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Immune response to ocular gene therapy with AAV8

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1 Abbreviations

AAV – Adeno-associated Virus

ACAID – Anterior-chamber-associated Immune deviation

AMD – Age related Macular Degeneration

α-MSH – α-melanocyte stimulating hormone

APC – Antigen presenting cell

CGRP – calcitonin gene-related peptide

EAU – Experimental autoimmune uveoretinitis

EMA – European Medical Agency

FC – Fold change

GFAP – Glial fibrillary acidic protein

GFP – Green fluorescent protein

INF – interferon

ITR – inverted terminal repeat

LCA – Lebers Congenital Amaurosis

NHP – non human primate

NK cell – natural killer cell

NKT cell – natural killer T cell

NOD – nucleotide-binding oligomerization domain

OCT – optical coherence tomography

PAMP – pathogen-associated molecular pattern

PE cell – pigment epithelium cell

PFA - paraformaldehyde

PRR – pattern recognition receptors

RIG – retinoic acid inducible gene 1

RP – Retinits Pigmentosa

TGFβ - transforming growth factor β

TSP - thrombospondin

VIP – vasoactive intestinal peptide

XLRS – X-linked retinoschisis

2 Introduction

2.1 Aim of the study

Adeno-associated viral vector (AAV) have established themselves as powerful tools for retinal gene therapy. Multiple clinical trials have shown that AAV mediated gene therapies can be safely administered to the subretinal space, efficiently deliver the therapeutic gene to the cells of the retina and ultimately improve the visual function of the patient.

The safety of the AAV mediated ocular gene therapy has been attributed to the non-immunogenic properties of AAV and the special anti-inflammatory mechanisms that constitute the immune privilege of the eye.

This assumption is recently being challenged by an increasing number of clinical trials reporting cases of intraocular inflammation following AAV mediated gene therapy treatment. These findings suggest that the immune privilege mechanisms of the eye can be overstressed and evoke the question whether the immunogenic potential of AAV has been underestimated. Although the inflammation was in most cases manageable under steroid treatment, in some cases the local immune response not only impaired the treatment effect but lead to a decline of visual function of the patient[1, 2].

Preventing these serious adverse events is of highest interest to patients and researches alike. To do this, a profound understanding of the immune response is essential. However, although the ocular immune privilege has been studied intensively, many questions remain unanswered and little is known about how the retinal immune system reacts upon the contact with AAV. It is unclear which part of the vector particle leads to inflammation, how the vector is sensed by the innate immunity and which cells are involved in the immune reaction.

It was therefore the aim of this dissertation to shed a light on the mechanisms of the ocular immune response to subretinal AAV8.

To elucidate the mechanisms involved in AAV immunity, we used the data and the material of a study that was primarily designed as a good laboratory practice (GLP) conform toxicology and biodistribution study for an investigational new drug (IND) application with the national competent authority (Paul Ehrlich Institute). Data from

nonhuman primates that were treated with different doses of subretinal und intravitreal AAV8 were compared to clinical data from three patients treated with the same vector.
In order to approach different aspects of AAV immunity, several assays were used: Systemic humoral immunity was assessed by capsid-antigen ELISA and the local immune response was investigated by immunohistochemistry. To study the local immune response on a molecular level, an expression profile of whole retinal tissue was conducted. This gene assay included receptor molecules as well as downstream proteins of the different signaling pathways.
In this study we present data suggesting that AAV8 is sensed by innate antiviral receptors and activates immune competent cells of the innate and adaptive immune system in the retina. This study will guide future investigators in conducting studies to enhance safety and efficacy of AAV vector mediated gene therapies.

2.2 Gene therapy overview and short history

The general concept of gene therapy is to deliver a therapeutic nucleic acid into a target cell in order to treat the genetic condition that is causing the disease.
This concept promises a very elegant treatment solution for many genetic diseases. Moreover, it offers a one-shot treatment option for diseases where otherwise no treatment option is at hand or only extensive and time-consuming treatments are available. As appealing as this concept is, as many difficulties are there to face in order to develop such a treatment option. Challenges in gene therapy are the immune response to foreign bodies like viral vectors, the (limited) transduction efficiency, the (limited) specificity to the target cells and the need for a long-lasting treatment effect.
These multiple obstacles require a spectrum of different approaches comprised in the term “gene therapy”.
For example, gene therapy concepts can be divided into in vivo and ex vivo concepts. In vivo gene therapy is a concept where vector particles are injected into the blood stream or directly into to the diseased tissue. In *ex vivo* gene therapy the target cells are treated in vitro and afterwards reinfused into the patient’s body.
Other differences exist in the function of the nucleic acid. Currently, most gene therapy strategies in the clinical setting introduce a healthy copy of the affected gene into the

target cell, a strategy dubbed gene augmentation therapy. This works well for recessive gene mutations with a reduced or complete loss of function of the affected gene. For dominant or more complex genetic diseases new concepts exist that use nucleic acids for silencing or editing genetic expression.

A third category by which gene therapies can be divided by is the delivery system of the therapeutic nucleic acid into the target cell. With respect to the delivery system, two broad categories exist: Nonviral physico-chemical approaches and recombinant viral vectors. Viral vectors like AAV are currently the most popular approach and account for more than two thirds of all gene therapy trials today.[3] The popularity of viral vectors derives from their superior stability and efficacy in transducing target cells compared to nonviral vectors. The most commonly used viral vectors are adenoviral, retroviral and adeno-associated viral vectors (AAV).[3] Synthetical nonviral vectors, on the other side, offer lower risk of immunogenicity, no risk of mutagenesis, easier synthesis and greater capacities in packaging large molecules.[4] Nonviral vectors are most often naked plasmids or less often liposomal molecules.

All these different approaches have evolved in a period of around 40 years[5]. The translation to patient care came in 1990 when the FDA approved the first viral vector mediated gene therapy trial in humans. Two children with adenosine desaminase deficiency (ADA-SCID) were treated effectively and without severe adverse effects with a retroviral vector.[6]

The euphoria that these results created led to an expansion of the field. In the following years trials followed for other primary immune deficiencies[7], for lipoprotein-lipase-deficiency[8], hemophilia B[9] and Beta-hemoglobinopathy.[10] By 2018, 2300 gene therapy trials have been completed, are ongoing or approved worldwide.[3] The first commercially available gene therapy was approved for therapy by the EMA in 2012: an AAV based gene therapy for lipoprotein-lipase-deficiency, called Glybera, was the first gene therapy that made the way from bench to bedside.[11]

However, the optimism of the field was also repeatedly dampened by disappointing clinical trial results. The following 2 events of uncontrolled immune responses and carcinogenesis were major setbacks in gene therapy development.

10 years after the FDA had approved the therapy for ADA-SCID, another study using a retroviral vector for a similar disease, X-linked severe combined immunodeficiency

(SCID-X1) was enrolled and initially presented sustained correction of the immunodeficiency. In the 2-year follow-up though, the group had to report that the risk of insertional mutagenesis by the retroviral vector had been underestimated and 2 out of the 10 treated children had developed a leukemia-like syndrome.[12]

In a similar way, the risk of viral vectors eliciting a vigorous immune response was underestimated with tragic effects, when the 18-year-old patient Jesse Gelsinger died of systemic inflammatory response syndrome after adenoviral (AdV) gene transfer.[13]

These examples illustrate the importance of further research to ensure safety in viral vector mediated gene therapy development. Although recombinant AAV (rAAV) carries a dramatically decreased risk of insertional mutagenesis than retroviral vectors and is less immunogenic than adenovirus (AdV) the broad use of rAAV in multiple clinical studies and accumulating reports of inflammatory responses[1, 2] highlight the relevance of this research area.

2.3 Ocular gene therapy

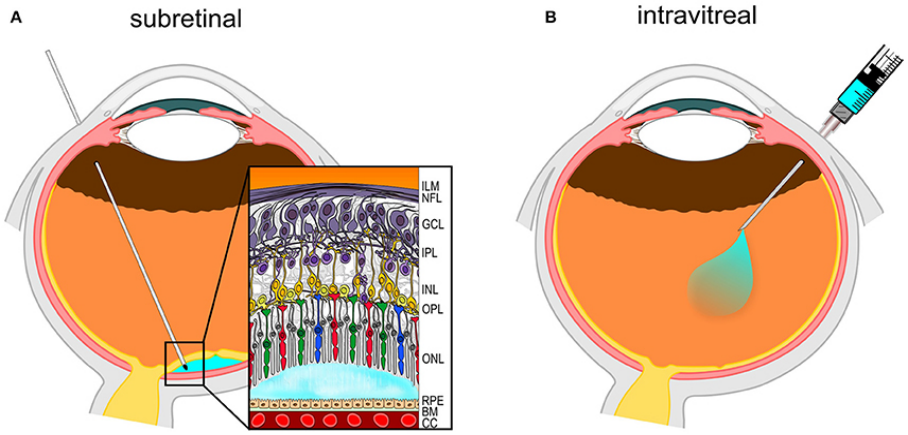
The eye has been at the forefront of gene therapy development for the following reasons: a) for blinding disorders like inherited retinal degenerations no or only insufficient medical treatment is at hand b) the eye as a bilateral organ offers a perfect internal control and is easily accessible for therapeutics and diagnostics c) the eye is immune-privileged, described first by Medawar in 1948[14] and therefore regarded as a relatively safe space to administer a foreign body like a virus.

The need for treatment options for inherited retinal degenerations is highlighted by the fact that, although the individual diseases are quite rare, as a group of disorders they affect over 2 million people worldwide.[15] In contrast to multigenic and multifactorial diseases like AMD, inherited retinal diseases are of monogenetic origin and therefore the optimal basis for gene replacement therapies. Another precondition for the advancement of ocular gene therapy development was the pace of scientific discovery of the underlying genetic mechanisms, made possible by the enormous technical development in genetic sequencing. In 1990, for the first time a point mutation in the rhodopsin gene for a form of retinitis pigmentosa (RP) was described.[16] Today the number of genes known to cause retinal diseases has risen to over 300 (RetNet: https://sph.uth.edu/retnet/)

Targeted monogenetic diseases include Leber's congenital amaurosis (LCA), Choroideremia, Achromatopsia, juvenile X-linked Retinoschisis, Stargardt disease, and other forms of RP.[17]

Since all of these diseases affect the retina, basically two different options exist for the application of ocular gene therapies: The vector solution can either be delivered to the retina by a subretinal or an intravitreal injection. For the subretinal delivery, a pars plana vitrectomy is performed, followed by the injection of around 200µl of volume into the subretinal space where a small bleb is created by the injection. The intravitreal injection on the other side is performed like the common intravitreal injection of medications for diseases like AMD. However, it was shown in the animal model that when cells of the outer retina are targeted, intravitreal injections are not as successful in restoring retinal function.[18] As reviewed by Gupta et al.[17], with one exception, all active and completed gene therapy trials for retinal degeneration by the end of 2017, used the subretinal approach.

The advantage of the subretinal approach is the intraoperatively vision-guided selection of treatment area and the proximity of vector solution to RPE and outer retina. Nevertheless, subretinal injections are the more complicated procedure with the inherent risks of temporary retinal detachment[19]. Complications like a macular hole formation, unresolved retinal detachment, choroidal effusions and the loss of foveal thickness have been described by the RPE65 trials[20-22]. Methods like the two-step procedure originally described by Bainbridge et al.[23], or intraoperative OCT[24] help to minimize the trauma of subretinal injections.



Gene therapy application through pars-plana injection into **A** the subretinal space between photoreceptors and the RPE and **B** into the intravitreal cavity.
Figure from Ochakovski et al.[25]

The first promising results for retinal gene therapy came from the RPE65 studies targeting Leber's congenital amaurosis (LCA). This condition was first described by Theodor Leber in the 19th century as a severe blinding disorder that accounts for 5% of all inherited retinal dystrophies. The mutation in the gene *RPE65* was identified in humans suffering of LCA in 1997[26]. Veske et al.[27] discovered only two years later that the blinded Briard dog[28] was also carrying a mutation in the canine homologue of *RPE65*. After it had been demonstrated that the *Rpe65* mutated Briard dog could effectively be treated with an AAV2 virus carrying the healthy *RPE65* gene[18], in 2008 three groups published initial reports of clinical trials in LCA patients using the same strategy. While functional gain in the treated patients was limited, no serious adverse events or systemic side effects were observed. [20, 22, 23, 29] The excitement that these results provoked gave the basis for future studies to follow. The scientific progress culminated in the first phase 3 study being successfully completed in 2017 and the approval of the first ocular gene therapeutic product for LCA by the FDA in 2018.[30]

However, the long-term follow-up of the LCA patients[1, 21, 31] also revealed the challenges of ocular gene therapies. Major challenges are efficacy and the longevity of the effect. The immune response was in general mild and manageable and will be discussed in more detail in the section "Immune response to AAV". In terms of efficacy the results somewhat varied. Whereas one group could report a gain in visual acuity for up to three years[32], the other groups could not show beneficial treatment effects in visual acuity but improved outcome measures of retinal sensitivity like microperimetry or pupillary responses[1, 21]. Concerning the longevity of effect, in the long-term follow up, the observation was made by several groups, that although function can be improved after injection, the degeneration of the retina is not necessarily abated and continues over the following years.[1, 33, 34] Functional benefits were found to decline after 4.5-6 years[34].

When looking into new target genes, a more general challenge of gene therapy which is not restricted to diseases of the eye, are the limitations imposed by the gene itself. The clinical trials of today rely on gene replacement of a nonfunctional protein. In cases where a mutation leads to a truncated protein with a negative effect on the functional protein, simple gene augmentation or replacement strategies may be insufficient. Here, new techniques of genome editing come into play that might be able to provide a solution for the treatment of these mutations in the future.

Secondly, the selection of target genes is limited through the packaging size of the delivery system. The AAV capsid is able to accommodate a 4.7kb genome. Techniques to overcome this problem are the use of viral vectors with larger capacity like lentiviruses, the minimization of promotors[35] or the simultaneous co-transduction with dual AAVs[36] with the aim of homologous recombination of overlapping nucleotide sequences.

2.4 Adeno-associated virus as vector

In order to introduce DNA into the host cell, the DNA molecule has to be protected from degradation before reaching the cell, then cross the cell membrane and escape the cells internal defense mechanisms to successfully advance into the cell nucleus. Synthetic nonviral particles are able to effectively transfect cells in-vitro but show minor efficiency in in-vivo experiments. Viruses, like AAV, have evolutionally perfected their capability to introduce their genome into the host cell over millions of years and are therefore regarded as highly effective and promising vectors for gene therapy.[37]

AAV was discovered in 1965 as a virus particle that can be found in the presence of an adenovirus but is itself replication defective.[38, 39] Because of its dependency on adenovirus to replicate, it was titled Adeno-associated virus. AAV belongs to the family of Parvoviridae, has a small non-enveloped icosahedral capsid and is about 25nm in diameter. In the last 50 years AAV became more and more experimentally characterized: different serotypes were identified[40], the 4.7 kb ssDNA was identified as the genome of AAV[41] and the two palindromic sequences flanking the genome, called inverted terminal repeats (ITR), were identified as the origin of genome replication.[42-44] Apart from the ITRs, the wildtype AAV genome encodes for four proteins required for replication called Rep Proteins, three capsid proteins (VP1-3) and the assembly-activating protein (AAP)[45]. In the absence of Adenovirus, AAV can persist in the cell in a latent stage.[46] This discovery led to the idea for the use of AAV in gene therapy.

For gene therapy the Rep and Cap genes are removed from the AAV genome and replaced by what is called a “transgene expression cassette” flanked only by the ITRs on each side.[47] The transgene expression cassette contains a promotor sequence and the open reading frame defined by the transgene. The selection of the promotor is carefully done

by the investigator, as the expression varies according to the different host cell types and its size impacts on the space left for the therapeutic gene.

A common production process for a recombinant AAV vector is to transfect HEK293 cells with three plasmids, one containing the transgene expression cassette flanked by the ITRs, one containing the rep and cap genes and another plasmid containing genes from adenovirus necessary for replication.

The cap genes define the capsid protein structure and the resulting serotype. The definition of a unique serotype is a virus that cannot be recognized by neutralizing antibodies generated against another serotype of the virus.[48] Pioneering work for the use of AAV in gene therapy was done with the serotype 2.[49]

Until today at least 13 different serotypes have been described and each of these serotypes presents a specific cell tropism.[50] The serotype can be purposefully selected to enhance transduction efficiency in the target cells. For example, AAV2, is very efficient in transducing RPE cells of the retina, a reasons for which it is used by the initial ocular gene therapy trials for RPE65 - LCA.[51] When targeting photoreceptors though, at least in the monkey retina, AAV8 for example was shown to be superior to AAV2.[51]

Further on, the efficiency and the tissue tropism can be enhanced by combining the genome from one serotype with the capsid from another, a process called "pseudotyping".[52, 53] Pseudotyped vector AAV2/5 for example is composed of the genome from AAV2 and the capsid from AAV5 and has been found to be very specific to the outer nuclear layer of the human retina.[54]

The uptake of AAV into the cells is mediated by various receptor molecules. The first to be described was heparan sulfat proteoglycan. This membrane-bound glycan facilitates the uptake of AAV2[55]. Different glycans were found to be responsible for other serotypes and various co-receptors were described.[56] In 2016 a universal AAV receptor (AAVR) was identified to serve multiple serotypes.[57] The relationship between all these receptors remains unknown.[56] After binding, AAV is internalized by endocytosis. This can happen in a clathrin-dependent or -independent manner[58, 59].

In the next step, endosomal escape is mediated by endosomal processing that involves conformational changes where the N-terminal domains of the VP1 and VP2 capsid protein are externalized.[60, 61] The complete vector particle then passes the nucleus membrane, followed by uncoating and genome release.[62]

Once in the nucleus the genome uses the host cells translational machinery to produce the viral proteins or in the case of rAAV, the transgenic protein. This process does not demand an integration into the host cells genome. Only approximately 0.1% of wild type AAV genomes integrate[63, 64], whereas the rest persists as double stranded episomal concatamers.[65] This integration rate is much lower in case of recombinant AAV particles utilized for clinical gene therapy as explained below.

In the case of dividing target cells, non-integration constitutes the disadvantage of genomic dilution as each daughter cell only inherits half of the non-integrated transgene copies. At the same time, lack of integration reduces the risk of mutagenic insertions. All available data show that the carcinogenic potential of recombinant AAV is extremely low, but is probably not zero. It is known that wildtype AAV integrates preferably on the AAVS1 site on chromosome 19 by an active Rep binding element of AAVS1[63, 64]. Recombinant AAV vectors, which are not provided with the Rep gene, can not integrate via the Rep binding element of AAVS1 but are able to integrate via non-homologous integration at sites of DNA damage or very specifically at homologous locations[66]. It has been shown in the mouse model that in some situations recombinant AAV can induce hepatocellular carcinoma by integrational mutagenesis, although this has never been observed in other animal models or humans[67].

2.5 Innate and adaptive immune response

Our immune system is a complex network of different defence mechanisms against pathogenic organisms like viruses, bacteria, fungi and parasites. Two broad categories of defence lines can be theoretically distinguished from another although they interact over multiple ways.

The innate immune response acts quickly, non-specific and does not generate immunological memory. It consists of anatomical barriers, antimicrobial proteins, the complement system and non-specific immune cells. The cells, mostly from the myeloid lineage, comprise macrophages, granulocytes, mast cells and dendritic cells. They are important sensor cells that express pattern recognition receptors (PRRs) which recognize foreign structures on the molecular surface of invading pathogens, also called pathogen-associated molecular patterns (PAMPs). Members of the PRRs are the transmembrane

proteins like the Toll-like receptors (TLRs) that sense extracellular or endo-vesicular foreign pathogens, or cytoplasmic proteins like the retinoic acid inducible gene 1 (RIG)-like and the nucleotide-binding oligomerization domain (NOD) -like receptors which have the ability to detect intracellular invasion of pathogens. The recognition of PAMPs by the PRRs leads over various signalling pathways to the activation of NFκB (nuclear factor κB) and interferon regulatory factors (IRFs) which are essential for the release of mediators like pro-inflammatory cytokines and chemokines. Cytokines are a way of communication between different immune cells and a connecting element to the adaptive immune response. Chemokines act as chemoattractants, guiding cells from the bloodstream to the infected tissue. Viral infection induces the production of cytokines called interferons (INF). Interferons can be distinguished into Type 1 (INF-α, INF-β) and Type 2 (INF-γ). Type 1 Interferons are released by many cell types after viral infection and lead to the induction of antiviral host cell activity, increased MHC class I expression and antigen presentation, activation of dendritic cells, macrophages and NK-cells. The antiviral host cell activity is mediated by Interferon-stimulated genes (ISG). Known antiviral ISGs are for example the Mx protein, which sequesters viral ribonucleoproteins, the proteinkinase R, which inhibits viral protein translation and OAS proteins that degrade viral RNA via the activation of Rnase L[68].

The presentation of viral antigens via MHC class I makes the infected cells susceptible to being killed by CD8 cytotoxic T cells of the adaptive immune response.

The adaptive immune response needs time to develop but is more efficient in eliminating a specific pathogen and has the ability to establish immunological memory.

When an antigen is presented for the second time, the immunological memory allows the adaptive immune response to launch a much faster immune response. The adaptive immune response consists of antigen-specific lymphocytes. Two major different types of lymphocytes exist: B- and T-Lymphocytes. B-lymphocytes or B cells will proliferate and differentiate into antibody - producing Plasma cells after contact with their antigen. When a T-lymphocyte or T cell recognizes its specific antigen, the T cell will proliferate and differentiate into different effector subtypes, like cytotoxic T cells (CD8 positive), helper T cells (CD4 positive) and regulatory T cells. Cytotoxic T cells kill infected host cells, helper T cells support other immune cells like antibody producing B cells and regulatory T cells are able to suppress the activity of other immune cells. The CD4 T helper cell can

further by divided into Th1 and Th2 cells. Th1 cells can be characterized by the production of INF-γ whereas Th2 cells mainly produce IL4 und 5. INF-γ, also produced by NK-cells and cytotoxic CD8 T cells is responsible for the classical activation of macrophages and is able to increase the expression of and the antigen presentation via MHC Class I and II.

2.6 Immune response of the eye

An immune response in the eye can be especially deleterious to the important sensory function of vision, as it can lead to opacities in any of the transparent structures or induce irreparable injury to the non-dividing cells of the retina. For this reason, the human body has developed a complex array of immune mechanisms that protect the eye against pathogens without intense and destructive inflammation. Structures like the cornea and lens are therefore poorly vascularized, lymphatic drainage of the eye is limited and the blood ocular barrier is preventing unselective exchange of molecules.

The blood ocular barrier features two components: the blood – aqueous barrier and the blood-retina barrier. The blood -aqueous barrier is formed by the non-pigmented layer of the ciliary body epithelium (PE) and by the endothelium of irideal capillaries.[69] The blood-retinal barrier is divided into the inner blood retina barrier, made up of adherens- and tight-junctions between the endothelial cells of the capillaries and the outer blood retina barrier composed of similar junctions between the RPE cells.[70]

Apart from these anatomical features, the eye has been found to exhibit a special down-regulatory immune environment. Peter Medawar who observed prolonged survival of tissue grafts placed into the anterior chamber of the eye was the first to describe this immune privilege.[14] The so called Anterior-chamber-associated Immune deviation (ACAID) was later extended to the intravitreal and subretinal space as well[71, 72]. The mechanisms that constitute this privileged immune response include anti-inflammatory molecules and immune suppressive cells.

The ocular-splenic axis of the ACAID involves antigen presenting cells (APCs) that leave the eye despite the absence of draining lymphatics and circulate to the spleen where specific tolerance against the presented antigen is induced. Once arrived in the spleen, the APCs, together with joining natural killer T cells (NKT cells) and B cells orchestrate the deviant systemic immune response by inducing antigen specific regulatory T cells. The regulatory T cells (Treg), positive for CD25 and FoxP3, can be of the CD4 “afferent” or the CD8 “efferent” type. They suppress the induction of a Th1 and Th2 cell response and inhibit B cells from switching to complement binding immunoglobulin isotypes. The delayed-type hypersensitivity (DTH) reaction to the introduced antigen is supressed.

Locally, an intraocular immunosuppressive microenvironment of TGF-β2, α-MSH, VIP, TSP, CGRP and MIF promotes the immune privilege by modulating the T cells into Treg

cells, preventing T cells to secrete INF-γ, suppress NK cell function and inhibit the production of nitric oxide by activated macrophages[73].

Ocular resident cells like PE cells of the iris or the retina possess the ability to actively convert effector T cells into regulatory T cells via the secretion of TGF-β2, TSP and cytotoxic T-lymphocyte-associated antigen 2α (CTLA2α)[74].

Beside the soluble immunosuppressive factors, membrane bound CD95L or FasL is expressed on all parenchymal cells in the eye and triggers the death of invading immune cells[75].

Despite these mechanisms there seems to be a difference between the intravitreal and the subretinal space in terms of antigen presentation to the host immune system that favours the subretinal space: Preclinical NHP studies with AAV mediated gene therapy for AMD and Leber's hereditary optic neuropathy have shown that intravitreal administered vector solutions can lead to mild and moderate inflammation at dose (2.4 x10^10 vg/ml) that is relatively low when compared to doses given in subretinal studies[76]. Secondly, animal studies in mice and NHP have shown that successful intravitreal or subretinal re-administration of vector is blocked by NABs after initial intravitreal but not after subretinal application[77, 78].

2.7 Immune response to rAAV

Generally, AAV is considered to be well-tolerable. In contrast to Adenovirus, which was also tested for retinal gene therapy but given up for its immunogenicity[79], AAV has been safely administered to the human subretinal space in various clinical trials.

Although epidemiological studies have shown that depending on serotype and country 30-80% of the population have been in natural contact with the ubiquitous virus AAV[80], the wtAAV has never been associated with any disease.

On the other hand, recombinant AAV vectors are known to be sensed by the innate immune system and have been observed to elicit adaptive humoral and cellular immune responses capable of inhibiting successful therapy. The immunogenicity of rAAV is hence controversially discussed. Many confounding factors contribute to the complexity of the question.

First, although nearly identical, the recombinant vector AAV is not the same as the wtAAV. Devoid of the capability to replicate and produce its normal viral capsid proteins, both of which are triggers of anti-viral immune responses, it is very likely that the immune response against rAAV has its own characteristics which can not be deducted from the immune response against wtAAV.

Secondly, as mentioned above, our immune system is able to react differently depending on the place of administration of the viral vector. Some organs like the eye are immunologically privileged, making the comparison between studies for different target diseases and different application routes difficult.

Finally, pre-clinical safety studies revealed that animal models do not perfectly predict the immune response to AAV in humans[81]. A possible explanation is the difference between the immune systems of rodents, larger animals or even NHP and humans. For example, the expansion of capsid specific T cells in humans treated with AAV for hemophilia B was unexpected, as it had not been observed in the pre-clinical cynomolgus monkey model[81]. This was later attributed to the difference in T cell activation. The T cells of humans are known to be more proliferative after activation due to the loss of inhibitory surface protein (SIGLEC) expression in human evolution[82].

Besides the immunogenic potential of the viral proteins, an additional question of rAAV immunogenicity is the role of the transgene. Principally, potential immunogenic parts of the AAV vector not only include the capsid epitopes but also the transgene product. Especially in cases of null-mutated patients, one could expect that the transgene product would be immunogenic to the host immune system. In the animal model this was shown in the case of F.IX gene replacement therapy for Hemophilia B. Cao et al. showed that the strength of the immune response correlated inversely with the degree of conservation of the endogenous F.IX coding information and thus null mutations leading to the strongest immune responses[83].

Luckily for the F.IX therapy development, it was also shown that hepatic gene transfer resulted in immunological tolerance induction which limited the transgene-directed immune response via Treg cells. In the eye, transgene immunity has not been reported making it unclear whether similar tolerance mechanisms to the transgene could play a role in ocular gene therapy.

Secondly, it is difficult to draw conclusive answers about transgene immunity from preclinical animal models as even in target genes which high homology, the actual antigenic potential of the respective protein product is usually not known. In humans, transgene immunity was neither observed against RPE65 in the initial LCA trials nor against F.IX in the hemophilia B trials[84, 85].

In order to monitor the immune response against rAAV, preclinical and clinical safety and efficacy studies for different target diseases have most often used the following assays[84]:

1. The humoral immune response has been assed using a Capsid protein ELISA to identify seroconversion and quantity capsid-specific antibodies. Additionally, the Goldmann-Witmer coefficient has been used to detect site-specific antibody production.
2. In vitro transduction assays in the presence of a test serum, called neutralizing antibody assays, have been used to functionally monitor the ability of antibodies in the test serum to inhibit successful transduction.
3. Cell-mediated immunity has been examined by enzyme linked immunosorbent spot (ELISPOT). This assay uses the secreted cytokines like INF-γ as a marker of activation upon antigen exposure.
4. Flow cytometry has been used as a way of quantifying the type and level of activation of immune cells via CD69 expression following exposure to antigen such as AAV capsid proteins.
5. Morphological changes of the tissue and infiltrating immune cells have been visualized in animals by histological methods.

2.7.1 Immune response to rAAV in the eye

For the eye, the results of the RPE65 trials for LCA provided the first patient data of immune responses to AAV. The results between the different groups varied a little which might be a reflection of the differences in vector and dose.

The group at the University College London (UCL, Bainbridge et al.) reported that intraocular inflammation and immune responses had been observed in 5 out of 8 patients of the high dose group (1x10^12 viral genomes), but in none of the 4 patients of the low dose group (1x10^11 vg) within three years of follow up after gene therapy with rAAV2.

Findings included posterior and anterior intraocular inflammation in three patients, one showing also elevated neutralizing antibodies and increased circulating T-cells reactive to the AAV2 capsid. No one presented antibodies reactive against the RPE65 protein. Except for one patient, where anterior uveitis was followed by macular pigmentary changes and persistent reduction in visual acuity, inflammation appeared none deleterious and responsive to glucocorticoid treatment.[1]

The group at University of Florida (Hauswirth et al.) reported that all eyes (n=6) recovered completely under steroid treatment and that the only adverse events were related to surgery. Neutralizing antibodies were observed in only two cases but interpreted as coincidence since other patients also showed similar titer elevations at timepoints where the contact with wtAAV would have been the more likely cause. T-cell responses to the AAV capsid were not observed.[21]

The group at the University of Pennsylvania (UPenn, Maguire et al.), which has published a phase III clinical trial including 20 patients, also did not report any serious adverse events related to the test item. Only 2 patients showed signs of intraocular inflammation which completely resolved under steroid treatment[30]. INF-γ ELLISPOT assays did not detect capsid directed T-cell responses[22]. The group has also been able to show that the re-administration of the same vector to the contralateral eye is safe and efficacious[86, 87]. Even high neutralizing antibodies at baseline had no negative effect on the efficacy of administration to the second eye[87]. Further on, this group investigated T-cell immunity against the RPE65 transgene product, which was found to be negative.[22, 31]

One can conclude different important facts from these trials. Immune responses were generally benign and resolved under steroid treatment. The fact that treatment of the second eye is so successful suggests that no relevant adaptive immune response is formed. On the other side, the need for immunosuppressive treatment was evident. Ocular inflammation was observed in multiple cases and the loss of efficacy over time might be explained (in part) by immune-mediated clearing of transduced cells over time. In summary, the role of the immune response in ocular gene therapy remained somewhat unclear although the extent of its magnitude seemed limited in comparison to the immune response against AAV after systemic application.

2.7.2 Immune response to rAAV after systemic application

A lot of what is known about rAAV immunity, especially cell-mediated immune responses, comes from trials where rAAV is delivered systemically (e.g. intravenously in order to transduce hepatocytes). In these trials, systemic application of rAAV elicited a systemic und local (target cell population) cellular immune response, which significantly limited the efficacy of the therapy.

The limitations imposed by cell-mediated immunity were first described by Manno et al[88]. They observed that the transgene expression diminished and liver enzymes rose approximately 4 weeks after systemic delivery of rAAV2 (expressing human factor IX). At the same time, capsid-specific T cells expanded[88, 89]. A similar study found the same results using an AAV8 vector and described that corticoid treatment was the most efficient strategy to prevent loss of transduced hepatocytes[9].

Subsequently, Pien et al. showed that capsid epitopes were presented on the cell surface via MHC-I class molecules, making the hepatocytes (or potentially any other transduced target cells) susceptible for clearance by CD8 positive T cells[81]. It was recently shown that this antigen presentation is also generated from empty capsids[90].

Some of the receptor pathways that connect the innate immune response against AAV with the adaptive immune response have been identified in animal models. The first one to be identified was the TLR 9-Myd88 pathway[91]. TLR 9 is an endosomal receptor that senses unmethylated CpG islands within the vector genome[92]. Confirming these findings, experimental transgene depletion of unmethylated CpG islands reduced CD8+ T cell responses[93]. Myd88 in turn initiates a signaling cascade, that ultimately leads to the activation of NF-κB and IL-6, both pro-inflammatory cytokines[94]. Secondly, it was shown that in response to AAV the TLR9-Myd88 pathway led to the release of type I INFs in peripheral dendritical cells (pDC)[91]. Self-complementary AAVs (scAAV) showed even higher immunogenicity via TLR 9 compared to single stranded AAV[95]. As mentioned earlier, type I INFs are important signaling molecules responsible for orchestrating an antiviral immune response and enhance via MHCI presentation the clearance of infected cells through CD8+ T -cells. Interestingly, the proteasome inhibitor bortezomib was shown to decrease AAV capsid antigen presentation and by inhibiting T-cell mediated clearance of transduced cells at the same time enhanced gene expression[96].

B cell intrinsic Myd88 signaling was also shown to be involved in the induction of INF-

γ producing CD4 T cells and the subsequent formation of Th1-associated IgG2 antibody responses to AAV[97, 98].

In non-parenchymal liver cells, the capsid of rAAV2 and rAAV8 was shown to be sensed by cell membrane-bound TLR2[99]. This is interesting, as for the eye TLR2 – TLR2ligand interactions of retinal APCs have been identified to play a role in the activation of uveitogenic T cells[100]. In the retina, not only the resident APCs present TLRs[101, 102]. Photoreceptor cells are also capable of expressing TLRs[103, 104].

Other intracellular receptors which have not yet been linked to AAV recognition but are known sensors of viral DNA, include the NOD-like receptors NLRP3 and AIM2. Both are known to interact with ASC and Procaspase 1 to form a complex called inflammasome, which in turn cleaves pro-inflammatory interleukines (pro-IL 1β and pro-IL18) into their active form in response to cytosolic DNA[105, 106]. Other receptors that might be involved in AAV innate immune response are cytosolic DNA sensors upstream of STING (stimulator of interferon genes), like DAI, IFI16, cGAS and DDX41[107]. The activation of STING leads into the Type I INF signaling and has been described to play a role in innate immune sensing of other DNA viruses like Herpes virus[108]. One study investigated whether AAV capsid directed CD8 T cell formation was dependent on STING signaling but found only the TLR9-Myd pathway uniquely capable of initiating this response[109].

In a nutshell, although generally considered as non-pathogenic, rAAV is sensed by the innate immune system as a foreign particle and can be presented to the adaptive immune system. NABs are able to inhibit successful transduction after systemic application of AAV, but the eye seems to be immunologically privileged concerning this issue. Cell mediated clearance of transduced cells plays a role after systemic application but has not been observed after application of AAV to the retina.

3 Results

3.1 Humoral Immune Response After Intravitreal But Not After Subretinal AAV8 in Primates and Patients

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Retina

Humoral Immune Response After Intravitreal But Not After Subretinal AAV8 in Primates and Patients

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See the appendix for the members of the RD-CURE Consortium.

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Purpose. To study longitudinal changes of anti-drug antibody (ADA) titers to recombinant adeno-associated virus serotype 8 (rAAV8) capsid epitopes in nonhuman primates (NHP) and patients.

Methods. Three groups of six NHP each received subretinal injections (high dose: 1×10^{12} vector genomes [vg], low dose: 1×10^{11} vg, or vehicle only). Four additional animals received intravitreal injections of the high dose (1×10^{12} vg). Three patients received 1×10^{10} vg as subretinal injections. ELISA quantified ADA levels at baseline and 1, 2, 3, 7, 28, and 90 days after surgery in NHP and at baseline and 1, 3, and 6 months after surgery in patients.

Results. Two out of 22 animals lacked ADA titers at baseline and developed low ADA titers toward the end of the study. Titers in the low-dose group stayed constant, while two of six animals from the high-dose group developed titers that rose beyond the range of the assay. All animals from the intravitreal control group showed a rise in ADA titer by day 7 that peaked at day 28. Preliminary data from the clinical trial (NCT02610582) show no humoral immune response in patients following subretinal delivery of 1×10^{10} vg.

Conclusions. No significant induction of ADA occurred in NHP when mimicking the clinical scenario of subretinal delivery with a clinical-grade rAAV8 and concomitant immunosuppression. Likewise, clinical data showed no humoral immune response in patients. In contrast, intravitreal delivery was associated with a substantial humoral immune response. Subretinal delivery might be superior to an intravitreal application regarding immunologic aspects.

Keywords: gene therapy, vitreoretinal surgery, retina, AAV, immune response, antibodies

Adeno-associated virus (AAV)-mediated gene therapies have been shown to be clinically safe and offer new possibilities for the treatment of genetic diseases, such as blinding retinal dystrophies. However, investigators have independently found evidence of immune reactions against AAV vectors, the transgene, or the transgene product.[1–4] These include the full range of active defense mechanisms including innate, humoral, and cellular immunity. As such, clinical trials in hemophilia patients have shown that circulating antibodies can effectively inhibit transduction even at low titers, and that AAV-directed $CD8^+$ cells target and remove successfully transduced hepatocytes as virus-infected cells.[5]

In contrast to the treatment for hemophilia, where AAV vectors are injected intravenously, relatively small doses are administered in the immune-privileged space of the eye for retinal disorders like Leber's congenital amaurosis, choroideremia, or achromatopsia. In 2008, different groups independently reported a modest beneficial treatment effect of AAV2 for the *RPE65* mutation in LCA patients.[6–8] In none of these trials were major adverse advents reported in the following 5 years. Clinically no inflammation unresponsive to steroids was observed, and only two patients developed a transient antibody reaction.[9] One study reported a single case in which anti-capsid antibodies emerged in a functional assay around day 14 but declined later,[6] and in another study some anti-capsid antibody titers increased toward day 90 but were still low compared to the overall mean.[8] Amado et al.[10] showed that subretinal readministration of an AAV2 vector elicits a humoral immune response against the viral capsid in large animals. However, transduction was still possible under these conditions. This is in line with the observation that readministration of subretinal AAV (to the contralateral eye) in three adult patients did not lead to a rise in antibody titer.[11] Importantly, though, intravitreal delivery of AAV2 has been shown to induce humoral immune response in mice and block transduction in subsequent subretinal or intravitreal injections.[12] Others have shown that intravitreal delivery of AAV in NHP results in an increase of anti-AAV antibodies and decreased transgene expression.[13] Although a rise in antibody titer does not seem to be necessarily harmful, safety of the patient is of paramount importance and

the role of a potential humoral immune response should be fully understood.

To further explore this we injected 22 NHP with different doses of recombinant adeno-associated virus serotype 8 (rAAV8) as part of a formal toxicology and biodistribution assessment toward regulatory approval of a phase I/II clinical trial (NCT02610582). Subretinal or intravitreal administration routes were used in a surgical setting identical to that in human subjects (including perioperative steroids). We aimed to elucidate whether a good manufacturing practice (GMP)-grade AAV8 vector would lead to a humoral immune response in a clinical scenario and whether route of delivery would make a difference. Additionally, the same assay was used subsequently to quantify anti-drug antibody (ADA) titers in human patients following subretinal gene therapy (NCT02610582) with the same vector.

Methods

Animals and Study Design

A total of 22 NHP (*Macaca fascicularis*) were allocated into four separate groups (Supplementary Table 1). Groups 1 to 3 consisted of six animals (three males/three females). Group 1 was treated with vehicle (balanced salt solution [BSS]; Alcon, Freiburg im Breisgau, Germany) with 0.001% PF-68 (BASF, Ludwigshafen am Rhein, Germany). Animals in groups 2 and 3 received the test item (rAAV8) in the left eye only via single subretinal injection. Four animals (two males/two females) were allocated to group 4 and received the same test item via intravitreal injection. Animals in group 2 were treated with low-dose (1×10^{11} vector genomes [vg]) and animals in groups 3 and 4 were treated with high dose (1×10^{12} vg). Animals used in these studies were cared for and handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and after approval by the local authorities (Regierungspraesidium) and in full compliance with the guidelines of the European Community (EUVD 86/609/EEC) for the care and use of laboratory animals, as well as in accordance with good laboratory practice (GLP) standards as defined by German GLP monitoring authorities and in compliance with U.S. Food and Drug Administration GLP regulations.

Vector and Vehicle

The AAV8 vector was produced according to GMP guidelines by cotransfection of human embryonic kidney cells followed by purification and concentration steps optimized for clinical use of vector solution as reported previously.[14]

Surgery and Perisurgical Care of NHP

Animals received general isoflurane (Forane; Baxter GmbH, Unterschleißheim, Germany) and local 2% oxybuprocain (Conjuncain; Bausch&Lomb GmbH, Berlin, Germany) anesthesia before preparing (peri-)orbital skin with 10% povidine iodine solution and rinsing the conjunctival fornices with 1% povidine iodine solution. Sterile surgical drapes and pediatric lid specula were applied before a temporal canthotomy was performed for improved access. Three 23-guage (G) transconjunctival sclerotomies were made approximately 1.5 mm posterior to the limbus and vitrectomy was performed as completely as possible without affecting the lens. A localized retinal detachment was induced through subretinal injection of BSS (Alcon) using a 41-G cannula (DORC 1270.EXT; D.O.R.C. Deutschland GmbH, Düsseldorf, Germany). Virus solution was injected into the preformed bleb using a foot pedal–controlled injection system (PentaSys II; Ruck GmbH, Eschweiler, Germany). Before recovery, subconjunctival cefuroxime (125 mg; ratiopharm GmbH, Ulm, Germany) and dexamethasone (2 mg, ratiopharm GmbH) were administered to the operated eye. Postoperative prophylactic treatment consisted of antibiotic (0.5% Moxifloxacine; Pharm-Allergan GmbH, Frankfurt am Main, Germany) and anti-inflammatory (1% Prednisolone; Pharm-Allergan GmbH) eye drops given three times a day each in the treated eye for 2 weeks and prednisone (Merck Pharma GmbH, Darmstadt, Germany) 1 mg/kg intramuscularly from day −2 until day 5. In the course of the study all animals received ophthalmoscopic screening (slit lamp, fundus biomicroscopy) for signs of inflammation at days 2, 3, 7, 22, 50, and 87.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples were collected from each animal prior to dosing and at days 1, 2, 3, 7, 28, and 90 post dosing. A total of 154 samples were analyzed using a sandwich ELISA strategy utilizing a ELISA kit for the determination of AAV serotype 8 particles in cell culture supernatants or purified preparations (PROGEN Biotechnik GmbH, Heidelberg, Germany; Art. No.: PRAAV8). The microtiter strips, coated with a monoclonal antibody specific for a conformational epitope on assembled AAV8 capsids, were incubated with GMP-grade rAAV8. This procedure completed the coating for the detection of the new analyte: anti-AAV8 antibodies. Captured anti-AAV8 antibodies in plasma samples were detected using an enzyme conjugate of a rabbit anti-NHP antibody (rabbit anti human [and NHP] IgG pAb Streptavidin Peroxidase Conjugate, Cat. No. 55221; MP Biomedicals, Santa Ana, CA, USA). An anti-AAV8 biotin-conjugated antibody, together with streptavidin peroxidase, served as positive control. This antibody was used in a serial dilution of 1:3 from 250 ng/mL down to 0.34 ng/mL. The highest concentration of 250 ng/mL showed a hook effect and was therefore regarded as out of the range of the assay. The remaining seven standard dilutions covered the complete range, returned from the plasma samples. Eight negative controls were included on every ELISA plate. For the negative controls, no plasma was added and the background was calculated from the mean absorbance of these blanks. The background mean was subtracted from the plasma samples.

After addition of substrate solution the color reaction was measured photometrically at $\lambda = 450$ nm. In order to avoid false-negative results due to very high concentrations of the analyte, the plasma samples were measured in serial dilutions (1:5, 1:25, 1:125).

To ensure the assay's validity, coating controls, a dilution sequence of positive controls, and a negative control with no analyte were included on every ELISA plate. The optimal assay setting was tested beforehand in a GLP confirming proof of principle study. The following criteria were implemented to ensure validity. Uncoated wells had to show a low absorbance value ($OD_{STD0} \leq 0.2$). Coating controls (1×10^9, 1×10^8, 1×10^7 vg/mL, no coating) had to show a dose dependency in mean absorbance values. Coated wells without analyte had to show a low absorbance value and give a good signal-to-noise ratio when using a short time for color reaction and using a blocking solution ($OD_{no\ Plasma} \leq 0.3$). The mean absorbance value of the positive control wells coated with 1×10^{10} vg/mL (standard 1, STD1) had to be ≥ 1.0. The mean absorbance value of the positive controls (standards 1–8) had to show a dose dependency (STD1 > STD2 > STD3 > STD4 > STD5 > STD6 > STD7 > STD8). Plasma from seroconverted animals had to show a clear dose–dilution relationship.

As these criteria were all met, the assay was considered to be appropriate for the detection of anti-AAV8 antibodies.

Analysis

For the NHP samples, the titer was defined as the reciprocal dilution of the plasma at which the linear, interpolated graph for individual plasma intersects a so-called titer intercept line (TIL). The range where the interpolated graph could intersect the TIL was defined between 5 and 160. In this assay, the TIL was defined as the 3.3-fold lower limit of quantification (LLOQ) of the assay. Limit of detection (LOD) and LLOQ were calculated according to German Institute for Standardization (DIN) 32654, using the standard deviation ($\sigma(x_0)$) of negative controls by the following approximation: LOD: $3 \times \sigma(x_0)$; LLOQ: $k \times 3 \times \sigma(x_0)$ (with $k = 3$ at relative confidence interval [CI_{rel}] = 33%). In the case of clinical samples, absolute absorbance values are reported of all dilutions tested and compared in a longitudinal fashion.

Some samples did not yield quantifiable results. In these, titers exceeded or stayed below the dilution range of 1:5 to 1:160. Because we calculated the titer using the slope between different concentrations, in cases where the absorbance values did not drop in line with the dilution series (see validation criteria), the intersection with the TIL was outside the dilution range (i.e., the reading did not meet the prespecified criteria of validity). The most likely reason for this is the oversaturation of the assay due to high titer concentrations or a technical error. Likewise, plasma samples where all dilutions gave absorbance values below the TIL were considered below the range.

Patients

Three patients (two male, one female) underwent the procedure after written informed consent was given and followed up according to the approved trial protocol (NCT02610582). The vector was applied via subretinal injection as described previously.[14] All three patients received 1×10^{10} vg of the clinical-grade vector rAAV8.hCNGA3. To monitor safety, clinical and ophthalmologic examinations were performed at screening, directly after surgery, and 1, 2, 3 ± 1, 14 ± 3, 30 ± 5, 90 ± 7, and 180 ± 7 days after surgery. Blood samples were taken in all patients at screening, as well as 30 ± 5, 90 ± 7, and 180 ± 7 days after surgery. The study was carried out in accordance with the ethical principles of the Declaration of Helsinki.

Results

Nonhuman Primates (NHP)

For 141 of 154 NHP samples, a titer for rAAV-specific antibodies could be calculated, while 13 samples (8%) were out of the range of the assay. Of these, eight were above the range of the assay (titer > 160), and five samples showed no seroconversion (titer < 5). In total, 20 of 22 animals were already seroconverted before application. The two seronegative animals (28011M, 28017M), both allocated to the low-dose group, developed very low antibody titers throughout the observation period (maximum titer: 19, 28011M, day 28). No sex-specific differences in the rAAV8 specific antibody titers were observed.

In the vehicle control group (Fig. 1A), the change of titer (day x − day 1) ranged from −28 in animal 28023M to +50 in animal 28062F. This represents the test variability and individual titer fluctuations, which occur without an ongoing inflammatory reaction since none of the animals received vector. In the low-dose group (Fig. 1B) we observed titer changes similar to the control group with a titer change range from −30 to +36. As such, the antibody titers of the subretinal low-dose group stayed constant over the entire observation period. Although no statistical analysis is applicable, no titer changes obviously different from those of the vehicle control group were observed. Interindividual differences as well as the time curve for the titers found in the low-dose group are similar to those of the vehicle control group. In the high-dose group (Fig. 1C), two animals had titers that were above the range of the ELISA assay (animal 28060F day 28, animal 28063F day 7 and day 28). Where titers exceeded the range of the assay (160), no titer change was calculated. With the three missing values put aside, the titer change in group 3 ranged from −25 to +68. The relevance of the missing values will be discussed later. In the intravitreal control group (Fig. 1D), which received the same dose as the high-dose group, the mean titer change was most pronounced compared to all other groups. All animals showed a tendency toward higher titers 7 to 28 days after surgery. In three out of four animals, titers began to rise by day 7, peaked at day 28, and declined toward day 90 but remained elevated above baseline. The titer change on day 7 ranged from +34 to +98 and on day 28 from +38 to +140. The maximum titer change was +140 (animal 28057F day 28). Individual titers for each animal and time point are shown in Supplementary Figures S1 through S4.

Patient Samples

We tested plasma samples from the first three patients with *CNGA3*-linked achromatopsia undergoing gene therapy (NCT02610582), which applies the same vector construct used in the NHP study above. The same ADA test was applied and showed no humoral immune response within the first 6 months following subretinal delivery of 1×10^{10} vg (Fig. 2). All three patients had quantifiable absorbance measurements at baseline, which did not change significantly at 1, 3, or 6 months after subretinal vector delivery.

Discussion

Through contact with wild-type AAV, humans develop antibodies against the different serotypes in their first years of life.[15,16] Depending on the study, seroprevalence for AAV8 ranges from 15% to 30% of the population (AAV2: 30%–60%)[16,17] to 82% in Asian adult humans (AAV2: 97%).[18] Seroprevalence for AAV8 in NHP is considered to be as high as in humans or even higher.[19] Accordingly, in our study, 20 of 22 animals were found to be seropositive for anti-rAAV8 antibodies, indicating a seroconversion before the first treatment. Likewise, all patients from the first cohort ($n = 3$) of the clinical trial (NCT02610582) had quantifiable absorbance values in the ADA assay at baseline.

In general, after infection with a virus, the immune system requires a few days to develop a specific humoral immune response. Although it is difficult to exactly predict the temporal dynamics of a humoral immune response against AAV epitopes, antibody titers in a clinical gene therapy trial for hemophilia using rAAV8 rose after 1 to 2 weeks.[5] Antibody titers rising in a similar time frame after rAAV8 delivery can therefore be attributed to a specific humoral immune response. This is what we observed in our intravitreal control group where antibody titers began to climb on day 7 and peaked at day 28 (no samples were taken in between, e.g., on day 14). Hence, a specific humoral immune response in NHP after intravitreal delivery of rAAV8 serves as a parsimonious explanation.

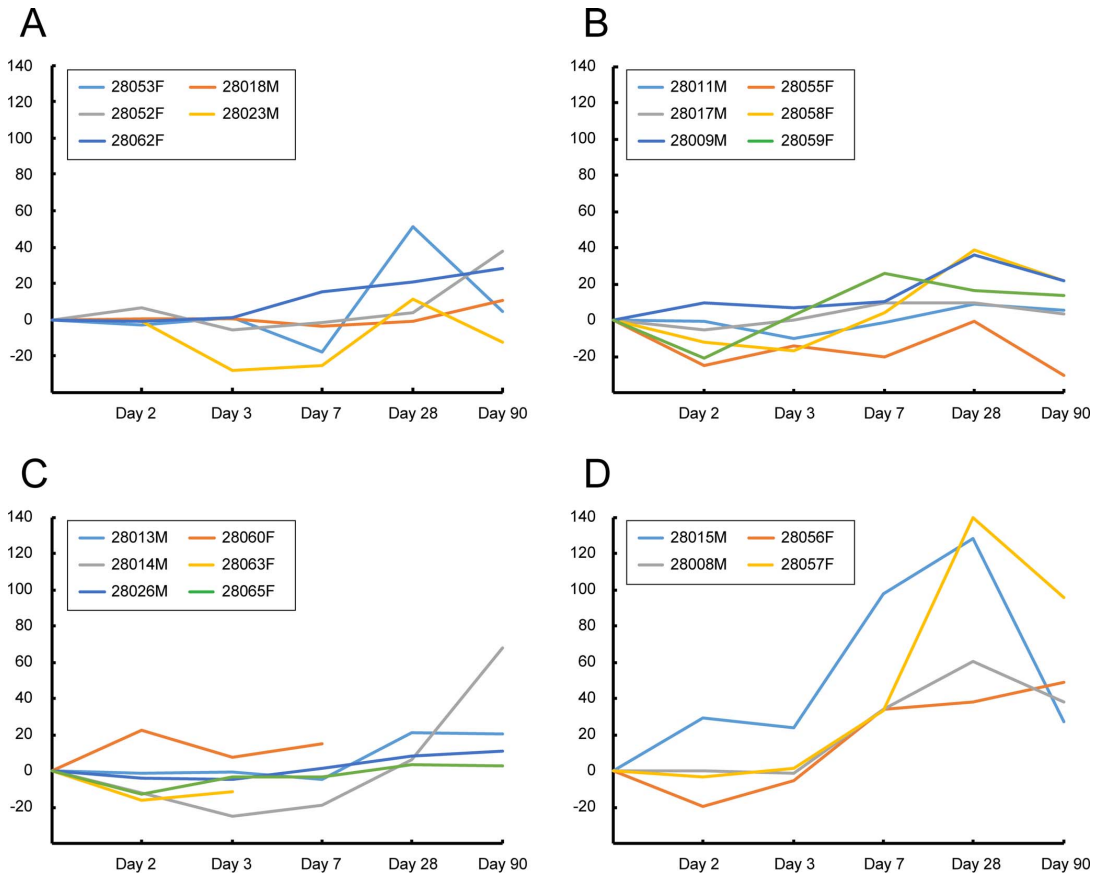

Figure 1. Individual titer change in individual animals presented as Δ to baseline (day 1). (**A**) Control group, (**B**) low-dose group (1×10^{11} vg), (**C**) high-dose group (1×10^{12}), (**D**) intravitreal high-dose group (1×10^{12}). Titers are calculated as described in Methods. Where titers exceeded the range of the assay (upper range of titer calculation: 1:160), no titer and no titer change could be calculated ([**C**] 28060F day 7 and 28063F days 7 and 28; [**A**] 28010M at all time points and therefore not included in graph).

Although no evidence is at hand for the missing values of the two animals of the high-dose group, 28060F and 28063F, having the previous considerations in mind, one could interpret these titers above the range of the assay on day 28 (28060F) and days 7 and 28 (28063F) as a humoral immune response. In both animals, titers declined toward an elevated level above baseline on day 90, which is consistent with what we observed in the intravitreal control group. Apart from these two animals, all other rAAV antibody titers in the high-dose group, as well as all rAAV antibody titers of the low-dose group, stayed constant over the 90-day observation period.

Since ELISA values for antibodies against AAV serotypes are not comparable between different studies it is difficult to make a decision on what titer change is considered a relevant change—especially since high titers do not translate into clinical findings. In our study we used the variability of control group data to assess which change in titer was numerically significant. It is important to remember, however, that this does not equate to clinical significance. Indeed, we observed no clinically relevant, test item–related changes in ophthalmologic assessment throughout the in-life phase of the study. Findings observed (limited and temporary anterior chamber flare and cells, drusen, and pigment clumping) were either also present before dose and thus regarded as background lesions (e.g., drusen), or equally evident in groups 1 to 3 and therefore related to the surgical procedure rather than the test item. The fact that none of the 22 primates presented signs of a clinically relevant inflammation shows that a rise in antibody titer cannot be directly correlated to a clinically significant pathogenic process. It may, however, become relevant in a scenario of multiple injections and/or intravitreal applications.[3,18] Intravitreal may be considered the preferred route of administration when targeting inner layers or wide areas of the retina.[20] However, some authors have suggested that after intravitreal injections, neutralizing antibodies are more likely to be generated than after subretinal injections and that these antibodies have the potential to inhibit effective gene transfer.[12,13] Part of the explanation for this enhanced humoral immune response might be the fact that the shedding and biodistribution of vector after intravitreal injections is considerably higher.[21]

This study has certain limitations, including absence of absolute thresholds of clinical relevance for levels of antibodies against AAV8. Furthermore, there is no international standard

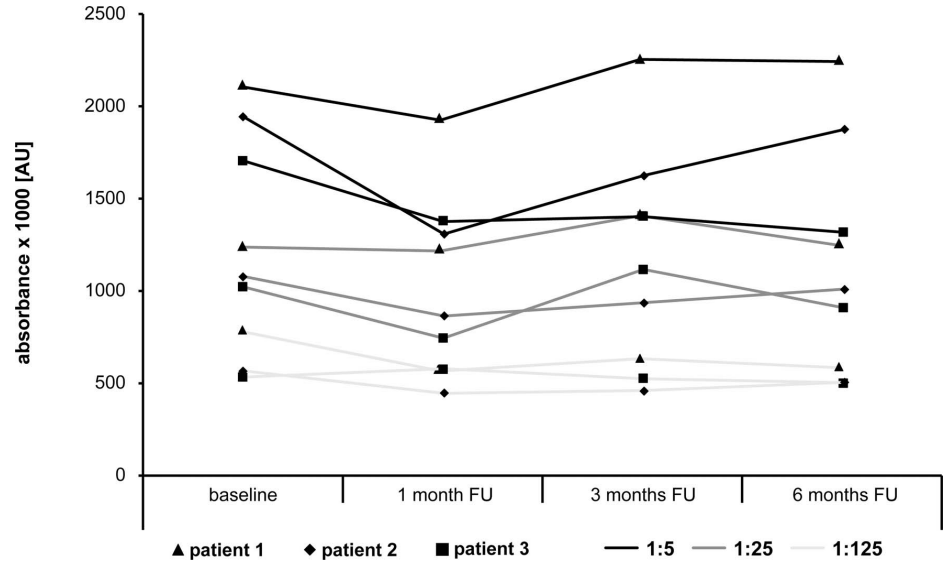

FIGURE 2. Anti-AAV8 antibody titers in patients before and after subretinal gene therapy. Samples from the first three patients were analyzed at baseline and 1, 3, and 6 months after subretinal delivery of 1×10^{10} rAAV8 vector genomes. Longitudinal analyses of plasma samples in serial dilution (1:5, 1:25, and 1:125) show no prominent intraindividual change in absorbance values, suggesting that there is no humoral immune response in these patients. AU, arbitrary units; FU, follow-up.

for benchmarking across studies. As such, dilution series and longitudinal follow-up are important aspects in these investigations. Another limitation is the lack of true technical repeats in the ELISA. Instead, each sample was measured in a dilution sequence and the titer calculated by using the slope instead of single values. This has the added benefit of accounting for differences in antibody affinity as a function of epitope concentration.

One needs to be cautious when extrapolating results from studies in NHP to the clinical situation because the response of the immune system of NHP to the therapeutic vector may differ from that of human subjects. However, we believe that our findings may help guide study designs of future clinical gene therapy trials. We argue that this assay can specifically detect antibodies against AAV8 epitopes and is appropriate for the comparison of pre- and postdose plasma specimens. Since our results rely on rAAV8, we can only speculate about the effects of other serotypes. It seems possible, though, that the time course of the humoral immune response as well as the effects of different routes of administration may be similar for the commonly used vector AAV2 and other serotypes.

In conclusion, our results show an excellent safety profile, especially regarding the low dose (1×10^{11} vg). This is important, as this dose was chosen as the highest dose used for the clinical trial in achromatopsia patients (NCT02610582). Groups 3 and 4, having received 1×10^{12} vg, showed more equivocal results, with some samples exceeding the range of the ADA assay. In general, the route of administration seems to have dictated the humoral immune response against AAV8: While an intravitreal approach promises the potential of panretinal transduction without the challenges of subretinal surgery, this study adds evidence to the observation that intravitreal injections are associated with a higher risk for humoral immune responses compared to subretinal delivery of AAV vectors. An ongoing trial with intravitreal application of AAV8 for X-linked juvenile retinoschisis (NCT02416622) will help to further clarify this observation.

More research is needed to understand the complex reaction to AAV in the immune-privileged eye. The ocular immune response against AAV beginning with innate mechanisms and leading to specific humoral/cellular immunity is still poorly understood. It is of eminent importance to gain a good knowledge of the mechanisms underlying the antiviral defense mechanisms of the visual system. This will allow further improvement of safety and enhancement of efficacy of AAV-mediated gene therapies in the eye.

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Appendix

RD-CURE Consortium

The members of the RD-CURE Consortium are the following: Bernd Wissinger, Martin Biel, Eberhart Zrenner, Karl Ulrich Bartz-Schmidt, Dominik Fischer, Susanne Kohl, Stylianos Michalakis, Francois Paquet-Durand, Tobias Peters, Mathias Seeliger, Marius Ueffing, Nicole Weisschuh, Barbara Wilhelm, Ditta Zobor, Stephen Tsang, Laura Kühlewein, Christian Johannes Gloeckner, and Nadine A. Kahle.

3.2 AAV8 Can Induce Innate and Adaptive Immune Response in the Primate Eye

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AAV8 Can Induce Innate and Adaptive Immune Response in the Primate Eye

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Ocular gene therapy has evolved rapidly into the clinical realm due to promising pre-clinical proof-of-concept studies, recognition of the high unmet medical need of blinding disorders, and the excellent safety profile of the most commonly used vector system, the adeno-associated virus (AAV). With several trials exposing subjects to AAV, investigators independently report about cases with clinically evident inflammation in treated eyes despite the concept of ocular immune privilege. Here, we provide a detailed analysis of innate and adaptive immune response to clinical-grade AAV8 in non-human primates and compare this to preliminary clinical data from a retinal gene therapy trial for *CNGA3*-based achromatopsia (ClinicalTrials.gov: 02610582).

INTRODUCTION

Only one decade has lapsed since the first ocular gene therapy in humans.[1–3] Since then, the field has evolved rapidly for three reasons. First, blinding disorders demonstrate high unmet medical need with no treatment available. Second, pre-clinical proof-of-concept studies using adeno-associated virus (AAV) showed convincing efficacy in the absence of significant toxicity. Third, the eye is regarded as an attractive target organ due to the availability of an internal control (fellow eye), its small size, and its diagnostic and therapeutic accessibility and not least due to the concept of ocular immune privilege initially described by Medawar[4] 70 years ago.

The initial reports from clinical trials confirmed the excellent safety profile of AAV and showed some evidence of efficacy. However, the endpoints used were psychophysical measures such as global light sensitivity and mobility tests in an open-label trial. Objective measures of efficacy such as electroretinography (ERG) were not able to reproduce the results gained from pre-clinical studies in dogs. The long-term data on the first two independently led retinal gene therapy trials suggested a decline of therapeutic efficacy with time.[5,6] Different hypotheses have been brought forward to explain this decline of visual function, including silencing of the episomal transgene, continuous degeneration despite transduction of cells past the point of no return, and clearance of transduced cells by immune mechanisms.

Immune-mediated clearance of cells transduced with AAV has been observed in clinical trials targeting hepatocytes, which are not immune privileged and are in plain sight of the immune system.[7] In contrast, the eye features passive and active mechanisms to counteract inflammation, such as the blood-retina barrier; lack of anatomically defined lymphatic drainage; abundance of local anti-inflammatory agents (e.g., transforming growth factor β [TGF-β], α-melanocyte stimulating hormone [α-MSH], and somatostatin [SOM]); and monocytes actively counter-acting adaptive immunity (e.g., F4/80$^+$ antigen-presenting cells [APCs] and CD8$^+$ T_{reg} cells).[8] It hence came as somewhat of a surprise that Bainbridge et al.[1,6] reported some degree of intraocular immune responses in five of eight high-dose patients following subretinal treatment with AAV2, of which one showed a persistent reduction of visual acuity following a mild anterior uveitis. Independently, MacDonald et al. (2016, Invest. Ophthalmol. Vis. Sci., abstract) presented a case of clinically evident intraocular inflammation after subretinal gene therapy. Following the application of serotype 2 AAV, intraretinal hyper-reflective spots appeared in optical coherence tomography (OCT) scans of the treated retina reminiscent of cellular infiltration by glia or other immune-competent cells. These changes dissolved after a course of systemic steroid treatment, adding to the notion that immune-competent cells played a role.

Here we report data suggesting activity of both innate and adaptive immunity in eyes of non-human primates (NHPs) that had received subretinal injections of clinical-grade AAV8 under concomitant

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Table 1. Study Design

Group	Dose Level	Route of Delivery (Volume)	Number of Animals	Sex F/M
90-Day Study (n = 22)				
1	vehicle	subretinal (200 μL)	6	3/3
2	low, 1×10^{11} vg	subretinal (200 μL)	6	3/3
3	high, 1×10^{12} vg	subretinal (200 μL)	6	3/3
4	high, 1×10^{12} vg	intravitreal (200 μL)	4	2/2
28-Day Study (n = 12)				
1	vehicle	subretinal (200 μL)	4	2/2
2	low, 1×10^{11} vg	subretinal (200 μL)	4	2/2
3	high, 1×10^{12} vg	subretinal (200 μL)	4	2/2

steroid treatment. Furthermore, we set this in context with preliminary clinical data from the first retinal gene therapy trial for *CNGA3*-based achromatopsia (ClinicalTrials.gov: 02610582).

RESULTS

Successful Delivery of Clinical-Grade AAV8 in 34 Cynomolgus Monkeys

All animals successfully received intraocular surgery mimicking clinical application, including concomitant systemic and local steroid treatment, transconjunctival 23G pars plana vitrectomy, and either intravitreal or subretinal injection of a predefined dose of clinical-grade AAV8 in vehicle (buffered saline solution [BSS] with 0.001% Kolliphor P188 Micro surfactant) or vehicle only. The study design, specifics of dosing, and sex distribution are summarized in Table 1. First, a 90-day study was conducted in 22 animals randomized into groups receiving either subretinal vehicle (group 1), subretinal low dose (1×10^{11} vector genomes [vg]; group 2), subretinal high dose (1×10^{12} vg; group 3), or intravitreal high dose (1×10^{12} vg; group 4). The rationale for including an intravitreal control group was to assess the biodistribution and toxicity following an inadvertent injection into the vitreous cavity and/or major reflux through the retinotomy secondary to a subretinal delivery. Dosing was performed without complication except lens touch in two animals, which subsequently developed lens opacifications. The pars plana was consistently found 2 mm posterior of the limbus. Introduction of consecutive trocars for vitrectomy was difficult due to the sturdy sclera and required extra care to avoid hypotonic collapse of the eyeball and inadvertent damage to ocular tissue such as the posterior lens capsule. The adhesive force between the retinal pigment epithelium (RPE) and the neuroretina was greater than in human patients, which necessitated high initial infusion pressures. To overcome the adhesive force and induce the neurosensory detachment with BSS in a two-step approach,[9] peak pressures reached $\leq$ 50 mmHg (6.7 kPa) in animals, compared to maximal infusion pressures of 30 mmHg (4.0 kPa) in human patients using the same setup (PentaSys II, Ruck). Placement of the retinotomy just inside the vascular arcades, halfway between optic disc and fovea centralis consistently led to a central macular bleb and promised efficient targeting of cone photoreceptors. Gravitational forces were insufficient to displace the bleb in an inferior direction.

All blebs had resolved by the first ophthalmological follow-up on day three. Clinical slit lamp and fundus biomicroscopy revealed limited numbers of white cells in the anterior or posterior chamber across the groups (Figure 1). Infiltration of the anterior chamber peaked three days after surgery. White cells in the posterior segment only became evident after one week or later. Cellular infiltrations found in anterior and posterior segments were less severe in the groups only undergoing the surgery without AAV8 exposure (group 1) or being exposed to AAV8 but being spared the subretinal injection (group 4), while subretinal surgery combined with AAV8 exposure (groups 2 and 3) led to the highest overall scores. Fundoscopy and imaging of the posterior pole showed only minor changes (Figure 2), such as the expected pigment displacement first reported by Nork et al.[10] Color photographs, infrared, and autofluorescence recordings all revealed the pigment displacement reflecting the area of detachment. Angiography showed that perfusion characteristics remained unchanged and the blood-retina barrier intact. Retinal thickness temporarily decreased due to transient loss of photoreceptor outer segments following retinal detachment and re-attachment. However, signal composition in the outer retina normalized over time and a formal non-inferiority analysis on the effect of subretinal versus intravitreal injection on the outer nuclear layer showed that subretinal surgery was not inferior to intravitreal application.[11]

At 90 days after gene therapy, there were no test item-related changes in organ weights, in macroscopic or in histopathological observations of ocular and extraocular tissue. Specifically, no degenerative, inflammatory, or hyperplastic abnormalities were found in any animal. Minor changes in the treated eyes consisted of irregularities of the pigment granules in the RPE, which were attributed to the subretinal dosing procedure, because they were equally evident in the vehicle control group.

To further exclude transient effects at an earlier time point, an additional 28-day study was conducted in 12 animals injected subretinally at dose levels of 0 (vehicle), 1×10^{11}, and 1×10^{12} vg (Table 1). No intravitreal control group was included in the 28-day study. Based on microscopic observations in retinal sections (Figure 3), subretinal administration of 1×10^{12} vg AAV8 resulted in marked mononuclear cell infiltration in the retina and choroid in two animals (both female) evident 28 days after surgery. These were associated with augmented cellular infiltrates into the subretinal space or with marked perivascular to diffuse mononuclear inflammatory cell infiltrates in the retina and choroid. One male animal from the subretinal high-dose group also showed a choroidal inflammatory cell infiltrate, but it was of much smaller magnitude. Animals from the subretinal low-dose group (1×10^{11} vg) demonstrated only minor infiltrates of mononuclear cells, which were also found in subretinal vehicle-treated animals and thus may constitute incidental background findings. Table 2 summarizes all relevant microscopic observations.

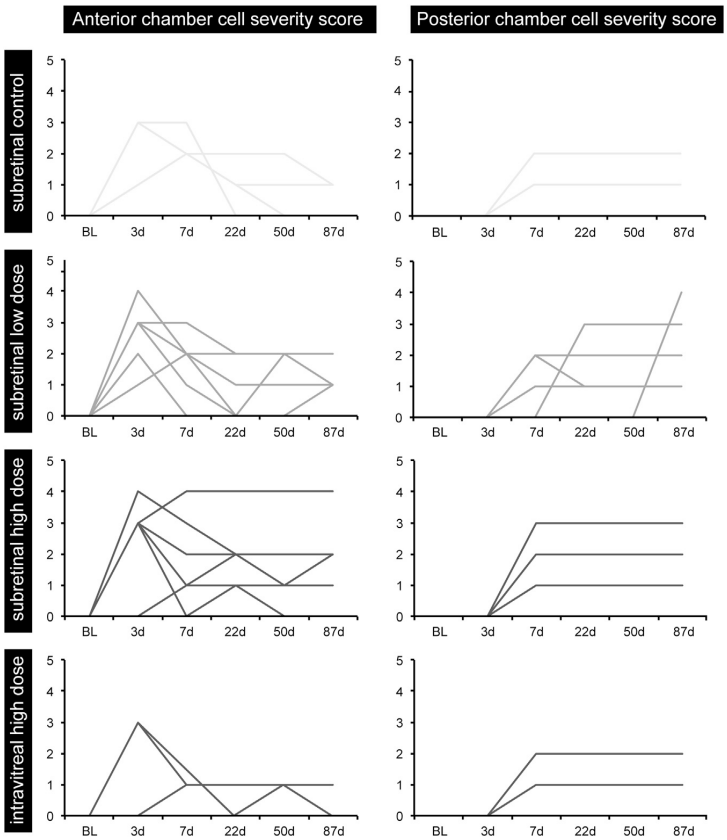

Figure 1. Grading Inflammation in the Anterior and Vitreous Chambers

Animals from all groups underwent slit lamp and fundus biomicroscopy before surgery (BL, baseline) and at five time points (3, 7, 22, 50, and 87 days) after surgery to grade the inflammation in the anterior (left) and posterior (right) segment of the treated eye following the classification of the Standardization of Uveitis Nomenclature (SUN) working group and the NIH classification.

Innate Immune Response following Retinal Gene Therapy

Retinal sections from all animals of the 28-day study (specifically including those with evident mononuclear cell infiltration in the retina and choroid) were selected for RNA extraction, reverse transcription, and quantification of antiviral response marker expression. Expression analysis comparing animals with inflammation evident in histological assessment (n = 3 from the high-dose group) to animals of the sham-injected group showed that innate immune response was activated in those animals and that the innate immune response was dominated by markers related to the pro-inflammatory T helper (Th) 1 pathway (e.g., interferon-gamma [IFNγ]-induced CXCL10) and LGP2, a retinoic acid-inducible gene 1 (RIG-I)-like receptor responsible for cytosolic viral DNA detection (Figure 4; Table 3).

Humoral Immune Response against AAV8 Capsids

Sandwich-ELISA was used to investigate longitudinal changes of antibody titers directed against AAV8 capsid epitopes in the 22 animals of the 90-day study before and after receiving subretinal injections (high dose, 1×10^{12} vg, n = 6; low dose, 1×10^{11} vg, n = 6; or vehicle only, n = 6) or intravitreal injections (1×10^{12} vg, n = 4) mimicking via falsa application. These data have been published elsewhere (Reichel et al., 2016, Invest. Ophthalmol. Vis. Sci., abstract). Briefly, titers of total antibodies (both neutralizing and binding antibodies) against AAV8 capsid epitopes remained constant in all animals of the subretinal dose groups over the 90-day observation period. Animals from the intravitreal group showed some response with elevated titers at 4 weeks post-treatment. Data on humoral immune response in the 28-day study was not separately available, but the longitudinal analysis of titers from the 90-day study included time points at 7 and 28 days, which adhered to the general trend described earlier.

Adaptive Immune Response following Subretinal Gene Therapy

Cryosections from subretinally treated animals from the 28-day study featuring mononuclear cell infiltrates in the subretinal space and/or perivascular retina were subjected to immunohistochemistry to further define the profile of ocular immune activation following AAV8-mediated retinal gene therapy. Retinal sections traversing the treatment area demonstrated activity associated with adaptive immune response (Figure 5). Staining for ionized calcium-binding adaptor molecule 1 (IBA1), a hematopoietic marker for microglia and macrophages, showed substantially more signal in eyes, which had received AAV8. Similarly, $CD8^+$ staining indicated the presence of cytotoxic T cells in the outer and inner retina of AAV8-treated eyes. In line with a general immune response, these sections also showed positive staining for F4/80, human leukocyte antigen-antigen D related (HLA-DR), and much higher levels of major histocompatibility complex class I (MHC I) expression, suggesting active antigen presentation with the potential for T cell activation. Next to microglia and macrophages, cytotoxic T cells, and APCs, we also found $CD20^+$ B cells associated with the inflammation following AAV8 application. The presence of cytotoxic T cells and B cells in the AAV8-injected areas, coupled with microglia and macrophages and APCs, supports the notion that both innate and adaptive immune responses play a role in the retina after AAV vector deployment.

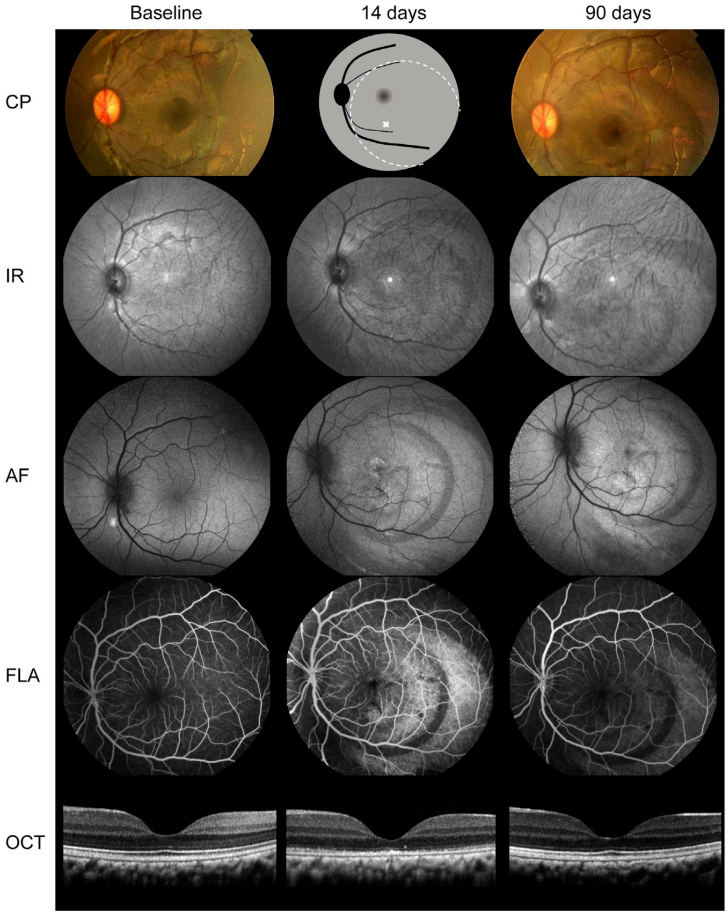

Figure 2. Change in Retinal Structure after Subretinal Surgery for Retinal Gene Therapy

Pictures from a representative case at baseline (left), 14 days (middle), and 90 days (right) after subretinal injection. Top panels show the color photographs (CP) and a diagram (middle panel) indicating the bleb size and location (dotted line) and retinotomy site (cross) in this animal. The infrared (IR) images show mild changes in pigment distribution, which are more clearly highlighted in the autofluorescence (AF) recordings. Fluorescein angiography (FLA), however, shows an intact blood-retina barrier and no leakage. Optical coherence tomography (OCT) demonstrate temporary changes in the signal composition in the outer retina that seem reversible by day 90.

Preliminary Data from Clinical Applications

Clinical application was initiated following unconditional approval by the relevant regulatory agency (Paul Ehrlich Institute, Germany) and the ethical review board of the University of Tübingen. This was based on the observation that there were no test-item-related changes found in the NHPs 90 days after surgery. The observation of potential, transient inflammation at four weeks in NHPs prompted regulators to define a minimum of 4 weeks between enrollments of trial subjects and ask for go or no-go decisions from an independent data-monitoring committee before each dose escalation. The first patient was injected in November 2015, and blood samples were taken one week before and 3, 14, 30, 90, and 180 days after surgery. C-reactive protein (CRP), total immunoglobulin M (IgM), and immunoglobulin G (IgG) did not show clinically significant changes in the first cohort of patients (n = 3, receiving 1×10^{10} vg), and patients remained clinically healthy. Stimulation of isolated peripheral blood mononuclear cell (PBMC) subfractions from subject 103 with AAV8 particles at different concentrations (Figure 6) led to higher activation rates when assessed before compared to after surgery (which can be partly explained by concomitant steroid treatment at 1 mg/kg bodyweight from the day before surgery until day 19). In the other two subjects, cytotoxic T cells ($CD3^+/CD8^+$) showed the most marked changes in the activation pattern around 30 days after surgery (despite the same steroid regimen). These preliminary clinical data suggest activation of cytotoxic T cells, supporting the results shown in the NHPs on activation of cytotoxic T cells in the AAV-injected areas.

None of these three patients showed clinically evident cellular infiltration of the anterior chamber and/or vitreous cavity at any time. No patient complained of symptoms or showed clinical signs associated with inflammation of uveal tissues, such as anterior uveitis or panuveitis, and visual function reached baseline again two weeks after surgery. However, one of the patients (subject 102) demonstrated hyper-reflective spots in the virtual cross sections of the treated area with a peak two weeks after surgery, which resolved under the concomitant steroid treatment as per protocol (1 mg/kg bodyweight [70 mg] for three weeks, followed by weekly reduction to daily doses of 50, 40, 30, 25, 20, 15, 10, 5, and 0 mg) without sequelae (Figure 7; Figure S1).

DISCUSSION

Gene therapy offers great hope to patients with hereditary diseases of the eye and could lead to treatment modalities for more common diseases of the eye (e.g., age-related macular degeneration) and beyond. However, this potential can only be leveraged if risks and benefits are well understood so that they can be weighed against each other by regulators, investigators, and patients. The immune system can play a vital role in gene therapy, and the assumptions that AAV is

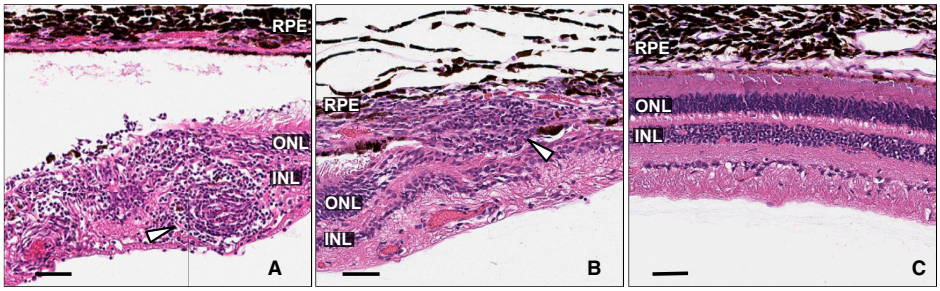

Figure 3. Mononuclear Infiltrates 28 Days after High-Dose Subretinal Gene Therapy with AAV8

(A and B) Representative findings from animals of the high-dose (1×10^{12} vg) group showing (A) perivascular, intraretinal infiltrates (arrowhead) and (B) choroidal, subretinal infiltrates (arrowhead). In contrast, retinal architecture is essentially recovered one month after surgery in the vehicle control group (C). Scale bar, 50 μm.

non-immunogenic and the immune privilege of the eye is complete are not supported by current evidence. Both early and late inflammatory reactions have been described in patients by independent investigators following AAV2-based ocular gene therapy (MacDonald et al., 2016, Invest. Ophthalmol. Vis. Sci., abstract).[5,6,12,13] Only a small subset of patients also suffered from functional consequences, and no life-threatening condition has been reported. However, it seems sensible to further our understanding of underlying risks related to immune response and ocular gene therapy in an effort to maximize safety and efficacy of clinical gene therapy.

Vandenberghe et al.[14] reported on the transduction pattern and efficiency of the AAV2 and AAV8 pseudotyped vector in cynomolgus monkeys and showed a dose-dependent humoral immune response (increases in neutralizing antibodies to the vector capsid) when using vector batches produced in the lab under non-guanosine monophosphate (GMP) conditions. In animals treated with the highest dose (1×10^{11} vg), they demonstrated no T cell activation in response to vector capsid after injection, while T cell responses to the GFP transgene product in the peripheral organs (such as blood and spleen) were detected in two of 14 injected animals. The eyes exposed to the same dose demonstrated focal spots of retinal inflammation, retinal thinning, and disrupted retinal architecture.[14]

Maclachlan et al.[15] documented an interesting pre-clinical safety and biodistribution study in the same species using clinical-grade AAV2 as vector containing a synthetic transgene encoding for a modified soluble Flt1 receptor (sFLT01) designed to neutralize vascular endothelial growth factor. In this study, injection of the maximal dose (2.4×10^{10} vg) into the vitreous cavity was associated with an immune response involving lymphocytes or plasma cells infiltrating ocular tissue. PBMCs were shown to react to AAV2 capsid protein, but not to sFLT01.

Although these studies supported the view that AAV has the potential to stage an adaptive immune response in the eye, they left several important aspects relevant for the treatment of monogenic disorders of photoreceptors to be addressed. In contrast to AAV2, which mainly transduces ganglion cells after intravitreal application, subretinal delivery of AAV8 leads to efficient photoreceptor transduction.[14] Our study therefore explored the effects of subretinal AAV8 gene therapy at a dose range relevant for clinical gene replacement therapy (up to 42 times higher than in the Maclachlan et al.[15] study).

Ye et al.[16] reported significant chorioretinitis following subretinal AAV5 supplementation gene therapy in *CNGB3* mutant dogs and dose-dependent ocular inflammation after subretinal delivery of either AAV5 or AAV2tYF in healthy cynomolgus monkeys. One macaque in the lower-vector dose group developed clinical endophthalmitis, and multiple neutrophilic infiltrates in ocular tissues, including the retina, were found in an undisclosed number of animals. NHPs from the high-dose group showed moderate or severe inflammation of the anterior and/or posterior eye segments associated with whitish subretinal foci, which correlated microscopically with mononuclear cell infiltrates of the choroid and/or retina.

Ramachandran et al.[17] assessed the novel vectors AAV7m8 and AAV8BP2 expressing GFP after intravitreal or subretinal delivery in NHPs and showed inflammatory responses at the highest dose (1×10^{12} vg). Specifically, they reported some degree of glial activation and lymphocytic infiltrates in the retina following application of 1×10^{12} vg AAV7m8. These findings were more pronounced after subretinal versus intravitreal delivery. In addition, perivascular inflammation in the retina, loss of RPE, and chronic choroidal inflammation was observed after subretinal delivery of 1×10^{12} vg AAV7m8, but not after intravitreal application of the same dose. Up to 1×10^{12} vg AAV8BP2 was applied via either route of delivery without significant retinal infiltrates.

Clinical evidence for a dose-dependent inflammatory response to ocular gene therapy was first reported by Bainbridge et al.[6] While no participant of their low-dose (1×10^{11} vg) cohort that received AAV2 encoding RPE65 showed any clinical sign of inflammation, five of eight participants from the high-dose group (1×10^{12} vg) presented with intraocular inflammation. Specifically, findings included anterior uveitis, focal chorioretinal pigmentary changes, mild vitritis,

Table 2. Microscopical Findings in Subretinally Treated Eyes 28 Days after Gene Therapy

Finding	Group 1 (Vehicle)				Group 2 (1×10^{11} vg)				Group 3 (1×10^{12} vg)			
Individual animal no.	28364M	28379M	28380F	28383F	28370	28376M	28384F	28390F	28367M	28375M	28381F	28383F
Cellular infiltrate in the sclera	–	–	–	–	f	–	–	–	–	–	–	–
Cellular infiltrate in the ciliary body/ora serata	–	–	–	–	–	f	–	f	–	–	–	–
Cellular infiltrate in the choroid	–	–	–	–	–	–	–		m	–	–	–
Cellular infiltrate in the retina	–	–	–	–	–	–	–	–	–	–	m	m
Cellular infiltrate in the subretinal space	–	f	f	f	f	–	–	f	f	–	f	f

f, focal; m, multifocal.

optic disc swelling, retinal vascular tortuosity, and sheathing of vessels. Apart from the focal pigmentary changes, all findings resolved under steroid treatment, and only one patient showed sustained reduction of visual function. In a different trial, MacDonald et al. (2016, Invest. Ophthalmol. Vis. Sci., abstract) showed a case of hyper-reflective spots in the treated area of a choroideremia patient weeks after subretinal AAV2-mediated gene therapy (1×10^{11} vg) that resolved under steroid treatment, bringing the patient back to his baseline visual acuity.

Although it is clear from these studies that AAV-mediated gene therapy has the potential to trigger an immune response in the eye, a number of questions highly relevant to the field are still to be answered. These mainly concern the nature of the immune response (e.g., regulatory versus cytotoxic immune-competent cells and Th1- versus Th2-dominated response), the causality (e.g., capsid versus transgene and null versus missense mutations), and the possibility of modulating the response (e.g., by dose or duration of concomitant steroid treatment, excluding pre-existing immunity, and method of delivery).

In a first attempt to address immune responses to AAV in the retina, we analyzed data from good laboratory practice (GLP) conform toxicology studies in 34 cynomolgus monkeys that were subjected to clinical-grade AAV8 vectors and preliminary data from the first clinical trial for *CNGA3*-based achromatopsia. We were able to show that surgical delivery of the vector was safe and did not result in a significant loss of photoreceptors or in other anatomical changes apart from pigment displacement. However, some animals from the high-dose group (1×10^{12} vg) demonstrated mononuclear infiltrates in the retina and choroid. In addition, expression profiling revealed upregulation of IFNγ-mediated cytokines of the pro-inflammatory Th1 pathway four weeks after subretinal injection. Immunohistochemical analysis showed that antigen presentation is augmented in the treated areas, glial activity is increased, and cells of the adaptive immune response populate the treated retina. Specifically, $CD8^+$ T cells and $CD20^+$ B cells were evident in the retina following application of 1×10^{12} vg AAV8 one month after the surgery. No inflammation was seen histologically in other animals that were sacrificed three months after the surgery, opening the possibility of a transient reaction. One may speculate that active components of the deviant ocular immune response (e.g., regulatory T cells or APCs, TGF-β, α-MSH, and SOM) contain such an inflammatory response. This raises the question of whether concomitant steroid treatment is beneficial (limiting a harmful immune response) or harmful (incapacitating the cellular regulatory component of the deviant immune response), a question worth addressing in future studies.

We observed some degree of discrepancy between the cellular and the humoral adaptive immune response. There was minimal change of antibody titers against AAV8 capsid after subretinal delivery, while the intravitreal group showed an increase in titers (Reichel et al., 2016, Invest. Ophthalmol. Vis. Sci., abstract). This may reflect our previous observations of a more favorable biodistribution profile after subretinal versus intravitreal application of AAV8 (Seitz et al., 2016, Invest. Ophthalmol. Vis. Sci., abstract). Intravitreal delivery leads to several orders of magnitude more AAV particles in the systemic circulation and lymphatic tissue compared to subretinal delivery, which in turn may explain a more robust humoral immune response (Seitz et al., 2016, Invest. Ophthalmol. Vis. Sci., abstract).

There was no clinically relevant inflammation of either the anterior or the posterior segment at any time point in our patients that would not respond to standard medical care. The previously described patient with discreet hyper-reflective spots in the spectral domain OCT (SD-OCT) virtual cross sections of the treated retina four weeks after surgery never showed functional loss. The hyper-reflective dots resolved under steroid treatment until the next scheduled visit (month 3) without sequelae. It is unclear what the microscopic equivalent of these hyper-reflective spots is, but inflammatory cells or displaced pigment epithelium is likely a candidate.

The study was primarily designed as a GLP conform toxicology and biodistribution study in preparation of an investigational new drug (IND) application with the relevant regulatory authority. As such, limitations of the study include the low number of time points investigated (28 and 90 days), the fixation of the tissue, and the lack of PBMC analysis and antigen-specific immune response (ASR) assays. These could have proved or ruled out immune reactivity against human CNGA3. However, the high homology between the orthologs in macaques and those in humans (>96%) and the lack of any previous ASR in the eye of healthy macaques and/or human patients with

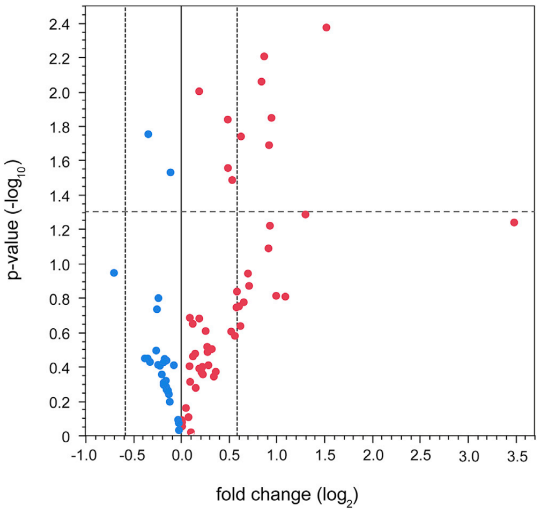

Figure 4. Differential Gene Expression in Primate Retina following Subretinal Gene Therapy

Expression profile from whole retina comparing samples from sham-injected animals versus animals from the high-dose group. Upregulated genes (red) are predominantly associated with the inflammatory Th1 pathway.

either missense or null mutations following gene supplementation make this rather unlikely.[5,6,12,15,16] The primary goal of this study was to provide GLP conform data on toxicology and biodistribution in the advent of a clinical trial, and any ASR in animals against a human antigen would not have been predictive of the risk in a first-in-man trial.

The immune response in NHPs can be different from that observed in human patients, and the seroprevalence for AAV8 capsid proteins is higher in NHPs versus the human population.[18–20] As such, the implications of the results in the clinical setting have to be substantiated by further investigations and most importantly by careful observations in ongoing and future clinical trials. The NHP study supported our strategy of subretinal delivery in the clinical trial but cautioned us to monitor our patients carefully in this first-in-man study and prompted the regulatory body to insist on a one month delay between individual surgeries.

The experimental data presented in this paper suggest the presence of innate and adaptive immune responses following AAV administration. The innate immune response seems to activate all three main pattern recognition pathways (Toll-like, NOD-like, and RIG-I-like receptor pathways) and initiate a Th1 response. This is supported by the evidence of microglia activation, the recruitment of cytotoxic T cells and $CD20^+$ B cells into the retina. The eye was long considered an immune-privileged organ favoring application of gene therapy; however, recent findings, together with the data presented here, suggest that more research is necessary to identify the temporal dynamics and extent of inflammation upon AAV delivery, its impact on the safety and efficacy of ocular gene therapy, and ways to use this knowledge to improve future therapeutic applications.

MATERIALS AND METHODS

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were treated and taken care of at the Covance Preclinical Services test facility in Münster. The study was approved by the local institutional ethics board and conducted in accordance to GLP standards as defined by German GLP monitoring authorities, as well as in compliance with U.S. Food and Drug Administration (FDA) GLP regulations. The age of animals ranged between 2 and 5 years; the weight of animals was between 3.0 and 15.0 kg in males and 2.0 and 6.0 kg in females before treatment. An animal health assessment was performed by a qualified veterinarian before the start of the pre-dose phase to confirm the suitability of each animal for the study. The animals were not prescreened for pre-existing anti-AAV8 antibodies. They were assigned to two studies: 22 animals to a 90-day study and 12 animals to a 28-day study (Table 1). Animals were regularly assessed by clinical observation, monitoring of food consumption and body weight, and ophthalmic examination (fundus and slit lamp examination, fundus photography, scanning laser ophthalmoscopy, OCT, and angiography). All animals were fasted overnight before scheduled surgery and necropsy. Before exsanguination, animals received an intramuscular injection with ketamine hydrochloride followed by intravenous sodium pentobarbitone.

Surgery

Before surgery, periorbital regions were thoroughly cleaned with povidone iodine, sterile surgical drapes were applied, and a pediatric lid speculum was applied to left eye. A temporal canthotomy was applied to facilitate access where necessary. Three sclerotomies were made 1–2 mm posterior to the limbus after transillumination confirmed the location of pars plana. The inferotemporal port was used to fix a perfusion cannula. A pars plana vitrectomy was performed, and 4.0 mg/0.1 mL triamcinolone acetonide (preservative-free formulation) were used to visualize vitreous or posterior hyaloid membrane where appropriate. Localized retinal detachment was induced through injecting 50 µL of BSS (Alcon) in the subretinal space using a 41G cannula (DORC 1270.EXT). A total of 200 µL of vector solution was injected subretinally or intravitreally to achieve the designated dosing. The surgery was performed the same way in the clinical trial with two exceptions: no canthotomy was necessary in patients, and all sclerotomies were sutured in the clinical trial, but not in the animals, in which additional irritation due to sutures would have caused more oculodigital manipulation.

Dosing

The high-dose group animals received a total of 1×10^{12} vg, and the low-dose group animals received 1×10^{11} vg. Subsequent to surgery, subconjunctival cefuroxime (125 mg) and dexamethasone (4 mg) were administered to the operated eye. Postoperative local treatment

Table 3. Genes Overexpressed in Treated Animals with Inflammation versus Sham-Injected Animals

	Pattern Recognition Receptor Pathways				
	Symbol	Function	FC	p Value	FDR of 0.2
Toll-like Receptor Pathway					
1	IRF5	member of the interferon regulatory factor (IRF) family of transcription factors, responsible for activation of genes encoding key pro-inflammatory cytokines	2.0	0.0001	significant
2	TRAF3	member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family, a key regulator of non-canonical nuclear factor κB (NF-κB) signaling	1.4	0.0378	significant
3	IRAK1	member of the interleukin-1 receptor-associated kinase (IRAK) family; the phosphorylation of IRAK1 leads to subsequent activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways	1.1	0.0246	significant
RIG-like Receptor Pathway					
4	LGP2	member of the RIG-I-like receptor family, activating downstream interferon signaling	3.2	0.0024	significant
Nod-like Receptor Pathway					
5	AIM2	member of the pyrin family that serves as a cytosolic sensor for double-stranded DNA and a component of inflammasome	2.1	0.0001	significant
6	PYCARD	encodes for apoptosis-associated speck-like protein (ASC), which in association with caspase-1, forms NLRP3 inflammasome	2.2	0.0215	significant
7	Casp1	member of the cysteine-aspartic acid protease (caspase) family; sequential activation of caspases plays a central role in the execution phase of cell apoptosis	1.9	0.0268	significant
Cytokine Signaling and Other					
88	CXCL10	member of the CXC subgroup of cytokines that binds to CXCR3 and plays pivotal roles in the chemotaxis of inflammatory cells, being involved in Th1-oriented immune responses	19.3	0.0130	significant
9	CXCR4	α-chemokine receptor specific for stromal-derived-factor-1	2.2	0.0386	significant
10	STAT1	member of the signal transducer and activator of transcription (STAT) family that mediates cellular responses to interferons, cytokines, and other growth factors	3.5	0.0024	significant
11	APOBEC3G	component of innate antiviral defense system	2.1	0.0167	significant
13	MX1	interferon-induced dynamin-like guanosine triphosphatase (GTPase) that participates in cellular antiviral response by antagonizing the replication of viral RNA and DNA	2.2	0.0303	significant
14	CTSB	cathepsin B, a lysosomal cysteine protease that participates in intracellular proteolysis	1.6	0.0110	significant
15	CD40	receptor on antigen-presenting cells, mediating a variety of immune responses	1.6	0.0158	significant
16	IFIH1	encodes for MDA5, an innate immune receptor that acts as a cytoplasmic sensor of viral nucleic acids	2.1	0.0092	significant
15	L0C708080	cathepsin S, a lysosomal cysteine proteinase participating in degradation of antigenic proteins	2.1	0.0152	significant

FC, fold change; FDR, false discovery rate, as described by Benjamini and Hochberg.[23]

included eyedrops 3 times a day for one week: Dexamytrex, Dexa-Gentamicin, and Pred Forte (1% prednisolone). Systemic immunosuppression in the form of prednisone at 1 mg/kg (intramuscular) was administered from day 2 until day 5.

Vector and Vehicle

The AAV8 vector (expressing the human *CNGA3* cDNA sequence and driven by the cone-specific human arrestin 3 [0.4 kb] promoter[21,22]) was produced according to GMP guidelines by Atlantic BioGMP in Nantes, France. The manufacturing process of the vector relied on a transient double-transfection protocol of an HEK293 Master Cell Bank (MCB) fully characterized according to the European Pharmacopeia. Both plasmids were produced from two high-quality characterized *E. coli* MCBs (DH10B strain). Following expansion, the HEK293 cells were seeded in CellStacks 10 chambers for the double-transfection step. The transfected cells and supernatant were then harvested in a BioProcess Container, and the lysate was clarified by low-speed centrifugation. The cell pellet was discarded and the supernatant was PEG-precipitated overnight at 2°C to 8°C and then stored frozen at a temperature $\leq -70°C$. After thawing, the product was treated with Benzonase to digest nucleic acids and purified by two rounds of Cesium chloride gradient ultracentrifugation, followed by a tangential flow filtration step for diafiltration and concentration. After formulation in vehicle (BSS [Alcon] with 0.001% Kolliphor P188 Micro [Sigma]), the vector was stored at a temperature $\leq -70°C$ until use.

Histology

Eyes were fixed in 4% paraformaldehyde for 24 hr at 5°C ± 3°C. After fixation and removal of cornea, iris, and lens, the eyecup was

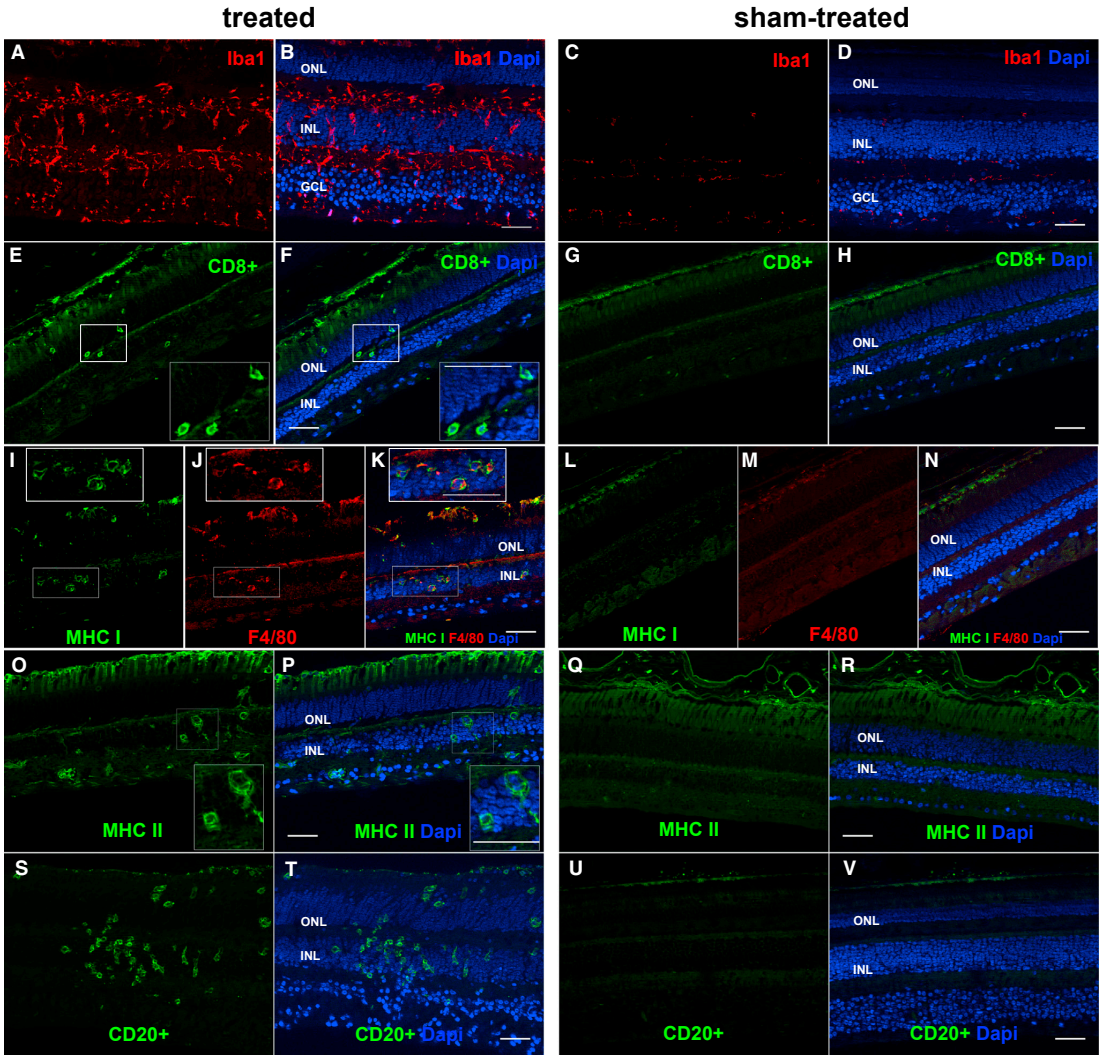

Figure 5. Retinal Immunohistochemistry Suggests Adaptive Cellular Immune Response in Eyes Treated Subretinally

Left panels show an eye treated with high-dose (1×10^{12} vg) AAV8 injection; right panels show the vehicle-treated control. (A–D) Staining for microglia and macrophages with ionized calcium-binding adaptor molecule 1 (IBA1) shows marked response in the treated eye. (E–H) $CD8^+$ cytotoxic T cells are evident in the AAV-treated eye, but not after surgery with vehicle. (I–R) Antigen-presenting cells are observed in the AAV-treated eye, as are $CD20^+$ B cells (S–V). Scale bar, 50 μm.

dehydrated in ascending concentrations of sucrose (10%, 20%, and 30% each for 2 hr) diluted in 0.15 M PBS. Then, the eyes were embedded in OCT and frozen in dry-ice-cooled isopentane (1 hr). Eye cryosections (20–40 μm) were made from the nasal to the temporal aspect in the sagittal direction and mounted on SuperFrostPlus glass slides.

qRT-PCR

RNA was extracted from 40 μm cryosections of treated eyes of the 12 monkeys in the 28-day study. Care was taken to select sections traversing the treated area in every animal. Using a sterile scalpel, the tissue was scraped off the slide and RNA was extracted after Proteinase K digestion through silica-membrane spin column technique

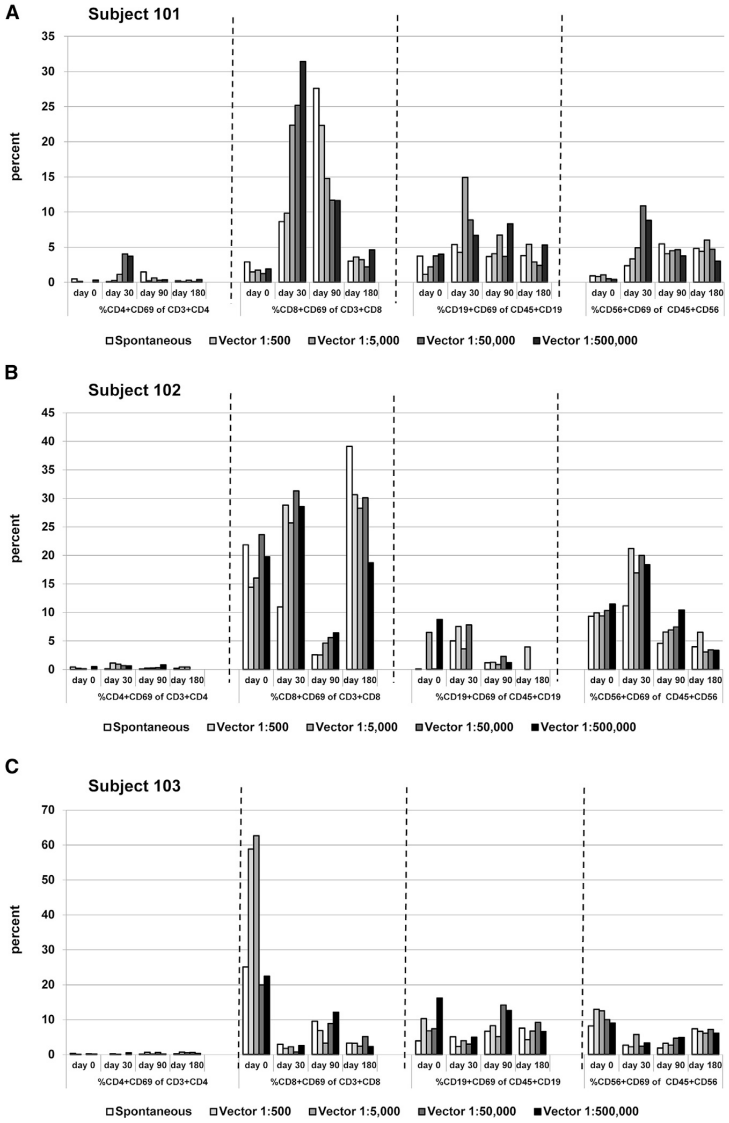

Figure 6. Activation Assays of PBMC Fractions

CD69 expression levels as surrogate markers for activation were quantified in PBMC subgroups $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD45^+/CD19^+$, and $CD45^+/CD56^+$ either spontaneously or after exposure to a dose range of AAV8 particles before and after treatment in three human patients. Two of three patients show marked changes in the activation pattern of cytotoxic T cells ($CD3^+/CD8^+$), with a peak 30 days after surgery. Different levels of spontaneous activity across time and across cohort demonstrate inter- and intraindividual variability. Absolute peaks of reactivity can be observed at different dilutions due to the non-linearity of the antigen-leukocyte response.

(RNeasy FFPE kit, QIAGEN). Concentration and quality of eluted RNA were analyzed by Infinite 200 NanoQuant (Tecan) and Bioanalyzer 2100 (Agilent); RNA was deemed of sufficient quality when the RNA integrity number (RIN) was above 7. Immediately after extraction of RNA, concentration differences were equalized, and reverse transcription was performed with the RT^2 First Strand Kit (QIAGEN) to prevent loss of RNA due to degradation. The cDNA was stored at −20°C until further processing. qPCR was conducted using the 96 gene Rhesus Macaque PCR Array (RT^2 Profiler PCR Array, QIAGEN) in a CFX96 C1000 Touch Thermal Cycler (Bio-Rad). The PCR cycling conditions were as follows: 10 min of 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a melt curve program at the end of each assay. Array quality was controlled by the following criteria: (1) for PCR array reproducibility, the average C_t of the array built-in PPC (positive PCR control) was 20 ± 2, and no two arrays had an average PPC C_t > 2 from each other, indicating that no amplification-inhibiting factors were present; (2) no inhibition of reverse transcription was determined by the average of the built-in RTC (reverse transcription control) − average PPC ≤ 5; and (3) no genomic DNA contamination was determined by a C_t of genomic DNA control (GDC) > 35. Data analysis was done by the $\Delta\Delta C_t$ method, in which every gene of interest (GOI) was normalized to the arithmetic average of expression of selected housekeeping genes (HKGs) using the following formula: $\Delta C_t = C_t^{GOI} - C_t^{AVG\ HKG}$. The following HKGs were used for normalization: β-actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein L13A. To compare expression of genes across the dose groups of animals, the $\Delta\Delta C_t$ for each gene was calculated as $\Delta\Delta C_t = \Delta C_t$ (dose group) − ΔC_t (control group). The fold change is then expressed as $2^{(-\Delta\Delta CT)}$. The p values are calculated based on Student's t test of the replicate $2^{(-\Delta\Delta CT)}$. The false discovery rate associated with multiple testing of expression data was controlled for by the false discovery rate method published by Benjamini and Hochberg.[23]

ELISA

A sandwich-ELISA strategy using an ELISA kit designed for titration of AAV8 particles (Progen, Germany) was applied to detect

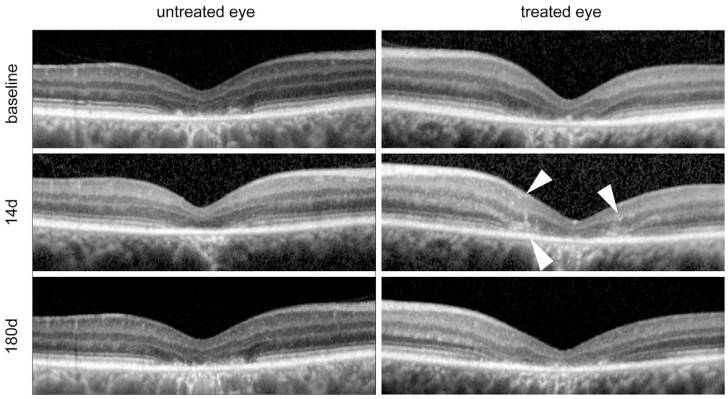

Figure 7. Virtual Cross-Sectional OCT Imaging
The treated retina (right) shows hyper-reflective spots (white arrowheads) two weeks after surgery compared to the untreated retina (left). Baseline imaging shows symmetric pre-existing foveal atrophy in this achromatopsia patient before surgery (top panels). Middle panels show the same area two weeks (14 days) after surgery to one eye (right panel). Six months (180 days) after surgery (lower panel), all hyper-reflective spots have resolved. However, some degree of structural loss can be seen that is not evident in the untreated contralateral eye.

anti-AAV8 antibodies. The microtiter strips with AAV8-specific antibodies from the kit were pre-incubated with clinical-grade AAV8. Plasma samples collected from each animal before and at days 1, 2, 7, 28, and 90 after surgery were then tested, and anti-drug antibodies (ADAs) were quantified via an enzyme conjugate of a rabbit-anti-NHP antibody (MP Biomedicals). An anti-AAV8-biotin-conjugated antibody, together with streptavidin peroxidase, was used as positive control, with both being added into the AAV8 titration strips. Once the substrate solution was added, the color reaction was measured photometrically at λ = 450 nm. To avoid false negatives, plasma samples were diluted serially (1:5, 1:25, and 1:125).

Immunohistochemistry

Sections were left to dry at room temperature for 30 min and then rinsed three times with PBS for 1 min (washing step). Sections were blocked with 10% donkey serum in PBS + 0.1% Triton X-100 for 1 hr, followed by the same washing step. Primary antibody was incubated for 1 hr at room temperature, followed by another washing step and application of secondary antibody for 1 hr at room temperature. The following antibodies were used: anti-human CD8 (1:500, AbD Serotec, MCA1226T), anti-HLA-DR (major histocompatibility complex class II [MHC II], 1:500, Abcam, ab136320), anti-Iba1 (1:700, Wako, 019-19741), CD20cy (1:500, Dako, M0755), anti-human HLA ABC (MHC I, 1:500, AbD Serotec, 136320), anti-F4/80 (1:250, Abcam, ab15285), donkey anti-mouse IgG H&L (1:1,000, Abcam, ab150105), and donkey anti-rabbit IgG H&L (1:1,000, Abcam, ab175470). After three rinses with PBS, the sections were mounted with an antifade reagent containing nuclear stain DAPI (Prolong Gold, Molecular Probes). Images of immunostaining were taken using an Axio Imager Z1 microscope (Zeiss, Oberkochen, Germany), and processed using Fiji (v.2.0.0-rc).

Patients

The three patients (two male and one female) were diagnosed with complete achromatopsia, and homozygous, disease-causing mutations in *CNGA3* were confirmed by a certified reference laboratory (CeGaT, Tübingen). Patients underwent the procedure after written informed consent was given and were followed up according to the approved trial protocol (ClinicalTrials.gov: 02610582; see also Supplemental Materials and Methods) approved by the local institutional ethics board. All patients in this first dosing group received 200 µL containing 1×10^{10} vg of the clinical-grade vector rAAV8.hCNGA3. Safety assessments included clinical and ophthalmological examinations (including OCT imaging) at screening, directly after surgery, and 1, 2, 3 ± 1, 14 ± 3, 30 ± 5, 90 ± 7, and 180 ± 7 days after surgery. All patients in the study received a prophylactic regimen with oral prednisolone at 1 mg/kg as per study protocol (from day 1 until day 19, followed by tapering off as deemed appropriate by the investigator).

Assessment of Cellular Immune Response in Patients

Blood samples were taken in all patients at screening and 30 ± 5, 90 ± 7, and 180 ± 7 days after surgery to isolate PBMCs and test activation potential upon stimulation with AAV8 in PBMC subpopulations, as previously described.[24] Briefly, 120 mL of heparinized blood were drawn from subjects, and PBMCs were isolated by centrifugation through Ficoll-Hypaque gradient within 24 hr. In previous studies, we showed that within this time, interval cells are still viable and reliable, and reproducible results without significant changes can be obtained.[25,26] PBMCs were adjusted to 1 million cells/mL (5×10^5 cells/well) in RPMI 1640 medium supplemented with 25% decomplemented autologous serum and gentamycin. PBMCs were incubated with medium only (spontaneous activity) or with serial dilutions of clinical-grade AAV8 vector (1:500, 1:5,000, 1:50,000, or 1:500,000) for 24 hr and then assessed for subgroup identity and CD69 expression levels as a surrogate marker for activation. Antibody cocktails were used for the demonstration of activated PBMC subpopulations (activated $CD4^+$ T cells, FastImmune CD4/CD69/CD3; activated $CD8^+$ T cells, FastImmune CD8/CD69/CD3; activated $CD19^+$ B cells, FastImmune CD19/CD69/CD45; activated $CD56^+$ natural killer [NK] cells, FastImmune CD56/CD69/CD45; Becton Dickinson, San Jose, CA). Furthermore, an IgG isotype control antibody was used (BD Biosciences, San Jose, CA; Pharmingen). At least 10,000 PBMCs were counted. Quadrants were set based upon

the isotype controls for each antibody. Results were expressed as the percentage of CD69-expressing cells of the respective cell types.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and one figure and can be found with this article online at https://doi.org/10.1016/j.ymthe.2017.08.018.

AUTHOR CONTRIBUTIONS

F.F.R. planned and executed experiments, analyzed data, and wrote the manuscript. D.L.D. analyzed data and wrote the manuscript. R.K. planned and executed experiments and analyzed data. T.P. planned and executed experiments. G.A.O. and I.P.S. analyzed data and edited the manuscript. B. Wilhelm, M.U., M.B., B. Wissinger, and S.M. edited the manuscript. K.U.B.-S. planned and executed experiments and helped to interpret data. M.D.F. planned and executed experiments, analyzed data, and wrote the manuscript.

CONFLICTS OF INTEREST

No competing financial interests exist for F.F.R., D.L.D., R.K., T.P., G.A.O., I.P.S., B. Wilhelm, M.U., B. Wissinger, K.U.B.-S., or M.D.F. M.B. and S.M. and other members of the RD-CURE Consortium are named inventors on a patent filed on behalf of EyeServe GmbH, relating to the expression cassette. M.D.F. is a consultant to NightstaRx Ltd., Casebia Therapeutics LLC, Regenxbio Inc., and EyeServe GmbH.

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4 Discussion

With the data presented in the two papers we attempted to approach the immune response to subretinal AAV from the broadest possible angle. The chances and limitations of this approach will be discussed in the following order: The data presented in the paper "Humoral immune response after intravitreal but not after subretinal AAV8 in primates and patients" will be discussed in the first paragraph "Humoral immune response". The clinical inflammation, the histology from the nonhuman primate retinas and the gene expression profile, all published in the paper "AAV can induce innate and adaptive immune response in the primate eye" will be discussed subsequently.

4.1 Humoral immune response

To summarize the results of the humoral immune response, our study demonstrated that antibodies were generated in the intravitreal NHP group but not in the subretinal NHP group. No antibody formation was observed in the three patients. Antibody titers started to rise from day 7 onwards, with a peak on day 28 and declined towards day 90.

These results mirror findings from other studies. It was already assumed that the intravitreal space somehow is more accessible to the immune system after two groups had described that AAV administered to the intravitreal but not the subretinal space can lead to antibody production which can prevent re-administration[77, 78]. It has been hypothesized that the enhanced biodistribution of viral vector over the blood and lymphatic tissue after intravitreal administration could serve as an explanation[110].

In contrast to the results of the mentioned studies, subretinal injections of rAAV2 expressing CNGB3 (in cynomolgus monkeys) showed a dose-dependent antibody formation[111], suggesting that an antibody reaction after subretinal AAV delivery is possible if a certain threshold is exceeded. Further support for this theory stems from the observation of a dose dependent humoral immune response after subretinal delivery in NHPs in a study by Vandenberghe et al. and in another study by Ramachandran et al. (although not as prominent as after the intravitreal application)[51, 112].

However, an antibody reaction in the subretinal high dose group would not necessarily mean that the assumption of a superior immune privilege in the subretinal space would

have to be suspended, it could also simply represent a reflux through the retinotomy into the intravitreal space.

In this context it has to be noted that in our study antibody formation in the subretinal high dose group can not be completely ruled out as some values in this group are missing. These missing values could not be calculated because they exceeded the range of the assay. Nevertheless, an antibody reaction in the subretinal high dose group of the NHP (1x10^12vg) would not have had direct implications for the human trial as the highest dose planned in the clinical trial corresponds to the low dose group of the NHP preclinical trial (1x10^11vg).

Concerning the question of relevance, it has to be kept in mind that the rising antibody titers in our study did not correlate with clinical findings in the ophthalmic assessment. For instance, the intravitreal group showed the highest rise in antibody titer but the lowest clinical severity score. Puzzlingly, in our results high antibody titers therefore could not be correlated with inflammation in the eye.

But, as studies have shown that pre-existing humoral immunity is able to reduce therapeutic efficacy of ocular or systemic gene therapies [77, 113], clinical relevance could arise in the situation of re-administration of vector.

Another limitation to this study is the lack of absolute thresholds for the level of significance of specific titer values. As ELISA values are not comparable between different studies it is difficult to judge which change of titer is to be deemed relevant.

Further, titers showed variability in the sham injected group over the observation period, suggesting the occurrence of normal fluctuations in titer and/or test-retest variability of the assay. It is possible that the rise of antibody titer observed in some animals are in fact fluctuations of the titer that arise from the contact with wtAAV8 as wtAAV8 was originally isolated from and is commonly found among macaques[114]. High seroprevalence was also seen in our study. 90% of the monkeys showed pre-treatment antibody titers against AAV8. Interestingly, the antibody reaction of the two seronegative animals did not differ from the other animals.

Another limitation of the study is the absence of technical replicates in the ELISA assay. Instead, the titers were calculated using the slope of a dilution sequence.

Finally, the study's significance is limited by the small number of animals in each group and the presumable differences in antibody reaction between the monkeys and the human immune system.

In summary, despite the mentioned limitations, our results suggest that AAV capsid antigen are processed by the adaptive immune response leading to the formation of specific antibodies. The histology data from the NHP, with infiltrating antigen presenting cells in the retina supports this thesis and further proposes the retina as a possible place of antigen presentation. These findings are of high value to the developers of ocular AAV mediated gene therapies in general and especially to the ones that use the intravitreal approach. These currently include AAV gene therapy studies for X-linked Retinoschisis (XLRS) or AMD[115, 116].

4.2 Ocular inflammation following AAV

4.2.1 Ocular inflammation in non-human primates

Clinical signs of inflammation like cells in the anterior or posterior chamber of the eye, were observed in all NHP groups. While cells in the anterior chamber peaked at day three and gradually declined afterwards, intravitreal cells persisted at low levels.

The subretinal control group which received a sham injection with balanced salt solution (BSS) also showed a limited number of cells at a lighter scale than the other groups indicating that some degree of inflammation is due to the surgical procedure by itself.

This kind of ocular inflammation following the application of subretinal or intravitreal AAV has been observed in several other preclinical and clinical studies.

Ye et al. described a dose dependent inflammation of the anterior and posterior segment with vitreous cells that persisted over the full observation period (90 days) after intravitreal application of rAAV2tYF (a modified version of AAV serotype 2) for X-linked juvenile Retinoschisis (XLRS).[111] These results were reproduced in a second study by the same authors for a subretinally applied vector based on the AAV 2 serotype (rAAV2tYF-PR1.7-hCNGB3 to treat Achromatopsia).[117] Both studies used a capsid containing three tyrosine-to phenylalanine mutations to enhance transduction efficacy. The implications for immune reactivity of the capsid are unknown.

One animal in the CNGB3 study developed a severe ocular inflammation (endophthalmitis) at day 5 and had to be sacrificed subsequently. Microbiological tests showed no bacterial growth in cytology or aerobic culture. These findings could suggest a test-item related immune response but Ye et al. argued that an occult bacterial endophthalmitis could not be ruled out completely. In all other vector-injected animals of the study histological examination showed minimal mononuclear cell infiltrates around retinal blood vessels.

Dose dependent ocular inflammation in NHP has also been reported by two other groups (AAV vectors expressing GFP): Vandenberghe et al. and Ramachandran et al. both showed that high dose subretinal AAV8 can lead to focal retinal inflammation[51, 112]. Ramachandran et al. additionally reported an upregulation of GFAP expression as a marker of glial activation. Vandenberghe et al. further described retinal thinning and disrupted retinal architecture as well as a T cell mediated transgene toxicity against GFP but no T cell response against the AAV8 capsid. The group hypothesized that the transgene immunity against GFP could be attributed to the fact that GFP is a foreign protein (derived from jellyfish) and thus potentially immunogenic in the NHP.

Although this reflects the situation of patients with null-mutations receiving gene addition therapy (which would lead to expression of a 'novel', if human, protein), transgene-directed immune response has not yet been described in human clinical trials (including in patients with predicted null mutations).

4.2.2 Ocular inflammation in patients

The patients of our study did not present any signs of inflammation in the clinical slit-lamp examination. It has to be noted though, that one patient presented hyper-reflective spots in the OCT. Although multiple theories exist for the pathological correlation of these spots, the most likely correlate are activated microglia or other infiltrating immune cells[118, 119]. Our observation in the NHPs support this interpretation. Under steroid treatment these spots resolved completely.

In the pivotal phase III trial for voretigene neparvovec (AAV2-hRPE65v2), 6 out of 20 patients were reported to have presented mild signs of ocular inflammation. All signs of inflammation resolved under concomitant steroid treatment[30].

A more vigorous inflammatory response in a patient has been reported by Bainbridge et al. in 2015. In their RPE65 trial, 5 out of 8 high dose group patients responded with signs of clinical inflammation and one patient showed a persistent reduction in visual acuity together with a rise of neutralizing antibodies around week 4 and a slight increase of circulating T cell against the AAV2 capsid[1].

Similar to the findings by Bainbridge et al., Dimopoulos et al. reported recently that in their AAV2-mediated choroideremia trial one out of six patients developed a serious adverse event after the injection of subfoveal AAV2.REP1. The patient presented a localized intraretinal immune response (hyperreflective spots), that resulted in a decline of vision and loss of outer retinal structures[2]. It is yet unclear why some patients reacted with vigorous inflammation and others did not.

4.3 Histology

In the hematoxylin and eosin staining of the NHP retinal sections of our 28 day study, mononuclear cells infiltrations were observed in the subretinal and choroidal space. These infiltrations were mostly found in the high dose group. Less pronounced infiltrations were predominantly found in the low dose group, but also found in the sham injected control group and could thus represent incidental background findings and/or be due to the surgical procedure itself (as opposed to the AAV). Interestingly, no histological abnormalities were found in the animals that were sacrificed after 90 days suggesting a transient nature of the immune reaction.

In order to further define the nature of the infiltrating immune cells found after 28 days, immunohistochemistry was performed on neighbouring sections of the same eyes. A technical limitation to overcome was, that the eyes had been fixed in 4% PFA leading to some difficulties in finding of the optimal staining protocol. As fixation in PFA is known to mask antigens and impair epitope binding, spleen tissue of the same monkeys was cut and stained beforehand with the same protocol to serve as a positive control (especially

for epitopes relating to immune competence). The sham-injected eyes of the control group served as the negative control.

4.3.1 Iba1

Iba1 (ionized calcium-binding adapter molecule 1) was used as the most reliable staining marker for microglia[120] Retinal microglia in the normal retina populate the plexiform layers[121]. They are stationary phagocytic sentinel cells that have migrated into the retina during embryonic development and early postnatal phase. In the retina, microglia are responsible for the control of retinal homeostasis and the phagocytosis of cell debris. Therefore, they inhibit a variety of surface proteins and long motile processes to scan their local environment[122]. Age-related and multifactorial retinal diseases as well inherited degenerative retinal diseases are associated with an activation of microglial cells[123-125]. In the case of activation microglia start to proliferate and change their appearance from a ramified form into an amoeboid form that migrates from the plexiform layer into other layers and to the site of damage[121].

When comparing the treated eye to the sham injected eye, staining with Iba1 showed a marked increase in signal. Also, the distribution pattern differed and treated eyes showed Iba1 signal not only the plexiform layer, but also the nuclear layers of the retina.

The signal increase was restricted to the area of treatment.

Microglia represent the cellular innate immune response in the retina. The activation we observed could therefore be due to the recognition of either vector particles themselves or a change of microenvironment that leads to proliferation of microglia. It is improbable that the activation visible 30 days after treatment is attributed to the temporary retinal detachment as the slides were compared to eyes that received a sham-injection of the same volume with BSS. The fact that the Iba1 signal is observed in the expected location (plexiform layers and after activation in all layers) further supports the thesis that microglia are indeed observed.

Microglial activation is regulated by several inhibitory factors expressed by other cells of the retina to maintain the balance between the beneficial phagocytic functions of the microglia and possible auto-destructive responses that damage neurons[126, 127].

Form the histological observation of activation we cannot tell whether this balance is compromised. The activation of microglia could also represent the reestablishment of the

homeostasis after some sort of damage caused by the treatment. What can be concluded from the observation, though, is that homeostasis is at least temporarily impaired leading to an activation of microglia.

4.3.2 MHCI

As mentioned in the introduction, the classic pathway of innate antiviral defence mechanisms of host cells leads to the presentation of viral particles via MHCI molecules, which -in turn- enable cytotoxic CD8 T cells to kill the virus infected cell.
Staining with MHCI gave a strong signal of the RPE and some singular cells in the retina that were not found in the sham injected eyes.
In the central nervous system MHCI is generally expressed only by glia cells and not by neurons in order to protect neuronal tissue from cytotoxic T cell responses. Nevertheless, this view has been challenged by the observation that in some special inflammatory situations in vivo and under artificial conditions in vitro neurons are able to present MHCI[128]. However, this has not yet been described for the adult retinal cells which is supported by our observation that did not include MHCI upregulation in retinal neural cells.
The RPE cell, as a phagocytotic cell is however expressing MHCI constitutively represented on our slides by a strong signal of MHCI over the RPE layer[129].
The MHCI positive cells observed within the retinal layers could therefore either represent microglia or other immune competent cells that have infiltrated the neural tissue.

4.3.3 MHCII

MHC II is expressed by antigen presenting cells (APC). In the retina these cells include microglia and macroglia, from which the latter can be subdivided into retinal astrocytes and Müller cells[121]. The RPE cells are known to express MHCII after stimulation with INF-γ and can therefore also be regarded as antigen presenting cells[130].
Immunohistochemistry of the AAV treated slides showed MHCII positive cells in the retina and in the subretinal space. Double-staining with Iba1 (unpublished images) showed that some cells co-express MHCII and Iba1 but not all cells do. Although no

evidence is at hand to the nature of these MHCII positive cells that do not co-express Iba1, they could, besides the other mentioned APCs, partly represent CD20 positive B cells, which were also found in the retina but could not be co-stained with MHCII for technical reasons.

4.3.4 CD8

CD8 positive cells that were found in the retina most likely represent infiltrating cytotoxic T cells. Capsid specific T cells have already been observed after AAV mediated gene transfer to hepatocytes[81]. In the eye they have not been described following AAV treatment but in models of uveitis like Experimental autoimmune uveoretinitis (EAU) CD4 as well as CD8 positive T cells are the main mediators of inflammation[131].
But CD8 positive cells could also carry out regulatory function. Although the main protagonist of regulatory T cells are $CD4^+$, $CD25^+$, $FoxP3^+$ T cells, $CD8^+$ regulatory T cells have also been described to be part of the immune suppressive mechanisms of the eye[132]. However, these cells did not stain for CD4, markers for regulatory activity (CD103b, FoxP3) or INF-γ as marker for a TH1 response.

4.3.5 CD20

Interestingly staining for CD20 showed that infiltrating B cells seem to be involved. This is another parallel to the pathological observations of EAU in a monkey model where infiltrating B cells have been described to be the main infiltrating lymphocyte[133]. In uveitis, B cells play a key role in the inflammatory process which is further evidenced also by the beneficial treatment effects of Rituximab, a CD20 antibody therapy[133, 134].
Tissue infiltrating B cells play a role in various autoimmune diseases, especially neurological autoimmune disorders[135]. They also play an important role as tumor infiltrating lymphocytes and take part in the rejection of renal allografts[136, 137]. Of relevance, they have also been observed in local immune infiltrations after intramuscular AAV1 gene therapy[138].
The function of the B cells in the retina is unclear. As reviewed by Smith et al. for non-infectious uveitis, there are multiple potential mechanisms by which B cells can initiate

or perpetuate inflammation in the eye. However, they can also contribute to immune suppression mediated by a subset of B cells called B regulatory cells[139].

The mere presence of B cells in the retina strongly encourages the idea of an antigen specific immune response mechanism. The induction of antibodies observed is in line with this theory. Unfortunately, no PBMC (peripheral blood mononuclear cell) activation assay data is at hand for the NHP. In the patient PBMCs no activation of $CD19^+$ B cell after stimulation with AAV8 was observed.

4.4 Expression Analysis

For the gene expression analysis, retinal tissue samples from the high dose group were compared to samples from the sham injected animals. The data showed enhanced gene expression of markers involved in the innate anti-viral immune response in the samples from the high dose group vs the sham injected animals. These included markers of the three major pattern recognition receptor pathways (TLRs, RIG-like receptors and NOD-like receptors) as well as markers involved in an INF-γ mediated TH1 response like the chemokine CXCL10.

Although TLR9 and Myd88 were not significantly upregulated, their downstream signalling molecules IRF5 and TRAF3 were. As mentioned in the introduction, the TLR9-Myd88 pathway has been shown to be part in the recognition of AAV and responsible for staging a Th1 mediated immune response. Although no answer is at hand why only some of these downstream molecules are overexpressed and others are not our findings could still be implicating that the TLR9 pathway is activated.

Other markers that were found upregulated in the high dose group have not yet been associated with AAV but could be interesting targets for future investigations. LGP2 (Fold change (FC) of 3.2, p value 0.0024) and AIM2 (FC of 2.1, p value 0.0001) are both sensor proteins for cytosolic dsDNA. LGP2, a DEHX box domain protein related to the RIG-I like receptors participates in the cellular response to cytosolic dsDNA[140]. Although the AAV genome traffics the cytosol via endosomes and is only released into the cells' nucleus, it can be hypothesized that some DNA molecules may be recognised, making these cytosolic DNA receptors interesting molecules for future investigations on AAV immunity.

Absent in melanoma 2 protein (AIM2) is a member of the PYHIN protein family and another sensor for cytosolic dsDNA. AIM2 forms together with ASC and caspase1 the inflammasome and drives INF gene transcription[105]. ASC (FC of 2.2 p value 0.0215) and Caspase 1 (FC of 1.9, p value 0.0268) were also both overexpressed which strengthens the assumption that this pathway could be activated.

The highest elevation (Fold change of 19, p value: 0.013) was shown for CXCL10. This chemokine is induced by INF-γ and attracts macrophages, T-cells and NK cells. It is further known to play a pivotal role in mediating the influx of effector T cells into the CNS in a number of viral infectious diseases and can be expressed by glia and neurons[141, 142]

Taken together, this data suggests that the subretinal treatment with AAV8 leads to an activation of the innate immune response in the retina.

Although INF-γ expression was not detected directly, the upregulation of several pathways connected to the release of INF-γ and the finding of T and B cells in the retina are highly suggestive of an INF-γ mediated TH1 response. Although INF-γ release is generally restricted to T cells and NK cells, in some situations it can also be expressed by microglia[143]. As the gene expression analysis data results from the whole retina tissue und not a specific cell line no conclusive answer to the question can be drawn from these results. In the future cell culture experiments might be able to shed more light on the actions of the different cells involved.

Limiting factors of the assay are the low number of animals included (high dose n=3, control group n=4) and the fact that the tissues had been fixed with PFA before RNA isolation. Paraformaldehyde leads to chemical modification and fragmentation of RNA[144]. For this reason, formalin-fixed samples are often poor material for molecular biology applications. Especially long RNA molecules are prone to fragmentation resulting in an altered expression profile. But as Wiegers and Hilz described in 1971, the use of Proteinase K prior to extraction digests the ribonucleases that degrade RNA during the extraction process[145]. After several attempts the use of Proteinase K was found to be crucial in receiving good quality RNA. This finding is well supported by existing literature[145-147]. Our results on RNA quality assessments after isolation and prior to the qPCR experiment showed that our data offers a valid expression profile.

4.5 Conclusion

Multiple studies have shown that subretinal AAV meditated gene therapy can lead to inflammation of the eye. This immune response is in most cases a transient phenomenon that is manageable with steroid treatment. But individual cases of clinically significant immune response effecting visual function illustrate that knowledge about the immunologic potential of AAV in the eye is not only essential to enhance safety but might also increase the efficacy of future therapies.

Our data suggest that certain amounts of subretinal AAV are able to overcome the anti-inflammatory immune privilege of the eye. This thesis is supported by gene upregulation of markers of the innate antiviral immune defense, the activation of microglia as the "immunological watchdogs"[121] of the retina and the infiltration of cells of the adaptive immune system like $CD8^+$ T cells and $CD20^+$ B cells. The role of these cells in the retina can only be hypothesized but $CD8^+$ cytotoxic T cells are known to play a major role in antiviral immunity against AAV transduced cells[81, 89]. It therefore seems likely that they adopt a similar function in the retina. The fact that NHP did not present histological signs of inflammation after 90 days matches the clinical observation of a transient immune reaction. This could be attributed to the anti-inflammatory features of the eye that in most cases are able to contain the immune response. It remains unclear, though, whether specific tolerance is induced or if the decline of inflammation is a sign of effective clearance of the remaining AAV vector particles. If the latter is true, future studies would have to determine if the immune response is only directed to free AAV8 particles or as well to vector transduced cells. As photoreceptors do normally not present antigens via MHCI, damage to these cells is probably only resulting as a side effect of activated infiltrating APCs that are not suppressed by the anti-inflammatory mechanisms of the eye.

It has to be kept in mind that all NHP and all patients received concomitant steroid treatment. As the inflammation responded to steroid treatment this measurement seems to be justified. Future studies will have to show if alternative or additional anti-inflammatory regimens could help to suppress the immune response even better. Care has to be taken when applying new anti-inflammatory drugs as studies have shown that the

immunomodulation is able to cause also unwanted detrimental effect to the tolerogenic properties of AAV[148, 149].

Limitations of the study include presumed differences between the human and nonhuman primate immune system, the limited timepoints, missing PBMC data from the NHP and missing antibody data for the 28 day NHP study.

However, this study adds valuable information to the question how AAV is able to elicit specific immune responses in the subretinal space. This information might be able to guide future studies in enhancing safety and efficacy of the therapy.

5 Summary

5.1 Introduction

Adeno-associated viral (AAV) vectors are commonly used in ocular gene therapy for inherited retinal degenerations. They are known for their excellent efficacy in transducing retinal cells and their benign immune profile. However, the observation that subretinal AAV can lead to ocular inflammation highlights the importance to re-evaluate the immunogenicity of AAV.

5.2 Methods and material

Cynomolgus monkeys (*Macaca fascicularis*) were treated with subretinal or intravitreal injections of AAV serotype 8. The high dose group received 1x10^12 viral genomes (vg) and the low dose group 1x10^11 vg. The control group received injections with vehicle only (balanced salt solution). Of the 34 animals included in the study, 12 were sacrificed after 28 days and 22 were sacrificed after 90 days. During the observation period, blood was taken at fixed timepoints for ELISA of antibodies directed against AAV capsid epitopes. After the inlife phase of the study, eyes were fixed in 4% PFA, embedded and frozen at -80°C. 20µm cryosections were subsequently used for expression profiling and immunohistochemistry.
These data were compared to clinical observations from three patients treated with the same vector subretinally. OCT images were taken and peripheral blood mononuclear cells were isolated from patient blood at defined timepoints to be analysed for AAV specific reactivity.

5.3 Results

The ELISA study of NHP derived blood samples showed an increase in anti-AAV8 titer at day 30 in the intravitreal high dose group but not in the subretinal high or low dose group. No antibody formation was observed in the three patients. Immunohistochemistry showed an inflammation of the NHP retina with activation of $IBA1^+$ microglia and infiltration of $CD8^+$ T cells and $CD20^+$ B cells at 28 days following surgery. Expression analysis of the retinal tissue showed an upregulation of genes related to the innate antiviral

immune response and associated with the release of INF-γ. None of the three patients showed clinically significant inflammation, but one featured hyperreflective foci in OCT images one month after surgery, which disappeared following steroid treatment. PBMC analysis showed changes in the activation pattern of $CD8^+$ T cells one month after surgery in 2 out of 3 patients.

5.4 Discussion

The data presented in this study suggests the activation of the innate and adaptive immune response following AAV administration. This hypothesis is supported by the activation of markers of antiviral immune pathways (Toll like, RIG1 like and NOD like receptor pathways) and the increase of capsid specific antibodies as well as retinal infiltration of cells of the adaptive immune response. Future studies will have to determine the exact dynamics of the infiltrating immune cells and how this immune response can be effectively inhibited.

6 Zusammenfassung

6.1 Einführung

Gentherapeutische Ansätze zur Behandlung erblicher retinaler Degenerationen basieren zum Großteil auf rekombinanten adeno-assoziierten Viren (AAV) als Vektor zur Transduktion der Netzhautzellen. AAV sind im Vergleich zu anderen viralen Vektoren bekannt dafür, dass sie bei guter Transduktionseffizienz gleichzeitig eine gering ausgeprägte Immunogenität aufweisen. Aufgrund sich mehrender Beobachtungen von Immunreaktionen auf AAV vermittelte Gentherapien in präklinischen und klinischen Studien wurde immanent, dass der bisherige Kenntnisstand zur Immunogenität diese Vektoren lückenhaft ist und einer umfassenden Klärung bedarf. Das Ziel dieser Doktorarbeit war über die Erforschung der Mechanismen auf molekularer und zellulärer Ebene die Tür in diese Richtung etwas aufzustoßen.

6.2 Material und Methoden

Im Rahmen einer präklinischen Toxizitäts und Sicherheitsstudie wurden Javaneraffen (Macaca fascicularis) mit einem AAV8 vector entweder intravitreal oder subretinal behandelt. Unterteilt wurde in eine Hochdosisgruppe (1x10^12 virale Genomkopien (vg)), eine Niedrigdosisgruppe (1x10^11 vg) sowie eine Kontrollgruppe, welche eine gepufferte Kochsalzlösung erhielt. 12 Versuchstiere wurden über einen Zeitraum von 4 Wochen, 22 Versuchstieren über einen Zeitraum von 90 Tagen nach Behandlung beobachtet bevor die Organentnahme durchgeführt wurde. Während des genannten Zeitraums wurden Blutproben entnommen und Antikörpertiter mittels ELISA Technik berechnet. Danach wurden die Augen in 4% Paraformaldehyd fixiert, in einem Gefrierschneidemedium eingebettet, und in 20µm Schnitten bei -80 C° eingelagert. Diese Schnitte wurden dann wiederum für immunhistochemische Färbungen und zur Erstellung von Expressionsprofilen genutzt. Diese Daten wurden verglichen mit klinischen Observationen dreier, mit dem gleichen Vektor subretinal behandelter Patienten. Hierzu wurden OCT Bilder und die in-vitro Reaktion peripherer Immunzellen auf den Vektor analysiert.

6.3 Ergebnisse

In den Vektor-injizierten Javaneraffen stiegen Antikörpertiter gegen des AAV8 Capsid um den Tag 30 nur in der intravitreal behandelten Hochdosisgruppe aber nicht in den subretinal injizierten Gruppen. In den Patientenproben waren keine Antikörperreaktionen sichtbar. Die immunhistochemischen Färbungen der Affennetzhäute fand sich bei den nach 4 Wochen getöteten Tieren eine entzündliche Infiltration von Immunzellen, unter diesen $IBA1^+$ Mikroglia Zellen, $CD8^+$ T-Zellen und $CD20^+$ B-Zellen. In der RNA - Expressionsanalyse derselben Tiere zeigte sich eine Aktivierung von Genen für Signal und Rezeptormoleküle welche über die Aktivierung von INF-γ an der angeborene Immunantwort gegen Viren beteiligt sind. Keiner der Patienten präsentierte klinische Zeichen einer Entzündung. Allerdings waren im OCT fokale Veränderungen der Netzhaut sichtbar, welche auf eine Prednisolontherapie ansprechend, daraufhin verschwanden. Die Analyse der PBMC zeigte eine Aktivierung von $CD8^+$ T-Zellen nach einem Monat in 2 von 3 Patienten.

6.4 Diskussion

Die in dieser Doktorarbeit präsentierten Daten zeigen die Aktivierung einer lokalen und systemischen Immunantwort. Die Hochregulierung von antiviralen Genen bestimmter Pathways (Toll like, RIG 1 like und NOD like receptor pathways) zusammen mit dem Anstieg von spezifischen Antikörpern suggeriert die Beteiligung der angeborenen sowie die adaptiven Immunantwort. Welche klinische Bedeutung die einzelnen Beobachtungen haben und welche Implikationen für die Weiterentwicklung okulärer Gentherapien müssen weitere Studien herausfinden. Dies ist ebenso wichtig für die Sicherheit von AAV-vermittelten Gentherapien in der klinischen Anwendung wie für die therapeutische Effizienz dieses Ansatzes.

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8 Erklärungen zum Eigenanteil

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

8.1 Erklärung zum Eigenanteil der Dissertationsschrift in Bezug auf die Veröffentlichung:

AAV8 Can Induce Innate and Adaptive Immune Response in the Primate Eye.
Reichel FF, Dauletbekov DL, Klein R, Peters T, Ochakovski GA, Seitz IP, Wilhelm B, Ueffing M, Biel M, Wissinger B, Michalakis S, Bartz-Schmidt KU, Fischer MD; RD-CURE Consortium.
Mol Ther. 2017 Dec 6;25(12):2648-2660. doi: 10.1016/j.ymthe.2017.08.018. Epub 2017 Aug 31.

Die Arbeit wurde in der Augenklinik Tübingen unter Betreuung von Daniyar Dauletbekov, PhD und Prof. Dr. Dr. M. Dominik Fischer durchgeführt.

Das dieser Arbeit zur Verfügung stehende Material ist Teil der Achromatopsie Studie des RD-CURE Konsortiums. Die Koautoren Dr. T. Peters, Prof. Dr. med. B. Wilhelm, Prof. Dr. M Ueffing, Prof. Dr. M. Biel, Prof. Dr. M. Wissinger, PD Dr. S. Michalakis, Prof. K.U. Bartz-Schmidt und Prof. Dr. Dr. M.D. Fischer sind Mitglieder dieses Konsortiums welches verantwortlich ist für die Konzeption der CNGA3 Achromatopsie Studie.

Vorbereitende Arbeiten
Die Entwicklung des Vektors, die Operationstechnik, die Konzeption der Toxikologie Studie erfolgte durch Drs. Peters, Michalakis und Fischer in Zusammenarbeit und im Rahmen des RD-CURE Konsortiums.

Die Konzeption des Experiments zur Genexpressionsanalyse erfolgte durch Prof. Fischer. Methodische Verfeinerungen der RNA Isolation für die Genexpression wurden von mir konzipiert. Die Konzeption der Experimente zur Immunhistochemie erfolgte durch Prof.

Fischer, erweitert durch meine eigenen Ideen und Vorschläge zB zur Färbemethodik oder der Untersuchung bestimmte Oberflächenmarker.
Die Konzeption der Aktivitätsassay der PBMCs erfolgte durch Prof. Fischer in Zusammenarbeit mit Frau Prof. Klein vom Immunpathologischen Labor der Inneren Medizin II, Tübingen.

Die Versuche zur Genexpressionsanalyse sowie die Immunhistochemischen Färbungen wurden von mir eigenständig durchgeführt. Die Asservierung der Patientenproben für die Gewinnung PBMCs wurden von mir zusammen mit I.P. Seitz durchgeführt. Die Aktivitätsassays der Patienten PBMCs wurden im Labor von Frau Prof. Klein in Kooperation durchgeführt.

Die statistische Auswertung der Genexpressionsanalyse erfolgte eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

8.2 Erklärung zum Eigenanteil der Dissertationsschrift in Bezug auf die Veröffentlichung:

Humoral Immune Response After Intravitreal But Not After Subretinal AAV8 in Primates and Patients.
Reichel FF, Peters T, Wilhelm B, Biel M, Ueffing M, Wissinger B, Bartz-Schmidt KU, Klein R, Michalakis S, Fischer MD; RD-CURE Consortium.
Invest Ophthalmol Vis Sci. 2018 Apr 1;59(5):1910-1915. doi: 10.1167/iovs.17-22494.

Die Arbeit wurde in der Augenklinik Tübingen unter Betreuung von Prof. Dr. Dr. M. Dominik Fischer durchgeführt.

Erklärung zum Eigenanteil

Das dieser Arbeit zur Verfügung stehende Material ist Teil der Achromatopsie Studie des RD-Cure Gene therapy trials Konsortiums. Die Koautoren Dr. T. Peters, Prof. Dr. med B. Wilhelm, Prof. Dr. M Ueffing, Prof. Dr. M. Biel, Prof. Dr. M. Wissinger, PD Dr. S. Michalakis, Prof. K.U. Bartz-Schmidt und Prof. Dr.Dr. M.D. Fischer sind Mitglieder dieses Konsortiums welches verantwortlich ist für die Konzeption der CNGA3 Achromatopsie Studie.

Vorbereitende Arbeiten
Die Entwicklung des Vektors, die Operationstechnik, die Konzeption der Toxikologie Studie erfolgte durch Drs. Peters, Michalakis und Fischer in Zusammenarbeit und im Rahmen des RD-CURE Konsortiums.

Die Konzeption der Antikörper Elisa erfolgte durch Prof. Fischer in Zusammenarbeit mit Drs Peters, Michalakis und Specht.

Die Antikörper Elisa wurden durch Dr. T. Peters durchgeführt.

Die statistische Auswertung der ELISA – Daten erfolgte durch mich unter Anleitung von Prof. Fischer.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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