This was a surprising finding, since until then abundant literature suggested that filarial and other helminth infections are regulated by Th2 cell– and eosinophil responses [77, 82-85]. Three different approaches were used to demonstrate *Wolbachia*'s important role in corneal pathology:

Firstly, *Wolbachia*-depleted extracts from doxycycline treated onchocerciasis patients led to a diminished inflammatory response in murine corneas compared to untreated, i.e. *Wolbachia* containing antigen. The decreased cell recruitment observed with doxycycline treated extracts involved neutrophils, but not eosinophils. This finding demonstrated that the presence of *Wolbachia* increases neutrophil recruitment. Furthermore, these results made clear that the number of neutrophils is a determining factor for the degree of pathology, since *Wolbachia*-depleted extracts led to less neutrophil infiltrate with less stromal thickness and haze. This conclusion was consistent with previous observations in which inhibiting recruitment of neutrophils, but not eosinophils to the corneal stroma resulted in significantly reduced corneal disease [52, 53]. Similarly, experiments using IL-5 knock-out mice which do not produce any eosinophils showed a sustained neutrophil infiltrate together with an exacerbated keratitis [64]. The difference in inflammatory response evoked by the treated versus untreated *O. volvulus* extract is likely due to the *Wolbachia* compound, although the presence of endotoxin in both reagents might contribute to some and at this point not known degree of neutrophil recruitment to the cornea simply due to this contamination. At the time these experiments were performed, it was thought that *Wolbachia* contained LPS which was responsible for the observed *Wolbachia* related immune response and would explain both the decreased endotoxin level and pathology seen with doxycycline treated antigen. As mentioned earlier, genomic sequencing has, however, shown that *Wolbachia* lack the genes required for LPS biosynthesis [40, 80]. In the mean time, the search for the molecular nature of

Wolbachia's stimulatory activity has focused on other candidate molecules found on the surface membrane of the bacteria. *Wolbachia* surface protein (WSP) has been shown in multiple experiments to strongly activate the innate immune system via TLR (see below) and could well explain not only the above, but also the findings of the second step in this study:

Extracts from *Wolbachia*-containing *B. malayi* revealed markedly more pathology than endosymbiont-free *A. viteae* antigen. This again pointed at the role of *Wolbachia* in development of disease. The relatively high endotoxin content of the *B. malayi* extract is possibly part of the reason why this reagent attracted far more neutrophils than its *Wolbachia* containing OvAg counterpart in the prior experiment. The *A. viteae* extract was also found to have an endotoxin level almost as high as the untreated *O. volulus* antigen. Interestingly, despite the similar endotoxin level, the inflammatory reaction caused by *Wolbachia*-free *A. viteae* was very mild compared to the *Wolbachia* containing OvAg. This observation points to *Wolbachia* as inducer of pathology, rather than contaminant LPS. Another uncertainty in interpreting the above results is to what extent the filarial proteins derived from different helminthes contribute to these findings, although the same protein concentration was used for all experiments.

Thirdly, TLR4 dependence was shown to exist for the inflammatory response to *Wolbachia* harboring *O. volvulus* antigen. TLRs are a family of at least 10 transmembrane proteins that are highly conserved in vertebrates and invertebrates, and represent a first line of defense by detecting conserved pathogen-associated molecular patterns (PAMPs) [86]. Cell stimulation through TLRs triggers activation of the transcription factor NF-κB that results in activation of genes encoding proinflammatory cytokines such as TNF-α [87] and Il-8 or the murine homologue KC [73]. TLR4 is one of the most important TLRs that mediates signals for a broad spectrum of ligands including fatty acids, oligosaccharides, LPS and heat shock proteins. Mice deficient in functional TLR4 therefore produced only very mild inflammatory responses despite the presence of *Wolbachia*. Although this experiment was done with *O. volvulus* antigen containing a small amount of endotoxin and therefore the potential of confounding these results, Brattig et al showed that recombinant WSP activates TLR4 and TLR2 [62]. Several other reports also indicate that *Wolbachia* stimulates innate immune responses via TLR: *Wolbachia* activation of macrophages is decreased in

TLR4 – mutant C3H/HeJ mice [60]; mice deficient in myeloid differentiation factor 88 (MyD88), which is common to the signaling pathways of TLR2 and TLR4, do not develop keratitis in response to *O. volvulus* antigens or to isolated *Wolbachia* bacteria [88].

Looking further into *Wolbachia* mediated mechanisms of neutrophil recruitment to the cornea, this study showed that expression of the adhesion molecule PECAM-1 which mediates leukocyte passage across endothelial cells and basement membranes [89, 90] was dependent on the presence of functional TLR4 and *Wolbachia* respectively. TLR4 - mutant mice challenged with untreated OvAg, as well as C57BL/6 mice exposed to OvAg/doxy extracts demonstrated decreased PECAM-1 expression and therefore a decreased number of neutrophils with less pathology. As neutrophils also express PECAM-1, it is likely that homophilic interactions between PECAM-1 on leukocytes and PECAM-1 on endothelial cells [89, 91-93] are involved in neutrophil recruitment to the cornea.

Upregulation of the CXC chemokines KC and MIP-2 - essential mediators for neutrophil recruitment in mice and Il-8 functional homologues – was also found to be dependent on the presence of functional TLR4 and *Wolbachia*. This observation is consistent with a study by Gillette-Ferguson's et al [63], in which isolated *Wolbachia* organisms stimulated in vitro production of MIP-2 and KC by neutrophils in a dose-dependent manner. Similarly, these cytokines were induced by filarial extracts containing *Wolbachia* organisms, but not by *Wolbachia*–depleted extracts.

In summary, the results of this study indicate that the innate immune system and *Wolbachia* endobacteria play an important role in the inflammatory response associated with the pathogenesis of *onchocerca* keratitis which is probably consistent with the following sequence of events: microfilariae invade the cornea, where they eventually die and release *Wolbachia* endobacteria into the confined environment of the corneal stroma. *Wolbachia* products activate the innate inflammatory response through TLR4 and according to Gillette-Ferguson et al also through TLR2 on resident corneal epithelial cells and keratocytes. Activated keratocytes can mature into stromal fibroblasts which produce proinflammatory and CXC chemokines [66]. TLR4 and TLR2 activation also results in PECAM-1 expression on peripheral limbal vessels, facilitating recruitment of neutrophils to

the avascular corneal stroma. As shown in a recent study [63], infiltrating neutrophils in the cornea surround the microfilariae and ingest *Wolbachia* organisms. As neutrophils express functional TLR2 and TLR4, they can produce MIP-2 and KC in response to Wolbachia, which mediates further neutrophil infiltration, degranulation and release of cytotoxic products such as nitric oxide, oxygen free radicals, and matrix metalloproteinase. The cytotoxic effect on keratocytes and corneal endothelial cells, the cells responsible for maintaining corneal clarity, will lead to increased stromal thickness and haze, resulting in visual impairment and eventually blindness (Figure 17).

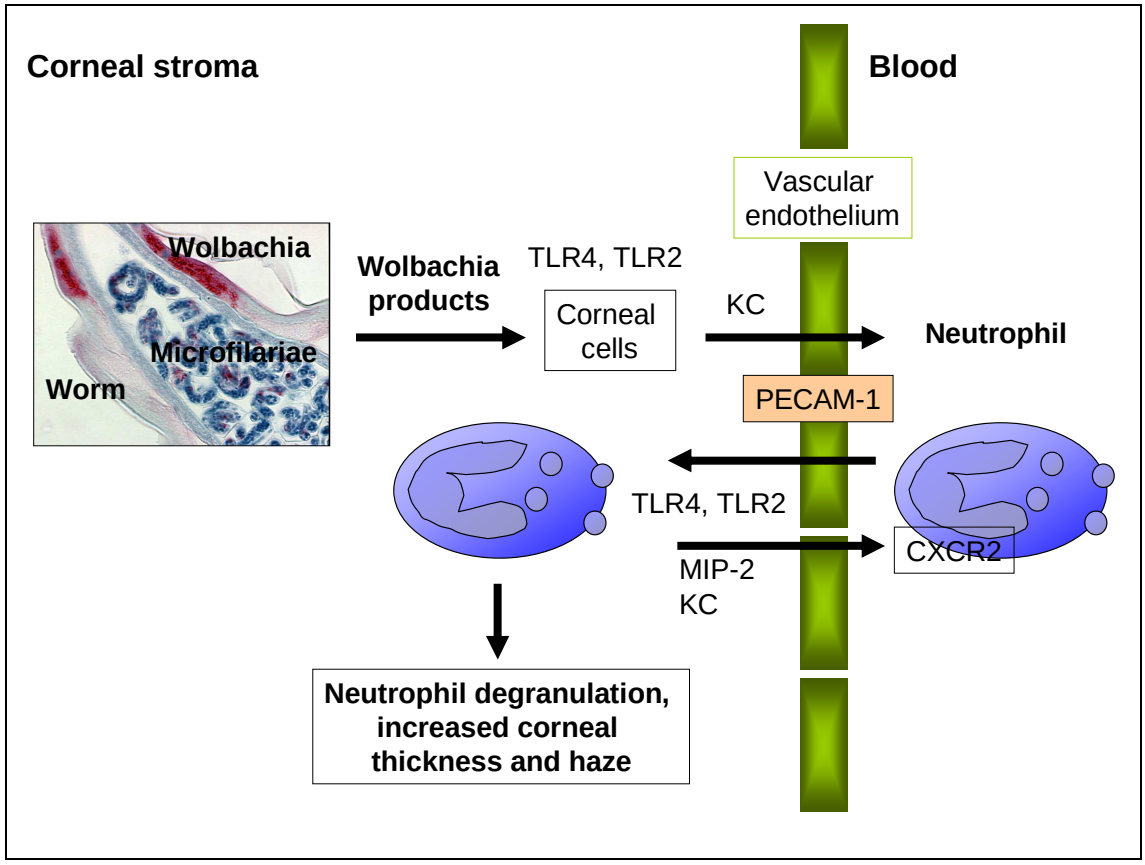

Figure 17 Proposed sequence of events of innate immune responses in *onchocerca* keratitis. See text for detailed explanation. Image by A. von Saint André.

To determine if TLR4 also regulates keratitis in the presence of an adaptive immune response, in a follow-up experiment (data not shown) C3H/HeN and C3H/HeJ were immunized with 3 weekly subcutaneous injections of untreated OvAg extract as a model for chronic infection [94]. Corneas were then injected intrastromally with the same *Wolbachia*-containing extract. Stromal thickness,

stromal haze, and neutrophil infiltration were assessed at 24 to 72 hours, and eosinophil infiltration was examined after 72 hours, when there is a pronounced eosinophil infiltration in immunocompetent mice [50, 52]. TLR4-mutant C3H/HeJ mice had significantly less stromal thickness, stromal haze, and numbers of neutrophils than wild-type C3H/HeN mice. However, there was no significant difference in eosinophil numbers between C3H/HeJ and C3H/HeN mice at 72 hours, indicating that in contrast to neutrophils, eosinophil recruitment to the cornea is not regulated by TLR4. When taken together, these findings demonstrate an essential role for *Wolbachia* and TLR4 in corneal pathology, even in the presence of an adaptive immune response.

Multiple other studies support an important role for *Wolbachia* and the innate response in humans and animals infected with onchocerciasis and lymphatic filariasis. Recombinant, purified *Wolbachia* surface protein (WSP), an abundantly expressed and highly conserved protein in filarial *Wolbachia*, acts as an inducer of the innate human immune system through both TLR2 and TLR4 dependent pathways [62, 95]. Interestingly, WSP was also reported to induce IL-8 production by neutrophils, consistent with an upregulation of its murine equivalents MIP-2 and KC in this study.

Wolbachia are released into the blood following anti-filarial chemotherapy of onchocerciasis, with peak DNA levels correlating with clinical reaction scores [96]. This so-called Mazzotti reaction is characterized by an acute papular onchodermatitis, lymphadenitis, pruritus, rash as well as fever, and sometimes, hypotension that may exacerbate to shock. In the early phase of the Mazzotti reaction a burst of circulating antibacterial acute phase reactants such as TNF-α and Il-6 as well as transient neutrophilia coincide with the above pathological manifestations [38, 77, 96, 97]. Interestingly, locally applied DEC to the skin of infected individuals causes abscess formation that is associated with an intense neutrophil rather than eosinophil infiltrate [98] – likely due to the release of endobacterial products. *Wolbachia* are also responsible for the recruitment and activation of neutrophils in the granulomatous response: Neutrophils infiltrate adult worm subcutaneous nodules in the presence of the endosymbiont, and disappear when *Wolbachia* are cleared using doxycycline [81, 99]. *Wolbachia*-induced cell activation may also result in the abundant binding of defensin, a major neutrophil

constituent, to the surface of adult female *O. volvulus* worms [100]. In addition, neutrophils are thought to be involved in the generation of a cyst at the anterior end of female worms, apparently as an aid for uptake of host nutrients and for facilitating mating with males. Thus, neutrophils recruited by endobacterial chemoattractants may contribute to the survival of the parasite [100, 101]. In tetracycline-treated patients, no accumulations of neutrophils in the vicinity of filariae are observed, and *O. volvulus* extracts from these patients show reduced chemotactic activity [102].

Similarly, in an animal model with the red deer (*C. elaphus*) which is often the host of several *onchocerca* species in the same animal, massive neutrophil infiltration was observed around the endobacteria–positive deer filaria O. *jakutensis*, while it was absent around the endobacteria-free deer filaria O. *flexuosa*. Since onchocercomas of both species were studied from the same animal, variation of host reactivity in different deer can be excluded as a cause for the difference in neutrophil chemotaxis [81].

A recent study comparing the different “forest” and “savanna” strains of *O. volvulus* of West Africa found to have a significantly greater ratio of *Wolbachia* DNA to nuclear DNA in the severe, ocular disease causing “savanna” strain, supporting the role of bacteria in the pathogenesis of ocular onchocerciasis [103].

In summary, all the above studies suggest that *O. volvulus* worms may release *Wolbachia* or *Wolbachia* products and thereby promote innate inflammatory responses both adjacent to the worm and systemically.

In lymphatic filariasis, similarly to the *in vivo* experiments in this study, soluble extracts of *B. malayi* adults or microfilariae also induced a potent innate inflammatory response *in vitro* [60, 63]. The activation of inflammation requires CD14 and TLR4 pattern recognition receptors and the activity is lost after antibiotic depletion of bacteria and is absent from soluble extracts derived from aposymbiotic species (*A. viteae* and *L. Loa*) [60, 104]. Similar to the Mazzotti reaction described above for onchocerciasis, inflammatory reactions also occur following anti-lymphatic filariasis drug treatment particularily in patients with high parasite burdens. Severe adverse reactions are associated with the increase in systemic proinflammatory cytokines and inflammatory mediators [97]. PCR and immunoelectron microscopy analysis of plasma samples following the treatment

of *B. malayi* with DEC showed the persistent presence of *Wolbachia* in patients with severe systemic inflammation [38]. Recently, clinical trials have provided further evidence to support the role of *Wolbachia* in the presentation of adverse reactions: In patients infected with *W. bancrofti*, prior treatment with a three week course of doxycycline to deplete *Wolbachia* prevented moderate adverse reactions to albendazole and ivermectin, whereas for individuals in the placebo group plasma *Wolbachia* levels were related to the incidence of adverse reactions, as well as the levels of pro-inflammatory cytokines and the pre-treatment microfilarial load [105].

In animal models of lymphatic filariasis, the production of TNF-α following the chemotherapy of *B. malayi* microfilariae only occurred in mice with an intact TLR4, suggesting that the release of *Wolbachia* is responsible for this inflammation [60]. Further effects of TLR4 mediated responses have been reported in mice infected with *Wolbachia* harboring *Litomosoides sigmodontis* [106]: In C3H/HeN mice infection results in adult female development including females containing mature microfilariae, but no detection of free microfilariae. Infection of TLR4 mutant C3H/HeJ mice produced worms with an increased fertility and the production of microfilariae, suggesting TLR4 mediated immune regulation of worm fertility.

Taken together, over the past several years there has been a considerable increase in the knowledge of the biological significance of *Wolbachia* for their filarial hosts, and the results of this and the above mentioned studies changed our understanding of the immunopathology of filariasis.

2 *Wolbachia* endobacteria – new targets for therapy

Onchocerciasis is the second most common infectious blinding disease in the developing world, responsible for an estimated 500,000 people with severe visual impairment and another 270,000 with blindness worldwide. A WHO Expert Committee in 1995 [1] estimated that over 120 million people are at risk with some 17.7 million infected, 99 % of whom live in Africa. Onchocerciasis is a disease of remote, rural, poor populations. People have in the past abandoned fertile land along the rivers that harbor the breeding sites of the *Simulium*, for fear of going blind. While the situation has improved due to the above mentioned control

programs in the savannah of West Africa [107], people with unsightly skin lesions still suffer major social and economical consequences [108] and according to a recent epidemiology study, approximately 5% of deaths in endemic areas of West Africa can be attributed to the effects of onchocerciasis [109].

The general idea in mass chemotherapy is to abolish microfilariae in skin and blood, in a large enough proportion of the population so that the cycle of transmission (see Figure 2) is inhibited. Using current tools, onchocerciasis appears to be ineradicable in Africa by both vector control and chemotherapy [7]:

Ivermectin, a macrocyclic lactone [110] acts through inhibition of glutamate-gated chloride channels of microfilariae, effectively immobilizing them. It rapidly reduces the number of skin microfilariae, but depletes them only for a few months, after which they reappear at amounts of 20% or more of pretreatment levels within a year [111]. This seems to be sufficient for transmission to continue [112]. Ivermectin does not kill the long lived adult worms, nor does is permanently stop microfilarial production since its embryocital activity seems to be mainly restricted to the late stages of microfilarial development, leaving early embryogenesis intact [111]. Repeated rounds of treatment reduce fecundity of female worms [113], but this effect is not complete and given the longevity of the adult worm (>14 years), interruption of mass treatment or too low coverage in the population, can quickly lead to reemergence of onchocerciasis [8].

Although the lack of ivermectin resistance is so far obviously encouraging, it is hardly surprising considering also the lack of any comprehensive monitoring strategies. The use of a single drug and the need of sustained treatment for decades have raised concerns over the potential development of resistance in onchocerciasis which were strongly endorsed at the *Conference on Eradicability of Onchocerciasis* in January 2002 [7]. Despite the lack of formal evidence for drug resistance, several cases of ‘non or poor responsiveness’ to treatment of onchocerciasis with ivermectin have been reported [7, 114]. Most recent observations suggest the development of resistant adult parasite populations in Ghana [10]. Also genetic selection of *O. volvulus* by ivermectin has been reported [9, 115-120] and drug resistance to ivermectin has already become a major obstacle to the control of nematode parasites of lifestock worldwide [121]. The need for new drugs to achieve elimination of transmission and hence elimination of onchocerciasis as a public health problem is undisputed. A list of drugs exists

[122-124] which show effects against microfilariae or embryos and thus might be developed for combination with Ivermectin or selected use. However, as reviewed by Awazi [125] development has been stopped since the effects are either inferior to Ivermectin or do not improve its efficacy. Similar to onchocerciasis, in lymphatic filariasis concerns regarding drug resistance exist not only for Ivermectin, but also for the other agents that are the mainstay of treatment, DEC and Albendazole [126].

Because of the essential role for the worms' reproduction and therefore their role as obligatory symbionts in filarial nematodes, *Wolbachia* have emerged as the only target for chemotherapy that results in long-term sterility of the worms in human onchocerciasis [127, 128], a priority research objective for the WHO for the last 20 years [1]. The fact that doxycycline is already registered allowed for a quick transition from animal to phase IIa studies on human onchocerciasis. Initial trials using a six week course of doxycycline led to depletion of *Wolbachia* and resulted in a block of embryogenesis in adult worms, which persisted for up to 2 years after start of treatment [31, 127, 129]. In contrast, patients treated with ivermectin alone showed an increase in microfilarial counts as early as four months after administration of ivermectin. The apparent permanent block in embryogenesis after doxycycline treatment was reflected in sustained reduction in skin microfilariae. A limited but significant macrofilaricidal effect was also observed, which had been reported earlier in bovine onchocerciasis [33]. Depletion of *Wolbachia* and sustained amicrofilaremia for almost 2 years after doxycycline treatment was also demonstrated in human lymphatic filariasis patients infected with *W. bangrofti* [130]. A recent placebo-controlled trial in humans infected with *W. bangrofti* has shown a clear macrofilaricidal effect of doxycycline when administered for 8 weeks at 200 mg/day, associated with 95% reduction of *Wolbachia* levels [131]. The results from human trials thus far have unequivocally demonstrated the superior pharmacological efficacy of doxycycline for both onchocerciasis and lymphatic filariasis. The long period of treatment (six weeks or more) and the known contraindications to doxycycline (pregnant or breastfeeding women, children up to 9 years of age) preclude its application for mass treatment at the moment. Further research is needed to exploit the principle of targeting *Wolbachia* in several ways, using other already existing antibiotics,

combinations and designing new drugs on the basis of the information provided by the *Wolbachia* and filarial genome data.

So far, as agreed at the conferences in Hamburg and Atlanta [7, 132], individual treatment of people with imported infection or those leaving an endemic area for a long time are indications for doxycycline (100 mg/ day for six weeks). Another indication could be in formerly endemic areas where a high level of control has already been achieved and re-emergence should be prevented. *Wolbachia*-targeting antibiotics may also improve compliance with mass treatment programs by reducing side-effects due to the release of intact bacteria and *Wolbachia* products from dying nematodes [38]. Pretreatment targeting *Wolbachia* may abrogate inflammatory responses and allow a wider range of antifilarial drugs to be used – not only for onchocerciasis, but also for other filarial diseases.

In conclusion, the discovery of endosymbiotic bacteria infecting most species of filarial nematodes that are pathogenic to humans has opened exciting new avenues of research into the pathogenesis and immunology of filarial infections. In fact, the results of this study suggest a complete alteration in our understanding of the immunopathology of onchocerciasis. It is not simply the worm, but the *Wolbachia* endosymbiont that is a major contributor to the development of ocular onchocerciasis. *Wolbachia* therefore seems to have a double role as a new target for therapy: Firstly, clearance of *Wolbachia* by antibiotic treatment may reduce and prevent ocular onchocerciasis, since *Wolbachia* plays an essential role in the pathogenesis of this disease. Secondly, targeting *Wolbachia* leads to long-term sterilizing and possibly macrofilaricidal effects that have the potential to eliminate onchocerciasis as a public health problem.

SUMMARY

Introduction: This study investigates the role of *Wolbachia* bacteria in the pathogenesis of *O. volvulus* keratitis in a mouse model. *Wolbachia* bacteria are essential symbionts of most filarial nematodes of importance for mankind.

Methods: Using a mouse model for river blindness in which soluble extracts of filarial nematodes are injected in the corneal stroma, changes in stromal thickness and haze of the cornea are observed by in vivo confocal microscopy, followed by immunohistochemical staining for neutrophils and PECAM-1, as well as ELISA of corneal chemokines. Reactions to filarial extracts containing *Wolbachia* are compared to those without the endosymbiont.

Results: The approach of characterizing *Wolbachia*'s role in river blindness in this study is threefold. Firstly, *Wolbachia*-depleted extracts from doxycycline treated onchocerciasis patients led to a diminished inflammatory response in corneas of C57BL/6 mice compared to untreated, i.e. *Wolbachia* containing antigen. The decreased cell recruitment observed with doxycycline treated extracts involved neutrophils, but not eosinophils. This finding demonstrated that the presence of *Wolbachia* increases neutrophil recruitment. Secondly, extracts from *Wolbachia*-containing *B. malayi* revealed markedly more pathology than endosymbiont-free *A. viteae* antigen. This again pointed at the role of *Wolbachia* in development of disease. Thirdly, Toll-like Receptor 4 (TLR4) dependence was shown to exist for the inflammatory response to *Wolbachia* harboring *O. volvulus* antigen by looking at the corneal pathology in TLR4-mutant C3H/HeJ mice, compared to the wild-type C3H/HeN strain. Investigating further *Wolbachia* mediated mechanisms of neutrophil recruitment to the cornea, this study also showed that expression of the adhesion molecule PECAM-1 in limbal vessels, as well as upregulation of the CXC chemokines KC and MIP-2 were dependent on the presence of functional TLR4 and *Wolbachia* respectively.

Conclusions: This study indicates that the innate immune system and *Wolbachia* endobacteria play an important role in the inflammatory response associated with the pathogenesis of *onchocerca* keratitis, suggesting a complete alteration in our understanding of the immunopathology of filariasis.

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ACKNOLEDGEMENTS

I thank Professor Eric Pearlman for his help and advice in the research and publication of this study.

I thank Professor Dr. med. Matthias Frosch and the Studienstiftung des Deutschen Volkes for their great support of this thesis.

Thanks to Helen Running for proof reading this work.

My thanks to: my parents Achim und Bettina von Saint Andre – von Arnim; my aunt Almut von Welck; my husband and son Seth and Achim Bagan.

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