A SYSTEMS APPROACH TO NON-INFECTIOUS UVEITIS

Fleurieke H. Verhagen

Colofon

Cover design: The unraveling of the immune system in this multifactorial disease is a complex process and sometimes it is hard to see the wood for the trees. The birch trees and scattered networks serve as a reminder that while each component of a network is important, a helicopter view is crucial to see the bigger picture.

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A SYSTEMS APPROACH TO NON-INFECTIOUS UVEITIS

Een systeem-brede benadering van niet-infectieuze uveitis
(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

Chapter 1	General introduction	*8*
Chapter 2	Prevalence and characteristics of ocular pain in non-infectious uveitis: a quality of life study. *Br J Ophthalmol. 2018*	*28*
Chapter 3	Potential predictors of poor visual outcome in human leukocyte antigen-B27 associated uveitis. *Am J Ophthalmol. 2016*	*44*
Chapter 4	Reduced number of relapses of human leukocyte antigen-B27 associated uveitis during pregnancy. *Acta Ophthalmol. 2017*	*62*
Chapter 5	Aqueous humor analysis identifies higher branched chain amino acid metabolism as a marker for HLA-B27 acute anterior uvietis and disease activity. *Adepted version accepted for publication (2018)*	*68*
Chapter 6	Systemic inflammatory immune signatures in a patients with CRB1 linked retinal dystrophy. *Expert Rev Clin Immunol. 2016*	*98*
Chapter 7	A disease-associated microRNA cluster links inflammatory pathways and an altered composition of leukocyte subsets to non-infectious uveitis. *Invest Ophthalmol Vis Sci. 2018*	*108*
Chapter 8	CCR6+ T cells are an early predictor of the need for systemic immunomodulatory treatment in non-infectious uveitis. *Submitted*	*154*
Chapter 9	A CD1c+ dendritic cell transcriptomic program is linked to human non-infectious uveitis. *Manuscript in preparation*	*186*
Chapter 10	Summary, general discussion and perspectives	*224*
Appendices	Samenvatting (Summary in Dutch) Dankwoord (Acknowledgements) About the author List of publications	*252* *260* *264* *265*

1

Chapter 1

GENERAL INTRODUCTION

BACKGROUND AND EPIDEMIOLOGY OF NON-INFECTIOUS UVEITIS

Uveitis comprises a heterogeneous group of intraocular inflammations that together account for ~10% of preventable blindness among the working-age population in western countries.[1,2] According to the Standardization of Uveitis Nomenclature guidelines[3], the anatomic site of the focal point of the inflammation determines the classification of uveitis into anterior-, intermediate-, posterior-, or pan-uveitis. In one out of every three uveitis cases an infectious cause can be identified, while in another (small) fraction another disease or malignancy is 'masquerading' as uveitis.[4–7] In the majority of uveitis patients, however, no infectious agent is found and they are classified as non-infectious uveitis (NIU). NIU is considered to be immune-mediated and frequently co-occurs with systemic inflammatory disease (e.g., Behcet's disease or sarcoidosis).[8] However, also various discreet forms of NIU manifest as an isolated (eye-restricted) inflammatory condition (e.g., Birdshot Uveitis).

NIU has been estimated to affect approximately 1.2-5 out of every 1000, which roughly translates to 25,000 individuals in the Netherlands and over a million in Europe.[9–12] Complications from immune activity in the delicate eye tissue continuously pose a threat to vision. Eventually this may lead to permanent visual impairment or blindness in up to 19% of all eyes.[13–17] In general, adult patients with non-anterior uveitis (i.e., intermediate / posterior or pan uveitis) are at greater risk for developing complications and permanent vision loss.[16,17] However, the visual prognosis, disease course and the treatment response all vary substantially between uveitis subtypes and individual patients.

The personal and socioeconomic consequences of NIU are immense since NIU affects relatively young (i.e., working age) people and often has a recurrent or chronic course, potentially demanding treatment for decades. Furthermore, NIU is associated with impaired performance of daily activities and work as well as an increased risk of depression.[18,19] Also, because it affects relatively young people, the total annual costs of blindness caused by uveitis are calculated to be roughly the same as the costs of blindness related to diabetes, despite the latter being more common.[2,20]

CLINICAL COURSE - THREE ARCHETYPICAL EXAMPLES OF NON-INFECTIOUS UVEITIS

Acute anterior (HLA-B27 associated) uveitis

Acute anterior uveitis (AAU) is the most common form of uveitis and accounts for about half of all patients in tertiary centers and up to 90% of all cases in primary settings.[5,9] Around half of the AAU patients is HLA-B27 positive against 8-10% of the Caucasian

population, depending on geographic location.[21–25] HLA-B27-associated anterior uveitis typically comprehends recurrent episodes of acute-onset, non-granulomatous inflammation of the anterior segment (i.e., iris, ciliary body) that is usually limited to one eye or alternating between the eyes.[26,27] Patients usually present between the age of 20-40, and complain of pain, photophobia and blurred vision.[28] Although this type of uveitis is usually self-limiting - resolving typically within three months - it is notoriously recurrent in nature and usually requires treatment for decades to prevent complications such as posterior synechiae, cataract formation, band keratopathy (opacification of the cornea), cystoid macular edema (CME) or secondary glaucoma.[24,29–32]

During an exacerbation it is not uncommon for visual acuity to drop dramatically.[33] In most cases the vision recovers with treatment and the long term visual outcome is relatively good, with 0-12% of all eyes meeting criteria for permanent visual impairment (Snellen equivalent ≤20/50).[33–37] However, the impact of HLA-B27 AAU on visual function might well be underestimated as most reports have a short follow-up time (<3 years), while this type of uveitis affects patients for decades. Also, with a recurrence rate of 1-2 times a year[32,35], it is a debilitating disease which highly impacts quality of life, the ability to work and mental well-being.[19,20,38]

HLA-B27 acute anterior uveitis is strongly linked to the HLA-B27-positive spondyloarthropathies, such as ankylosing spondylitis (AS), a seronegative arthritis in which up to 50% of patients will eventually develop anterior uveitis.[39] Vice versa, 42-71% of HLA-B27 positive anterior uveitis patients will develop AS.[40–43] In most cases AS develops before uveitis[44] and many patients who are first diagnosed with uveitis turn out to have a history of unrecognized back pain.[42,43,45] Other associated diseases include inflammatory bowel disease (IBD, 2%), psoriatic arthritis (3-4%), reactive arthritis (formerly known as Reiter's disease, 8-21%), and undifferentiated spondylarthropathies (5-21%).[28,33,37] The strong link between this uveitis entity and other (HLA-B27) systemic conditions suggests that studying this disease provides a unique gateway to dissect the pathology of anterior NIU in the context of common inflammatory mechanisms.

Idiopathic intermediate uveitis

Intermediate uveitis (IU) usually affects the anterior vitreous and peripheral retina (pars plana), but may involve also tissues in the area just behind the iris and lens or the vitreous membrane.[3] IU is associated with the HLA-DRB1*15 allele[46,47] and linked to multiple sclerosis (MS, 2-30%) and Sarcoidosis (~10%) - both HLA-DRB1*15-associated immune-mediated conditions.[48–55] The diagnosis of MS is based on the McDonald criteria, which

require typical central nervous system lesions evident by MRI.[56,57] It is important for the treating ophthalmologist to exclude any underlying demyelinating disease, because – paradoxically – MS can exacerbate upon treatment with anti-TNF treatment (Adalimumab), an agent used in the treatment of uveitis (see '***treatment***' below).[58] Distinguishing ocular sarcoidosis from idiopathic uveitis is usually achieved by chest imaging (X-ray, CT-scan or chest computed tomography) and increased serum levels of soluble interleukin(IL)-2 and/or angiotensin converting enzyme (ACE).[59]

About half of all IU patients will be considered idiopathic ('idiopathic intermediate uveitis' [IIU], also termed pars planitis) in the absence of an associated infection or evidence for systemic illness.[3] IIU has a prevalence rate of 1.4-5.9 per 100.000.[60,61] On ophthalmological examination vitreous cells and haze can be seen as well as the formation of inflammatory cell clusters known as snow balls and snow banks. Inflammation of the anterior segment, such as the development of corneal keratic precipitates, can occur in up to half of the patients.[62] However, unlike anterior uveitis, patients with IU rarely complain about pain or photophobia (<7%).[63] In fact, the affected children and adults (mean age of onset 23-33 years) often present with few complaints other than blurred vision and/or floaters.[48,63] Other typical features of IIU are peripheral vascular sheathing, optic disc edema and vasculitis.[62]

The disease burden of IIU can be attributed to its chronic disease course with frequent complications.[61,64] The predominant vision threatening complications are cystoid macular edema (CME) and optic disc edema, but also vitreous opacities frequently hamper vision.[22,53] Although long-term visual prognosis is relatively good, it is a potentially blinding disease. Typically, vision is lost gradually and eventually causes visual impairment in 13% of all eyes, most of which (10% of all eyes) will become legally blind, most often as a result of persistent CME.[13] This high inter-patient variability in visual prognosis illustrates the need personalized medicine to prevent under- and overtreatment of IIU.

Birdshot Uveitis

Birdshot Uveitis (also known as Birdshot chorioretinopathy or Birdshot chorioretinitis) is one of the most extensively characterized entities of posterior uveitis and involves bilateral multifocal inflammation of the choroid and retina. The term 'Birdshot' is referring to the scattered cream colored choroidal lesions which resemble the pattern of a shotgun.[65,66] With an estimated prevalence between 0.1-0.6 per 100,000, Birdshot uveitis is a very rare disease.[65] The most striking feature of Birdshot is the unusual strong association with HLA-A29 (MHC class I). All patients carry this class I allele and HLA-A29 positivity

has become a prerequisite for diagnosis.[67–71] Patients are usually over 45 years and of West-European descent and complain of blurred vision, floaters, night blindness, reduced contrast sensitivity, dyschromatopsia (difficulties with color vision) and reduced peripheral vision.[72,73] On ophthalmological examination (mild) vitritis and haze, CME, vasculitis, and –as disease progresses - the characteristic hypopigmented oval choroidal lesions become more pronounced.[74] Other typical features are significant retinal vascular leakage of the arcade vessels on fluorescence angiography, unique patterns of reduced perifoveal fundus auto fluorescence signals, and reduced retinal and choroidal thickness.[75–78] Changes in choroidal tissues are accompanied by focal infiltrates visible by extramacular enhanced depth optical coherence tomography (OCT), matching the focal paravascular T cell infiltrates reported in histopathology investigation of enucleated birdshot eyes.[79]

Although birdshot can be self-limiting, often this condition has an insidious onset and chronic course with poor visual outcome (despite long preserved central visual acuity), which - if untreated - may result in widespread retinal atrophy and visual field loss.[73,80] Birdshot commonly requires systemic treatment with immunomodulatory agents, which fail in up to 40% of patients (see '***treatment***' below). Thus, there is a pressing need for more effective treatment options for this severe form of NIU.

TREATMENT OF NON-INFECTIOUS UVEITIS

Glucocorticosteroid therapy is the first-line of therapy in the treatment of NIU.[81] Short-term treatment with local (topical or periocular) corticosteroids may be sufficient to dampen eye inflammation for some patients.[45] However, in many cases long-term systemic immunomodulatory therapy (IMT) is required to control eye inflammation and prevent vision loss.

Given the significant concerns with long-term safety of glucocorticosteriod therapy due to the side effects of long term use, it is essential to switch to corticosteroid sparing treatment in case prolonged IMT is required to control uveitis: non-corticosteroid systemic IMT includes conventional agents; methotrexate (MTX), mycophenolate mofetil and azathioprine, and T cell inhibitors such as cyclosporine and tacrolimus, or the more recently available biologicals, which target specific immune molecules (e.g., cytokine(receptors)).[82] Although the beneficial use of most (conventional) immunosuppressive therapies for the treatment of NIU have been established in various prospective and retrospective studies, double blind placebo controlled trials remain scarce. Consequently, a very limited number of IMT is officially registered for the use in NIU and until very recently, corticosteroids were the only officially registered systemic therapy for the treatment of NIU.[82]

In 2016, the anti-TNF-alpha monoclonal antibody Adalimumab was the first biological to be registered for the treatment of therapy-resistant intermediate, posterior or pan uveitis after being proven effective in randomized clinical trials.[83,84] Although the advent of anti-TNF treatment has revolutionized the treatment of NIU, it is not registered for anterior uveitis, which is the most common localization of NIU.[5,9] Also, treatment of IIU with Adalimumab remains controversial as it can cause or exacerbate MS (see '***idiopathic intermediate uveitis***' above).[58] Furthermore, anti-adalimumab antibodies develop in up to a third of all patients, which hinders mono-therapy and, thus, requires combined treatment with methotrexate.[85,86] Next to this, current therapies fail in up to 40% of cases, either due to inefficacy or because they are stopped because of side effects.[83,87] This is an alarming observation, because prompt effective treatment is essential to minimize damage from complications of NIU.

To summarize, the treatment of NIU is hampered by the limited registered options and high variability in response rate. It is currently unknown which patients will respond to which therapy or who will develop side effects. Nor is it known which patients will benefit from systemic IMT and who will only need local therapy. As a result, trial and error-based clinical assessment (e.g., wait to see therapy response) currently guides therapy choice. Consequently, there is a pressing need for tools to assess optimal treatment strategies for individual patients.

Caveats in the treatment of patients with non-infectious uveitis

The great variability in disease course, therapy response and prognosis among patients with NIU, as illustrated above, means there is a high need for more 'personalized' or 'patient-tailored' medicine. This approach implies that selection of treatment should be guided by clinical and biological features (i.e., biomarkers) rather than on diagnosis alone.[88] Also, the poor response to existing IMT options show a need for new therapeutic agents. However, to achieve this, a deeper understanding of pathophysiological mechanisms underlying the disease is crucial.

PATHOGENESIS - WHAT DO WE KNOW?

The exact cause of human non-infectious uveitis is poorly understood and has even led to the proposal to rename NIU as 'undifferentiated' uveitis.[3] NIU is considered to be an immune-mediated condition driven by (erroneous) inflammatory responses of both the innate and adaptive arms of the immune system.[89–91] Although a multitude of immune components are associated with NIU, autoreactive T cells are the protagonists in murine models of uveitis and most scientific evidence in humans also support T cell involvement (see '***T cells in NIU***' below).

The cause of derailed T cell activity in NIU is unknown, however, the classical view considers environmental factors to catalyze (eye) inflammation in genetically susceptible individuals.[89] Genetic studies have mapped part of the genetic susceptibility for NIU to immune genes[39,92–94], most commonly the major histocompatibility complex (MHC) region.[25,46,68,70,71,95,96] The association between NIU and MHC (also called human leukocyte antigen (HLA) in humans) has been long known. In fact, the association of HLA-B27 and acute anterior uveitis was the earliest association between HLA and disease to be discovered.[25] Other types of NIU have been linked to MHC as well, such as the above-mentioned full association between HLA-A29 and Birdshot.[69] However, the HLA haplotypes that are associated with uveitis are common in the (healthy) population but only a fraction of these individuals will develop uveitis. This suggests that HLA positivity alone is not sufficient to cause disease.[97,98] Indeed, recent Genome Wide Association Studies (GWAS) have discovered strong associations with the endoplasmic reticulum amino peptidase (ERAP) genes in various inflammatory conditions, including HLA-B27 associated anterior uveitis and Birdshot uveitis.[39,67,99] ERAP genes encode aminopeptidases that are involved in trimming of peptides before they are loaded onto the HLA class I proteins to be presented to the surveilling immune system.[100] HLA class I and ERAP functionally interact in antigen processing, which suggests a central role for antigen processing in the pathogenesis of non-infectious uveitis.[99,101] Yet, the role of antigen presenting cells in human NIU is relatively unexplored (see '***dendritric cells in NIU***' below). Other genes implicated in NIU disease pathogenesis include also IL23R (encoding the receptor for Th17-related cytokine IL-23), and other loci of significance have been found containing cytokine genes such as IL10-IL19, IL18-IL1R1, IL6R.[39]

Still, these genetic associations only explain a part of disease pathogenesis as there are only a few reports of monozygotic twins that both develop uveitis.[102–106] Currently, NIU is considered a complex (e.g., multifactorial) condition driven by an interplay of many genetic, epigenetic and environmental factors.

T cells in NIU

NIU is considered a T cell-mediated disease based on the observation of abundant intraocular T cell infiltrates in the inflamed retinas as well as in vitreous fluid of patients.[107–109] Consequently, most studies into T cells have focused on phenotyping immune cells in the blood or eye fluid (i.e., aqueous humor or vitreous fluid) and have revealed altered proportions of T helper (Th) cells type 1 and 17.[91,110] Both Th1 and Th17 are able to cause experimental autoimmune uveitis (EAU) in mice.[111] Other supporting evidence for the Th17 axis includes numerous reports on elevated levels of cytokines linked to these cells in eye fluid and blood, including interleukin (IL-)17, and IL-23.[112–115] Importantly, in human

NIU there is merely circumstantial evidence for the direct involvement of Th17 cells in uveitis pathology and consequently their function might also be regulatory in humans.

There remain many caveats in the understanding of the pathophysiology of NIU. For example, it is unknown where T cells are activated and subsequently infiltrate the eye. Studies on T cells in NIU show antigen reactivity to various ocular (primarily retinal) antigens, including Retinal S-arrestin (S-antigen) and interphotoreceptor retinoid-binding protein (IRBP). Immunization mice with these retinal antigens is used to mimic uveitis in mouse models mediated by pathogenic T cells that produce IL-17.[91] However, some of these ocular antigens can also be found in tissues outside the eye. More importantly the uveitis phenotypes in animal models based on T cell reactivity to these antigens do not fully cover the clinical variation in human uveitis phenotypes (e.g., unilateral or bilateral, acute or insidious of onset, recurrent or chronic, etc).[90] Also, it remains unknown where T cells can be activated, since the current dogmatic view states that ocular antigens remain sequestered behind the blood-retinal barrier.[91,116] Perhaps during infection the blood retinal barrier is compromised and consequently facilitates the exposure of ocular antigens to autoreactive T cells.[90] Alternatively, uveitogenic T cells could also be activated in the gut, indicating a role for the gut microbiome.[117,118] Interestingly, among the first models for anterior uveitis is the endotoxin induced uveitis in rats.[119,120] Also, experimental autoimmune uveitis (EAU) induced by immunization with ocular antigens is dependent on bacterial adjuvant (for example lipopolysaccharide) and activated dendritic cells.[91] Vice versa, 'spontaneous' uveitis models do not develop in germfree conditions.[117] This has slowly refocused scientific efforts back to the long-standing hypotheses of molecular mimicry with microbial peptides (perhaps presented via risk HLA alleles)[121,122] or bystander T-cell activation[123,124], perhaps through microbiome dysbiosis.
Further investigation of T cell subsets in well-defined clinically distinct types of human NIU will aid in answering some of the questions above. Also, the skewing of T cell responses towards an autoreactive Th1 or Th17 phenotype is determined by the interaction with antigen presenting cells (APCs).[111,125] Therefore, it is highly essential better understand the role of APCs, such as dendritic cells, in the pathogenesis of NIU.

Dendritic cells in NIU

Antigen presenting cells express MHC class II molecules, which activate T cells through presentation of antigen embedded in these MHC molecules together with costimulatory surface receptors, such as CD40, CD80 and CD86. Dendritic cells (DCs) are professional antigen presenting cells. Injection of eye-protein pulsed dendritic cells elicits uveitis in mice and demonstrates the ability of dendritic cells to cause intraocular inflammation.[126]

However, it is yet unknown where this DC-T cell interaction takes place in human uveitis: in the eye or in the periphery (as is suggested by the microbiome hypothesis).[127] Regardless, CD11c+ (myeloid) dendritic cells (mDCs) have been shown to be enriched in aqueous humor of anterior uveitis patients[128] and elevated proportions of circulating CD1c+ myeloid DCs (myeloid dendritic cells type 1, or mDC1s) have been associated with uveitis activity.[129]

SYSTEMS IMMUNOLOGY AS A TOOL TO BETTER UNDERSTAND NON-INFECTIOUS UVEITIS

Mouse models of uveitis have contributed greatly to the better understanding of NIU, but can only represent limited aspects of human uveitis.[90] It is therefore essential to study NIU using human tissues. Efforts to accomplish profiling of ocular fluids and blood samples have taken a giant leap in the last decade: with the rapid advancement in technologies, we have entered an era of 'systems'-scale biological phenotyping. Novel sensitive, high-throughput, multiplexed experimental techniques make it possible to extensively phenotype the immune system.[130] To deeply phenotype changes in composition and function, information about various distinct layers of biological information (e.g., DNA, mRNA, microRNA, proteome, metabolome) can be obtained and compared between tissue compartments (i.e., serum, plasma, immune cell subsets, eye fluid). These results can then be combined by powerful computational modelling approaches to identify the pathways that are most relevant for disease course. Furthermore, clinical end points can be matched to molecular data to provide diagnostic and prognostic clues which form the basis for personalized - or patient tailored - medicine.[131] This 'holistic' approach of mapping and combining several (molecular and clinical) data layers is known as '***systems medicine***' or '***systems immunology***'.[132] The approach for this thesis is explained further in **Figure 1**. Several tools to facilitate the immune phenotyping of patients with NIU are outlined in more detail below.

Flow cytometry

Flow cytometry is a technique used to phenotype and quantify cells based on the expression of surface and intracellular proteins, which can be detected by fluorescent-labeled antibodies.[133] The combination of multiple markers (i.e., flow 'panels') allows the characterization of discreet cell populations by manual or automatic gating of cells based on the relative intensity of expression for each surface marker. Manual gating is a process in which a researcher defines which cells are positive or negative for a marker in two dimensional scatter plots. However, as the surface protein expression pattern rarely yields two perfectly separable clusters, this approach is prone to user bias and, consequently,

may lead to inconsistent outcomes between different (experienced) researchers.[134,135] Furthermore, cell identification is biased towards known cell phenotypes. With the advent of automatic gating by computational algorithms, cell populations are identified based on hierarchical clustering of phenotype date obtained by the ensemble of cell surface marker intensities.[134,135] Detected clusters (e.g., cell populations) can then be analyzed and compared across predefined conditions (for example disease groups).

MicroRNA profling

MicroRNAs (miRNAs) are small non-coding RNAs that are abundantly found in almost all biological tissues. MiRNAs interfere with the translation of messenger RNA (mRNA) of more than half of the protein-coding genome and consequently orchestrate complex biological circuits including immunity.[136–139] Although binding of miRNA to mRNA generally has a modest effect on protein expression, changes in the expression of miRNAs can have a great impact on cellular signaling.[140] As a result, a large number of profiling studies have been conducted to find changes in the levels of miRNAs that could potentially be used to diagnose or monitor disease or serve as novel therapeutic targets.[141,142] MiRNA profiling studies can be conducted using sequencing technologies, microarrays or PCR platforms, all with their respective (dis)advantages.[143]

RNA-sequencing of immune cells

High-throughput sequencing of the whole transcriptome (RNA-seq) provides rich information on gene expression within a cell.[144] RNA sequencing produces large expression datasets of both coding and non-coding genes and their organization in the cellular circuitry. Although RNA levels do not always reflect cellular protein levels[145] transcriptomic profiling has become central to understanding disease mechanisms at the cellular level. Because RNA sequencing yields vast amounts of data computational modeling is crucial to unearth sophisticated structures of relevant information. Computational analyses, such as weighted gene correlation network analysis (WGCNA), are used to define clusters of co-expressed genes (e.g., pathways) linked to disease to identify targets for functional studies.

Metabolomics

Metabolomics refers to detection and quantification of a broad range of small metabolic products using mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy.[146] This rapidly emerging omics field is commonly used to detect disease specific metabolic signatures to better understand pathophysiology or to aid in diagnosis and reveal prognostic biomarkers. There are several techniques to measure metabolites, each with

their respective advantages and limitations. Direct Infusion Mass Spectrometry (DIMS) is a method particularly suitable for hypothesis generating (untargeted) approaches as it enables fast measurements on low sample (i.e., clinical) volumes. However, standard compounds are needed for accurate peak identification, which is commonly achieved by targeted ('low-throughput') Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS).[147,148]

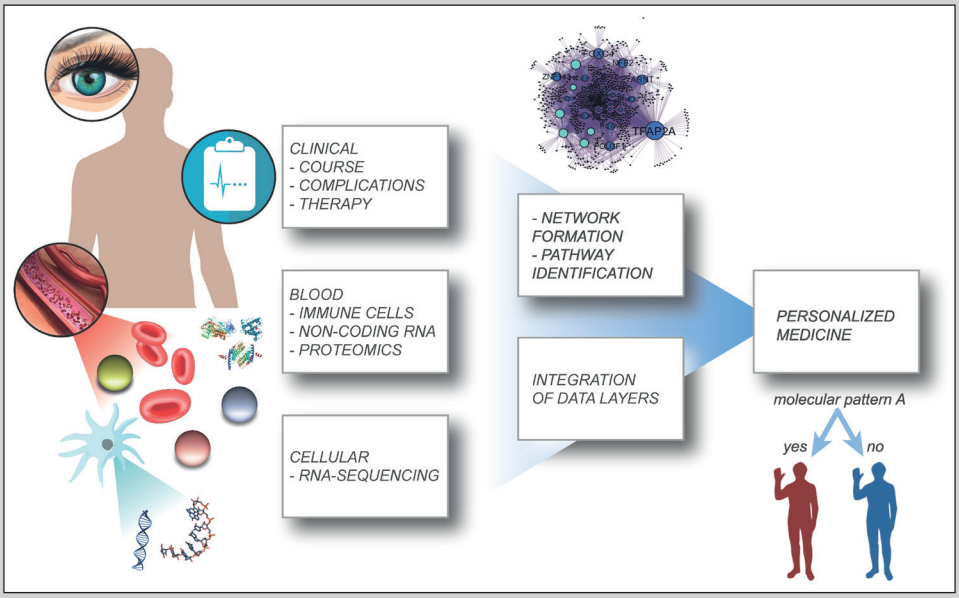


Figure 1. Work flow of systems immunology as a means towards personalized medicine.
Using high-throughput techniques several layers of the immune system (i.e., immune cells, circulating proteins, immune cell gene expression etc.) will be studied. Through computational modeling immune networks of uveitis associated factors are created that guide in the selection of key molecular disease routes. Integration of network data may aid in spatio-temporal regulation of various arms of the immune system.. Identified key mediators from systems data are further integrated with clinical outcome data and form the basis for omics-guided clinical decision tools.

THESIS OUTLINE

The aim of this thesis was to gain better insight the clinical and immunological aspects of non-infectious uveitis, to serve as a foundation for personalized medicine and find new options for therapeutic targeting. To better understand disease impact we conducted a quality of life study and focused specifically on (peri)ocular pain, an ill understood complaint of intermediate and posterior uveitis patients that has been prioritized by the Dutch uveitis patient organization (**chapter 2**). To be able to predict which patients have a higher risk of poor visual outcome we studied clinical parameters in HLA-B27 associated anterior uveitis (**chapter 3**). Also, because HLA-B27 associated anterior uveitis often

affects relatively young patients of a reproductive age and systemic IMT might harm an unborn child, we studied how this type of uveitis behaves during pregnancy (**chapter 4**). Next, we compared the metabolic profile of aqueous humor between HLA-B27 positive and negative acute anterior uveitis (**chapter 5**). In **chapter 6**, we combined clinical and molecular monitoring of a patient with ocular inflammation during treatment with a novel biological (Tocilizumab). In **chapters 7-9** we used a systems approach to deeply phenotype several layers of the immune system in the blood of patients with one of three archetypical uveitis subtypes: HLA-B27 associated acute anterior uveitis, idiopathic intermediate uveitis, and Birdshot uveitis. We mapped circulating serum microRNAs (**chapter 7**), we phenotyped circulating immune cells using flow cytometry (**chapter 8**), and we performed RNA-sequencing of CD1c-positive myeloid dendritic cells, an important but ill understood cell subset in the disease pathogenesis of non-infectious uveitis (**chapter 9**). The most important findings and their implications are summarized and discussed in more detail in **chapter 10**.

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Chapter 2

PREVALENCE AND CHARACTERISTICS OF OCULAR PAIN IN NON-INFECTIOUS UVEITIS - A QUALITY OF LIFE STUDY

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ABSTRACT

Purpose: To survey the frequency, character, severity and impact of ocular pain on quality of life in adult non-infectious uveitis patients

Methods: This patient requested cross-sectional survey study describes the results of three self-administered questionnaires (the National Eye Institute visual function questionnaire (NEI VFQ-25), the 36-Item Short Form Health Survey (SF-36) and the McGill Pain Questionnaire Dutch Language Version (MPQ-DLV)) from 147 non-infectious uveitis (NIU) patients from a university-based tertiary referral center in Utrecht.

Results: The mean VFQ Ocular Pain score of all NIU patients was 72 (± 24), which is significantly lower than an ocular disease free reference group (90 ±15, $p<0.0001$), indicating more ocular pain. This was true for all types of NIU, regardless of the localization: although ocular pain scores were lower in anterior uveitis (AU) patients compared with non-AU patients (mean 62 (±24) vs 74 (±24), $p=0.04$), non-AU patients still scored substantially lower than the reference group that had no ocular history ($p<0.0001$). Patients with NIU also scored significantly lower on all other VFQ-subscales as well as on the SF-36 subscales "Role Limitations due to physical problems", "Vitality", "General health" and "Bodily Pain" compared with controls. The VFQ Ocular Pain subscale correlated with other quality of life subscales (both VFQ-25 and SF-36), indicating a relationship between pain and quality of life.

Conclusion: This study shows that ocular pain is highly prevalent in non-infectious uveitis patients, regardless of the localization. Furthermore, ocular pain has an impact on quality of life.

INTRODUCTION

Non-infectious uveitis (NIU) is a collective term for a heterogenic group of intraocular inflammations. As a disease group NIU is among the major causes of vision loss of the working-age population in the western world.[1] However, the impact of the disease goes beyond the (threat of) vision loss. This is demonstrated by a decreased vision related quality of life and impairment in the daily activities, social life and work.[2–4]

Although pain is one of the hallmark features of acute anterior uveitis, it is not often mentioned in the context of chronic non-anterior uveitis. Still, there is a variety of studies that demonstrate the presence of ocular pain in patients with all kinds of NIU, indicating that ocular pain might have higher prevalence, in a wider variety of NIU patients, than has thus far been presumed.[4–9]

At this moment the prevalence and impact of ocular pain in non-infectious non-anterior uveitis are unknown. Upon the specific request of the Dutch uveitis patient society we surveyed the prevalence, character, severity and impact of ocular pain in patients with NIU using three standardized and validated questionnaires.

PATIENT VOICE (DUTCH UVEITIS SOCIETY)

In several contact moments with our members the question was raised what was known about the relationship between non-infectious uveitis and pain, and especially what this means for the quality of life. The reason for this question is that several patients experience ocular pain, but also problems at work: some of them are not able to work or lost their job. This strongly influences private life and their confidence, and thus, quality of life.

METHODS

We performed a cross-sectional survey study using three self-administered questionnaires. Patients of 18 years or older with NIU that visited the Ophthalmology outpatient clinic of the University Medical Centre Utrecht in the months May or September 2014 were invited to participate. Each patient underwent a full ophthalmological examination by an experienced ophthalmologist, routine laboratory screening, and a chest X-Ray. Laboratory screening included (but was not limited to) erythrocyte sedimentation rate, renal and liver function tests and screening for various infectious agents in the serum including syphilis, Lyme disease and an Interferon-Gamma Release Assay (IGRA) tuberculosis test. Common viral causes (Herpes zoster virus (HZV) or Varicella zoster virus (VZV), Cytomegalovirus (CMV), Rubella) were excluded by PCR and Goldmann-Witmer coefficients assessment of aqueous humor when viral causes were clinically suspected.

Patients were excluded if they had scleritis or if they had undergone eye surgery in the last month, under the assumption that ocular pain perception would be too much influenced. The study was approved by the Medical Ethics committee of the UMC Utrecht. All patients signed written informed consent.

Data collection

Three standardized and validated questionnaires were used: the National Eye Institute Visual Functioning Questionnaire (NEI VFQ-25) to asses vision related quality of life (VR-QoL), the 36-Item Short-Form Health Survey (SF-36) to assess the general health related quality of life (GH-QoL), and the Dutch version of the McGill Pain Questionnaire (MPQ-DLV).[10–15] Questionnaires were sent to all candidates. All patients were asked to fill in the questionnaires and return them by post. We also asked for the patient's medical history and medication use. In addition, the clinical records were reviewed in order to collect information about activity, localization, laterality, duration, and aetiology of the uveitis, ocular or systemic comorbidities and therapy. All these variables were recorded as outlined by the Standardization of Uveitis Nomenclature (SUN) criteria.[16]

The activity was scored as either quiescent (no signs of activity on the previous and adjacent visit) or active (cells in anterior chamber, increase of cells in vitreous fluid, new onset CME / vasculitis / phlebitis within two weeks of filling in the questionnaire). Because patients filled in the questionnaires at home – sometimes leaving weeks between the hospital visit and filling in the questionnaire – a third category of activity was added: recently active or low-grade activity (either active uveitis in the last six months before filling in the questionnaire or persistent cystoid macular edema /persistent low grade vasculitis). For analyzing, Snellen Best Corrected Visual Acuity (BCVA) was converted to the logarithm of the minimum angle of resolution (logMAR) equivalent. Counting fingers (CF) was converted to +1.98 logMAR and hand motion (HM) was converted to +2.28 logMAR, while light perception (LP, n=3) or no light perception (NLP, n=2) were excluded from statistical analysis.[17]

The NEI VFQ-25 measures VR-QoL across 12 subscales: general health, general vision, ocular pain, near vision activities, distance vision activities, vision specific social functioning, vision specific role difficulties, vision specific mental health, vision specific dependency, driving, peripheral vision, and colour vision.

The SF-36 measures the GH-QoL across eight subscales: physical functioning, role limitations due to physical health, role limitations due to emotional problems, energy/ fatigue, emotional well-being, social functioning, bodily pain, and general health. For both the SF-36 and the VFQ-25, answers to the questions are converted to a 0-100 point

scale in which 100 indicates best possible outcome and 0 represents the worst.
The MPQ-DLV contains a 100mm VAS score ranging from 0 (no pain) to 100 (worst pain imaginable) for three moments: pain at the moment of questioning, the minimal amount of pain and the worst amount of pain. The character of the pain is represented by an adjective list. The adjectives are grouped according to pain quality and are ranked by intensity. This results in a score for "the number of words chosen" (NWC) and a "pain rating index" (PRI) for each subscale (sensory, affective and evaluative) and a total score (sensory, affective and evaluative combined). Higher scores indicate more pain. We also used the chosen adjectives to make an inventory about the character of the pain. We asked patients specifically to fill in the MPQ-DLV about any ***present*** pain that they thought was ***related to the uveitis*** or else to leave the MPQ-DLV empty.

Statistics

Statistical analyses were performed in SPSS version 21.0 (SPSS Inc., Chicago IL). To compare data between groups Pearson $\chi 2$ test or Fisher exact test (in case of <5 cases in one group) was used for categorical variables, and a student-T test or Mann-Whitney-U test for continuous variables. Subscale scores were compared with previously published data on a reference group.[10,18] Spearman's rank correlation test was used to test for correlations. A proportional odds logistic regression was calculated to assess the influence of certain variables on the Ocular Pain and the Role difficulties subscales because these are ordinal in nature. To reduce the number of categories in the multivariate model we made dichotomous variables from categorical variables (example: AU vs non-AU). Variables were all entered into the model and removed in a stepwise manner based on significance until the final model was reached. A p-value<0.05 was accepted as indicating statistical significance. An FDR correction of 5% was used to correct for multiple testing.

RESULTS

In total 298 patients were asked to participate. Of these 147 (49%) replied and complete data were available. The demographic and clinical characteristics of the 147 responders, as well as of patients with and without ocular pain ("ever having experienced ocular pain" yes/no), are given in **Table 1**. The scores from the three questionnaires are reported in **Table 2**. Patients with NIU scored significantly lower on all other VFQ-25 subscales, compared with an ocular disease free reference group (N=122, 62% females, mean age 59 (SD 14), median BCVA of worst eye 20/20 (range 160-13/20).[10] NIU patients also scored significantly lower on the SF-36 subscales "Role Limitations due to physical problems", "Vitality", "General health" and "Bodily Pain" compared with a Dutch healthy reference group (N=1742, 44% females, mean age 47.6 (SD 18.0)).[18]

Table 1. Characteristics of study population

	All	Ocular pain + [A]	Ocular pain - [A]	p-value
N, No. (%)	147 (100)	47 (32)	99 (67)	
Male gender, No. (%)	55 (37)	26 (55)	29 (29)	**0.002**[#]
Age, mean (SD)	56.4 (15.1)	61.0 (13.7)	54.4 (15.4)	**0.01**
Disease duration, No. (%)				
< 5 years	64 (44)	23 (49)	41 (41)	
5-10 years	42 (29)	11 (23)	31 (31)	0.70
10-15 years	22 (15)	6 (13)	15 (15)	
>15 years	19 (13)	7 (15)	12 (12)	
BCVA best eye, median (range), logMAR	0.0 (-0.2-1.0)	0.1 (-0.2-1.0)	0.0 (-0.2-0.7)	**0.007**
BCVA worst eye, median (range), logMAR[B]	0.2 (-0.1-NLP)	0.2 (0.0-NLP)	0.2 (0.1-NLP)	0.09
Bilateral disease, No. (%)	125 (85)	37 (79)	87 (88)	0.15
Localization, No. (%)				
Anterior	21 (14)	5 (11)	16 (16)	
Intermediate	21 (14)	7 (15)	14 (14)	0.71
Posterior	58 (40)	21 (45)	36 (36)	
Pan	47 (32)	14 (30)	33 (33)	
Diagnosis, No. (%)				
Idiopathic	53 (36)	19 (40)	34 (34)	
Sarcoidosis [C]	31 (21)	6 (13)	24 (24)	
Birdshot	27 (18)	15 (32)	12 (12)	
HLA-B27+ AAU	12 (8)	1 (2)	11 (11)	
White dot syndrome	8 (5)	2 (4)	6 (6)	
Multiple Sclerosis	4 (3)	1 (2)	3 (3)	
Other [D]	12 (8)	3 (6)	9 (9)	
Disease activity, No. (%)				
Quiescent	64 (44)	23 (49)	41 (41)	
Low-grade or recent activity	65 (44)	14 (30)	51 (51)	**0.009**
Active uveitis	18 (12)	10 (21)	7 (7)	
Ocular comorbidity, No. (%)				
Blepharitis / conjunctivitis	14 (9)	3 (6)	11 (11)	0.55
Keratoconjunctivitis sicca	12 (8)	1 (2)	11 (11)	0.10
Corneal disease	7 (5)	3 (6)	4 (4)	0.68
Medical history, No. (%)				
Cardiovascular disease [E]	19 (13)	6 (13)	9 (9)	0.56
Hypertension	17 (12)	9 (19)	8 (8)	0.05
Depression	13 (9)	5 (11)	8 (8)	0.61
Diabetes type I or II	12 (8)	4 (9)	8 (8)	>0.99
Rheumatic disease [F]	11 (7)	3 (6)	8 (8)	>0.99
Cancer	9 (6)	4 (9)	5 (5)	0.47
Thrombosis /embolism	7 (5)	3 (6)	4 (4)	0.68
Migraine / cluster headache	6 (4)	0	6 (6)	0.18
Arthrosis	3 (2)	0	2 (2)	>0.99
Lumbal hernia	3 (2)	1 (2)	2 (2)	>0.99
Other [G]	22 (15)	6 (13)	16 (16)	0.59
Therapy, No. (%)				
Periocular injections	111 (76)	39 (83)	71 (72)	0.14
Systemic medication	76 (52)	26 (55)	49 (49)	0.51

Legend Table 1
[A] VFQ-25, question 4, answer 2-5. Missing n=1. [B] 5 patients (5 eyes) have light perception (LP, n=2) or no light perception (NLP, n=3) and are excluded from statistical analysis. [C] Proven sarcoidosis n=26, clinical diagnosis n=5. [D] Other: VKH n=3, Behcet's disease n=2, Punctate inner chorioiditis n=1, Sympathetic ophthalmia n=2, vasculitis n=4. [E] Cardiovascular disease: myocardial infarction, arrhythmias, cardiac failure, atherosclerosis and resulting strokes. [F] Rheumatic disease: ankylosing spondylitis n=4, arthritis / enthesitis n=1, Dupuytren's disease n=1, fibromyalgia n=2, polymyalgia rheumatic n=1, rheumatic fever n=1, reactive arthritis n=1. [G] Other: bladder stones, nephrectomy, generalized zoster infection, Meniere's disease, cirrhosis of the liver, thyroid disease, lichen sclerosis, psoriasis, epilepsy, endometriosis, osteoma, neuropathy, severe anemia, vasculitis, recurrent erysipelas.
[#] Significant after FDR 5% correction for multiple testing

Prevalence, severity and impact of ocular pain

The mean VFQ Ocular Pain subscale score, which measures severity and impact of ocular pain in addition to the mere presence of ocular pain, was 72 (± 24) for all NIU patients. This score was significantly lower than the ocular disease free reference group used for the development of this questionnaire (90 ±15, p<0.0001), indicating more ocular pain.[10] Ocular Pain subscale scores were lower in AU patients compared with non-AU patients (mean 62 (±24) / median 62.5 (range 25-100) vs mean 74 (±24) / median 75 (12.5-100), p=0.04). Still, non-AU patients scored substantially lower than the reference group without ocular history (p<0.0001).

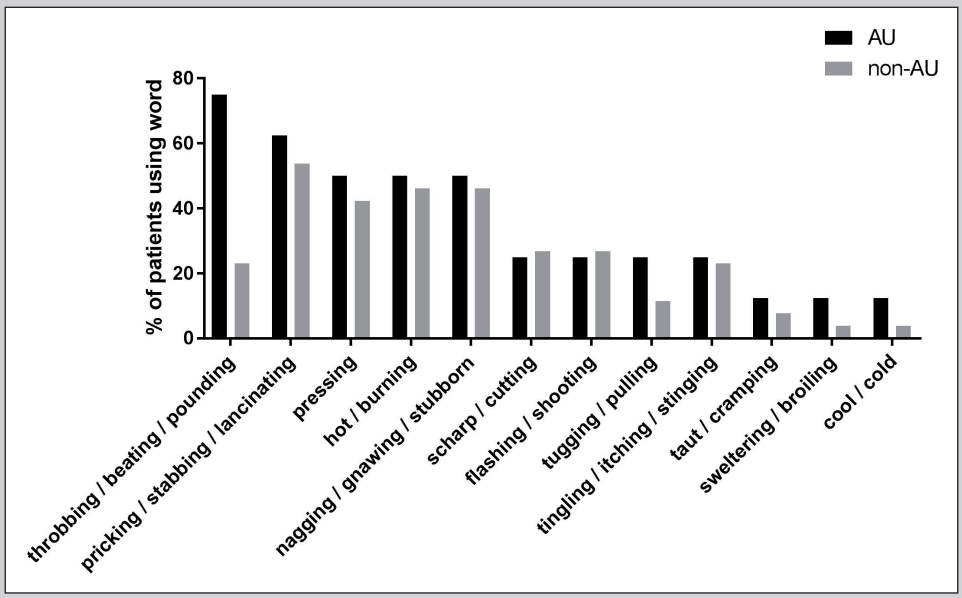


Figure 1. Description of ocular pain. Adjectives from MPQ-DLV Sensory Category used by non-infectious uveitis (NIU) patients to describe ocular pain. Words are grouped according to pain quality, each group containing three words ranked by intensity. Words that were used by NIU patients to describe the pain are represented in this figure, words that were never used are left out. Patients are grouped according to primary site of inflammation into anterior uveitis (AU, n=8) or non-anterior uveitis (non-AU, n=26) patients.

Table 2. Questionnaires

		Uveitis		Ref. *	
	Scale	Median (range)	Mean (SD)	Mean (SD)	p-value
NEI VFQ-25					
General health (n=144)	0-100	50 (0-100)	46 (23)	75 (17)	**<0.0001#**
General vision (n=146)	0-100	60 (20-100)	64 (15)	81 (13)	**<0.0001#**
Ocular pain	0-100	75 (13-100)	72 (24)	90 (15)	**<0.0001#**
Near vision (n=146)	0-100	75 (17-100)	72 (23)	93 (10)	**<0.0001#**
Distance vision	0-100	75 (17-100)	70 (23)	95 (8)	**<0.0001#**
Social functioning	0-100	100 (25-100)	88 (18)	99 (3)	**<0.0001#**
Mental health	0-100	75 (0-100)	72 (20)	91 (11)	**<0.0001#**
Role difficulties (n=146)	0-100	63 (0-100)	65 (27)	96 (9)	**<0.0001#**
Dependency (n=146)	0-100	100 (0-100)	88 (21)	99 (5)	**<0.0001#**
Driving (n=106)	0-100	75 (20-100)	74 (20)	89 (14)	**<0.0001#**
Color vision (n=146)	0-100	100 (25-100)	92 (17)	98 (8)	**<0.001#**
Peripheral vision	0-100	75 (0-100)	75 (27)	97 (10)	**<0.0001#**
SF-36					
Physical functioning (n=144)	0-100	90 (0-100)	81 (24)	83 (23)	0.32
Social functioning (n=145)	0-100	88 (0-100)	82 (24)	84 (22)	0.30
Role limitations - physical (n=145)	0-100	100 (0-100)	65 (43)	76 (36)	**<0.001#**
Role limitations - emotional (n=144)	0-100	100 (0-100)	83 (34)	82 (33)	0.73
Mental health (n=143)	0-100	84 (16-100)	78 (17)	77 (17)	0.50
Vitality / Energy (n=143)	0-100	65 (5-100)	63 (21)	69 (19)	**<0.001#**
Bodily pain (n=145)	0-100	50 (10-100)	47 (15)	75 (23)	**<0.0001#**
General health (n=143)	0-100	60 (10-100)	60 (21)	71 (21)	**<0.0001#**
MPQ-DLV**					
NWC-S (Sensory) (n=33)	≤12	3 (0-12)	3.5 (2.6)		
PRI-S (Sensory) (n=33)	≤36	5 (0-20)	5.4 (4.3)		
NWC-A (Affective) (n=33)	≤5	1 (0-5)	1.2 (1.4)		
PRI-A (Affective) (n=33)	≤15	1 (0-5)	1.4 (1.5)		
NWC-E (Evaluative) (n=33)	≤3	2 (0-3)	1.8 (1.3)		
PRI-E (Evaluative) (n=33)	≤12	3 (0-11)	2.9 (2.8)		
NWC-T (total) (n=33)	≤20	6 (0-20)	6.4 (4.6)		
PRI-T (total) (n=33)	≤63	9 (0-28)	9.7 (7.2)		
VAS (current pain)	1-10	0.5 (0.0-0.5)	1.2 (1.5)		
Vmin (least pain)	1-10	0.3 (0.0-2.2)	0.4 (0.6)		
Vmax (worst pain)	1-10	4.9 (0.5-9.6)	5.1 (2.9)		

* Reference group for NEI-VFQ-25: Mangione et al. Arch Ophthalmol 1998. / Reference group for SF-36: Aaronson et al. J Clin Epidemiol 1998

** **NWC:** Number of Words Chosen (NWC) per category of the McGill Pain Questionnaire (MPQ) for the Sensory Scale (NWC-S, questions 1-12), Affective scale (NWC-A, questions 13-17), Evaluative scale (NWC-E, questions 18-20), Total (NWC-T=NWC-S + NWC-A + NWC-E, questions 1-20). **PRI:** Pain Rating Index (PRI) of chosen adjective (per chosen adjective a rating of 1 to 3 or 1 to 4 can be given) per category of the McGill Pain Questionnaire (MPQ) for the Sensory Scale (PRI-S, questions 1-12), Affective scale (PRI -A, questions 13-17), Evaluative scale (PRI -E, questions 18-20), Total (PRI -T= PRI -S + PRI -A + PRI -E, questions 1-20).

significant after FDR 5% correction for multiple testing

Localization and description of the pain

Thirty-four/147 patients (23%) filled in the MPQ-DLV, reporting ocular pain, indicating they had pain at that specific moment that they attributed to the ocular inflammation. Twenty/34 (59%) also located the pain elsewhere than the periocular region: 13/34 patients (38%) reported headaches, 7/34 patients (21%) reported involvement of the ipsilateral temple, or the brow (n=4, 12%), and 6/34 patients (18%) felt the pain spreading to the neck and shoulder in a band shaped manner. Words that were used to describe the pain are given in **Figure 1**.

Impact of ocular pain on Quality of Life

The Ocular Pain subscale score correlated significantly with the VFQ-25 composite score, the VFQ-25 subscales "Vision Related Distance Activities", "Vision Related Mental Health", "Vision Related Role Difficulties" and "Driving" (n=106) (all rho>0.3 and p<0.001), and with the SF-36 subscales "Role Limitations due to physical problems" and "General Health" (both rho>0.3 and p<0.001). Furthermore, patients that had answered to worry more about their eyesight (VFQ question 3) also had significantly lower Ocular Pain scores (p=0.001) (**Figure 2**).

NIU patients scored particularly low on the VFQ subscale "Role Difficulties" (ie. problems with performing work related activities). Variables that were associated with lower Role Difficulty subscale scores (indicating more problems in this area) in a logistic odds model were a lower Ocular Pain score (OR=1.87 (95% CI 1.55-2.26), p<0.001) and a lower BCVA of the best eye (higher logMAR, OR=0.005 (95% CI 0.001-0.035), p<0.001) (after correction for age (p=0.97), gender (p=0.81), the use of systemic medication (p=0.11)).

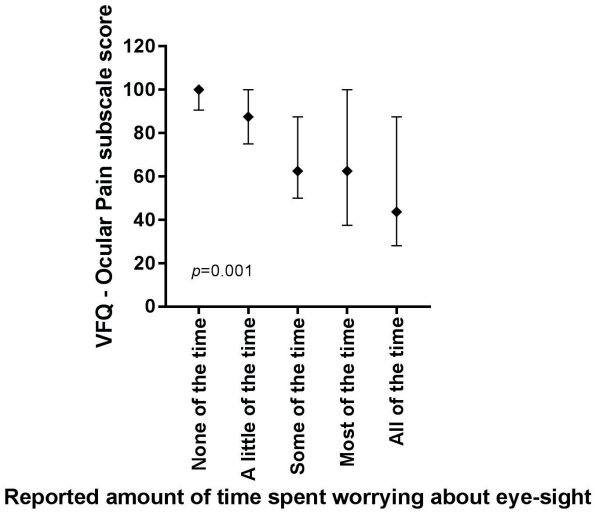


Figure 2. VFQ Ocular Pain subscale scores from non-infectious uveitis patients, according to the reported amount of time spent worrying about the patient's eyesight. Median and interquartile range are given. P-values are from Kruskal Wallis test.

Associations with ocular pain

We investigated possible associations of patient characteristics with presence and severity of ocular pain. All variables and their respective odds ratios are depicted in **Table 3**. In multivariate analysis, following adjustment for age, localization of uveitis, blepharitis, cluster headache and migraine, the following characteristics were found to be associated with lower ocular pain scores: female gender, recent or low-grade activity and blepharitis, cluster headache or migraine.

Exclusion of patients with blepharitis, cluster headaches or migraine (n=20) did not alter our results: with a mean subscale score of 74 (±23) (median 75, range 12.5-100) NIU patients still scored significantly lower on the VFQ-Ocular pain subscale score compared to ocular disease free controls (p<0.0001).

Table 3. Proportional Odds Logistic regression of ocular pain[A] in non-infectious uveitis patients

Predictor	OR[B] (95% CI) – crude	p-value	OR[B] (95% CI) – adj.	p-adj.
Male Gender	2.47 (1.35-4.54)	**0.003**	2.70 (1.40-4.84)	**0.003**
Age, increase of 5 years	1.09 (0.99-1.20)	0.09	1.10 (0.99-1.22)	0.07
Activity				
Inactive	*Ref*			
Recent / low-grade activity	0.55 (0.29-1.01)	0.05	0.40 (0.22-0.74)	**0.003**
Active uveitis	1.58 (0.61-4.06)	0.34		
Anterior Uveitis	0.39 (0.17-0.89)	**0.03**	0.49 (0.21-1.15)	0.10
BCVA worst eye (logMAR)	1.22 (0.69-2.16)	0.51		
BCVA best eye (logMAR)	1.03 (0.78-20.74)	0.10	ns[C]	
Unilateral disease	2.00 (0.88-4.50)	0.10	ns[C]	
Periocular injections	1.74 (0.89-3.39)	0.10	ns[C]	
Current systemic therapy	0.94 (0.53-1.67)	0.84		
Disease duration				
< 5 years	1.67 (0.36-2.22)	0.82		
5-10 years	0.85 (0.32-2.21)	0.73		
10-15 years	0.74 (0.25-2.19)	0.58		
>15 years	*Ref*			
Keratoconjunctivitis sicca	0.43 (0.15-1.22)	0.14		
Blepharitis	0.35 (0.13-0.93)	**0.04**	0.23 (0.08-0.65)	**0.006**
Migraine / cluster headache	0.24 (0.05-1.06)	0.06	0.14 (0.03-0.67)	**0.04**
Depression	1.08 (0.40-2.95)	0.88		

[A] Proportional Odds (Ordinal) Logistic regression for 6 categories of VFQ Ocular Pain subscale score (from worse to better): <50 (n=23), 50 (n=18), 62.5 (n=25), 75 (n=20), 87.5 (n=20), 100 (n=41)

[B] OR: Odds ratio of VFQ Ocular Pain subscale score lower than 100. An OR of <1.0 indicates higher likelihood of having a lower composite score.

[C] Variable entered into model, but removed due to insignificant contribution to the model

Abbreviations: (p-)adj.: adjusted (p-value), ns: not significant, OR: odds ratio, ref: reference in the model

DISCUSSION

This study shows that ocular pain is a highly prevalent complaint of non-infectious uveitis patients. The mean VFQ-25 Ocular Pain subscale score in our population was 72, which is in line with the scores of NIU patients reported in literature (mean scores of 70-75).[4–9] Although AU patients had lower VFQ-25 Ocular Pain subscale scores than non-AU patients, both groups had decidedly lower VFQ-25 Ocular Pain subscale scores than an ocular disease free reference group, indicating that ocular pain is not exclusive to anterior uveitis.[10]

In our study the presence of ocular pain had a profound impact on several aspects of quality of life, both vision related as well as general health. This is not surprising since pain in general is known to decrease quality of life and interfere with the performance of daily activities and ocular pain specifically has been related to decreased perception of general health.[3,4,19,20] Furthermore, our study shows a direct and considerable relation between ocular pain and the fear of vision loss.

Female gender and low-grade or recent uveitis activity were associated with worse ocular pain in our study population. The association between ocular pain and female gender has been described before, but the association between pain and low-grade or recent activity (and not active uveitis) is surprising.[9,21] Differentiation between low-grade inflammation and recent inflammation was not possible because patients often filled in the questionnaire at home, leaving weeks or even months between filling in the questionnaire and the hospital visit. We did not find ocular pain scores to be associated with duration of uveitis, treatment or visual acuity, which is in line with literature.[21,22] Surprisingly, we could not find a significant effect of localization on the ocular pain after adjustment for other factors. One of the reasons for this might be the limited number of patients with AU in this study.

We used the Dutch Language Version of the MPQ, which is different from the English version of the MPQ in the use of some words to describe the pain. Differences between language versions are based upon differences in the emotional value that is attributed to a word in a certain language. This means that some of the words could not be directly translated back to the English MPQ equivalent.

The main limitation of this study is the particularly expressed interest in ocular pain. This could have resulted in a selection bias. We were able to get an answer from 49% of the patients we contacted. Reasons for not participating may include personal, cultural or regional issues. Also, the amount of questionnaires (three) might have prevented some patients from participating. However, since the VFQ scores (in particular the VFQ-25 Ocular Pain subscale score) are comparable with literature we do not think that this has had much effect on the results.

Furthermore, the reference groups that were used [10,18] were not age and gender matched with our own population. Despite this, the ocular disease free reference group [10] was comparatively similar with our own group with respect to sample size (n=147 vs n=122), sex (63% female vs 62% female) and age (mean age 56 year (SD14) vs 59 year (SD 14)). Still, this group is not drawn from a Dutch population which might have influenced our results. However, since our results from the NEI VFQ-25 are in general comparable with other studies describing non-infectious uveitis in various regions across the world[4,5,7,9] we believe that any existing influence would not alter our main findings.

Although both VFQ and the MPQ are able to provide more insight into the character, severity and impact of ocular pain, they are not able to identify the etiology of the pain. In AU pain is mostly attributed to ciliary muscle contraction or stimulation of the nociceptor fibers of the ophthalmic nerve by inflammatory mediators.[23,24] The exact etiology of pain in non-AU remains more elusive, especially since it is not experienced by every non-AU NIU patient. As there are many clinically distinct types of NIU, etiology might vary per NIU type. Headaches might be the result of referred eye pain since the eyes share innervation by trigeminal nerve (of which the ophthalmic nerve is a branch) with adjacent tissues.[25]

To conclude, this study shows that ocular pain is highly prevalent in non-infectious uveitis patients, regardless of the localization. Ocular pain has a great impact on quality of life, making it highly relevant for the treating physician. Paying attention to pain in the care of NIU might be supportive for the patient. Although future studies still need to investigate etiology and possible treatments for ocular pain caused by uveitis, this study may provide the foundation for better recognition and appreciation of ocular pain in NIU patients.

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3

Chapter 3

POTENTIAL RISK FACTORS FOR POOR VISUAL OUTCOME IN HUMAN LEUKOCYTE ANTIGEN-B27 ASSOCIATED UVEITIS

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ABSTRACT

PURPOSE: To identify potential predictors for permanent vision loss in patients with human leukocyte antigen (HLA)-B27 -associated uveitis in a tertiary referral center.

DESIGN: Retrospective case control study

METHODS: The charts of 212 patients (338 eyes) with HLA-B27-associated uveitis that visited the University Medical Center Utrecht with a follow-up of at least 6 months were retrospectively studied. Clinical features at presentation and during follow-up were compared to final visual outcome in quiescent state. Eyes with (sub-) normal vision (>20/50) were compared with visually impaired (≤20/50) and blind (≤5/50, or a visual field of <10°) eyes, using survival analysis. A multivariate Cox proportional hazard analysis was performed to analyze potential predictors for permanent vision loss.

RESULTS: Median follow-up was 10.4 years (range 0.5-44.7). During follow-up 226 eyes (66%) experienced vision loss up to 20/50, but most recovered. Twenty patients (9%) became permanently visually impaired or blind in at least one eye because of uveitis, after a median of 9.7 years (0-20.9 years). The main cause was secondary glaucoma or related to glaucoma surgery (12/22 eyes, 55%). Survival analysis showed, after adjustment for age and gender, an ocular pressure of >21mmHg, hypotony and panuveitis to be potential predictors at presentation, and the development of secondary glaucoma or hypotony to be predictors for blindness or visually impairment during follow-up.

CONCLUSIONS: The long term visual prognosis of HLA-B27-associated uveitis is relatively good, but the true incidence of permanent vision loss is probably still underestimated. Our findings highlight the importance of proper control of intraocular pressure.

INTRODUCTION

Acute anterior uveitis is the most common type of uveitis (intraocular inflammation) in referral centers in het Western world[1–7] and is associated with human leukocyte antigen (HLA)-B27 positivity in 6-39%.[1,5,7–10] HLA-B27-associated uveitis typically comprehends recurrent episodes of acute onset, non-granulomatous anterior uveitis, usually limited to one eye or alternating between the eyes.[9,11] Onset of uveitis is usually between the second and fourth decade, leading to high disease burden in a relatively young population. Patients are exposed to recurrent uveitis episodes, each time conveying the risk of complications and vision loss.[11–16]

Reported visual outcome is relatively good with a surprising 0%-12.3% of all eyes developing a visual acuity of 20/50 or less.[13,15–20] However, the risk of visual loss might be underestimated since most of these studies have a follow-up time of less than 3 years. Reported potential risk factors for visual impairment or blindness (including transient vision loss) include posterior synechiae at presentation, active inflammation, corticosteroid sparing therapy, corticosteroid injections, chronic disease course, male gender and posterior segment involvement.[19,21] The aim of this study is to identify potential predictors for permanent visual impairment and blindness in a cohort of patients with HLA-B27-associated uveitis in a tertiary referral center.

METHODS

Patient selection

Medical charts of patients with HLA-B27-associated acute onset uveitis that were seen at the department of Ophthalmology, University Medical Center Utrecht (UMC-U), the Netherlands, between 1996 and December 2014, were retrospectively reviewed. All patients of 18 years and older with a minimum follow-up of 6 months and complete medical records were included. We excluded all patients in whom uveitis displayed highly atypical features, unlikely related to HLA-B27, such as an insidious onset of uveitis or posterior uveitis in the absence of inflammation of the anterior segment.

This study was conducted in compliance with the Helsinki principles. Ethical approval was requested and obtained from the Medical Ethical Research Committee in Utrecht, with a waiver of informed consent acquisition.

Clinical characteristics

Each patient underwent a full ophthalmological examination by a trained ophthalmologist, routine laboratory screening and an X-Ray of the lungs. Laboratory screening included

erythrocyte sedimentation rate, renal and liver function tests, angiotensin converting enzyme (ACE), HLA-B27 positivity, and screening for various infectious agents in serum including syphilis, Lyme disease and an Interferon-Gamma Release Assay (IGRA) tuberculosis test. In case of atypical uveitis aqueous humor was tested for infectious agents by means of PCR and Goldmann-Witmer coefficients.

For each patient the following characteristics were documented: age, gender, medical history including HLA-B27 related systemic diseases (ankylosing spondylitis (AS), reactive arthritis, psoriasis and Inflammatory Bowel Disease (IBD)) and date of onset of uveitis (defined as date of diagnosis by a (referring) ophthalmologist). Uveitis was classified and graded in accordance with the standardization of uveitis nomenclature (SUN) classification.[22] For each eye the following features were recorded at presentation and during follow-up: cell grade in anterior chamber, presence of hypopyon, fibrin, posterior synechiae, corneal edema (defined as the presence of corneal thickening or Descemet membrane folds), keratic precipitates (KPs), intraocular pressure, duration of inflammation (limited: duration of <3 months or persistent: duration of 3 months or more), course (acute: 1 episode with a duration of <3 months, recurrent: multiple episodes with a duration of <3months, chronic: duration of ≥3 months and/or reactivity within 3 months after cessation of treatment, or quiescent under therapy: chronic treatment, not meeting criteria for chronic course). For each eye the following complications were recorded: posterior synechiae, band keratopathy, cystoid macular edema (CME), secondary cataract requiring surgery, ocular hypertension (defined as intraocular pressure >21 mmHg without optic nerve damage or visual field abnormalities but requiring therapeutic intervention), secondary glaucoma (defined as ocular hypertension with optic nerve damage or glaucomatous abnormalities in visual field), and hypotony (defined as an intraocular pressure of <6 mmHg on at least two measurements and time points, excluding post-operative hypotony within 3 months after glaucoma surgery). Therapeutic interventions, such as periocular corticsteroid injections, systemic steroids, disease-modifying antirheumatic drugs (DMARDs) (Methotrexate, Hydroxychloroquine, Sulfasalazine, Leflunomide, Mycofenolate mofetil, Azathioprine, Ciclosporin, Cyclophosphamide, Mycophenolate Mofetil, Tacrolimus, chlorambucil) and biologicals (Adalimumab, Etanercept, Infliximab, Rituximab, Tocilizumab), were noted.

Vision

Best Corrected Visual Acuity (BCVA) was noted for each eye at presentation and when uveitis was quiescent, defined as no inflammatory cells in the anterior chamber, after the last inflammatory episode at our clinic. Also, the worst BCVA during follow-up was noted. Thresholds for visual impairment (VI) and blindness were used as recommended by the

SUN working group, with a BCVA ≤20/50 (Snellen equivalent) for visual impairment, and BCVA ≤20/200, or a visual field (VF) of ≤10 degrees for blindness respectively.[22]
Visual acuities are presented in Snellen equivalent. For statistical analysis Snellen BCVA was converted into the logarithm of minimal angle of resolution scale (logMAR) and converted back to Snellen BCVA for data presentation.[23] The most important cause of vision loss was noted for each eye, as well as the date that the threshold for blindness/VI was first reached.

For statistical analysis eyes were divided in two groups: (sub-) normal vision (BCVA>20/50) or visually impaired/blind (VI/blind). Fourteen eyes could not be placed in either group: five eyes because their final BCVA is very likely to improve (because of persistent uveitis activity (n=3) or pre-operative cataract (n=2)), and nine eyes (from 8 patients) because their vision loss was predominantly caused by a non-uveitis related ocular comorbidity. The respective causes of vision loss in these eyes are amblyopia n=2 (best corrected visual acuity before uveitis was 20/40 and 1/300 respectively), Prader-Willi syndrome (n=1), Fuchs endothelial dystrophy (n=2), age-related macular degeneration (n=2), secondary corneal decompensation after surgery (n=1) or after traumatic injury (n=1). These eyes were included in the description of the group as a whole, but excluded from comparison between VI/blind eyes versus eyes that retained (sub-)normal vision, as well as risk analysis. Eyes were also excluded from risk analysis if the criteria for permanent blindness/visual impairment were reached before presentation in the UMC-U, because these eyes were not "at risk" anymore since it had already developed.

Statistical analysis

Statistical analysis of the data was performed using SPSS version 21.0 (SPSS Inc., Chicago IL). To compare data between groups Pearson χ^2 test or Fisher exact test (in case of <5 cases in one group) was used for categorical variables, and a student-T test or Mann-Whitney-U test for continuous variables. A survival analysis was performed using Kaplan-Meier curves (with Log-rank test) to correct for varying lengths of follow-up. To correct for paired samples only one eye per patient was used in this risk analysis: either the eye with the worst outcome (BCVA or visual field) was chosen, or the eye was selected randomly in case of similar outcomes. A univariate and multivariate Cox proportional hazard analysis was conducted. Variables were divided in "present at presentation" or "developed during follow-up", and multivariate analysis was conducted for these two time-points separately. Variables with $p<0.05$ in the univariate Cox proportional hazard analysis, and potential confounders (gender, age of onset of uveitis), were entered into the multivariate analysis. P-values below 0.05 were considered statistically significant. All significances were 2-tailed.

RESULTS

The charts of 212 patients (338 eyes) with HLA-B27-associated uveitis, fulfilling the inclusion criteria, were analyzed (see **Table 1**). A total of 28 patients (13%) had significant permanent vision loss in at least one eye. This vision loss was directly caused by uveitis in 20 patients (9% of all patients) and was bilateral in two cases (1% of all patients). Eighteen out of the 22 eyes met the criteria for legal blindness and 4 eyes were visually impaired.

Table 1. Patient characteristics

	All patients		Permanent vision loss (visual impairment / blindness) in ≥ 1 eye [a]				
			No [a]		Yes [a]		
	N	%	N	%	N	%	p-value
Total number of patients	212	100	179 [a]	100	20 [a]	100	n.a.
Male gender	112	53	96	54	10	50	0.76
Follow-up years (median; range)	10.4 (0.5-44.7)		10.5 (0.5-44.7)		12.2 (0.5-30.7)		0.90
Age of onset (mean; SD) [b]	36.5 (14.5)		35.9 (12.8)		34.8 (17.5)		0.81
Age at referral (mean; SD)	38.7 (13.6)		37.9 (12.9)		40.4 (15.1)		0.41
Associated systemic disease							
Total	104	49	91	51	8	40	0.36
Multiple	6	3	5	3	1	5	-
AS	87	41	77	43	7	35	-
Reactive arthritis	11	5	9	5	0	0	-
Psoriasis	8	4	6	3	2	10	-
IBD	4	2	4	2	0	0	-
Therapy							
Peri-ocular steroids	118	56	96	54	18	90	**0.002**
Systemic steroids	83	39	63	35	16	80	**<0.001**
DMARDs	47	22	36	20	9	45	**0.02**
Biologicals	31	15	21	12	8	40	**0.001**
Surgery	67	32	50	28	15	75	**<0.001**

Abbreviations: AS: ankylosing spondylitis, IBD: inflammatory bowel disease, SD: standard deviation, DMARD: disease-modifying antirheumatic drug, n.a.: not applicable

[a] fourteen eyes from thirteen patients are not in either group: 3 eyes because of active inflammation at last follow-up which resulted in a vision ≤20/50, 2 eyes because visual impairment resulted directly from (pre-operative) cataract and post-operative vision is not known, and 9 eyes because vision loss is predominantly caused by an ocular comorbidity.

[b] missing N=34

Ocular signs at presentation

The characteristics of all eyes at presentation and during follow-up are summarized in **Table 2**. The VI/blind eyes presented significantly more often with an intraocular pressure of more than 21 mmHg, or less than 6 mmHg, than eyes that retained (sub-) normal vision (43% vs. 6%, p<0.001, and 10% vs. 1%, p=0.03 respectively). There was no difference between VI/blind eyes and eyes that retained (sub-) normal vision for severity of inflammation, defined as 3+ cells or more, or the presence of fibrin or hypopyon.

Ocular signs and complications during follow-up

During follow-up, VI/blind eyes significantly more often progressed to panuveitis (p=0.02), the course was more often of a persistent duration (p=0.002) and more often a hypopyon or corneal edema developed (p=0.01 and p<0.001 respectively). VI/blind eyes had developed more complications like CME (p<0.001), hypotony (p<0.001), secondary glaucoma (p<0.001), cataract (p<0.001), posterior synechiae (p=0.01), and band keratopathy (p=0.003).

Treatment

Patients with VI/blind eyes were treated more often with peri-ocular injections, systemic steroids, DMARDs, Biologicals, and underwent more ocular surgeries (**Table 1**). It is notable that patients with HLA-B27-associated systemic disease were significantly more often treated with DMARDs (34% vs 11%, respectively p<0.001) and biologicals (24% vs 6%, p<0.001) than patients without a related systemic disease.

Visual acuity

At presentation the median vision was 20/32 with 33% of all eyes having a BCVA of 20/50 or less. During follow-up 226 eyes (66% of all eyes) had a visual acuity of 20/50 or less at some point during follow-up (see also **Table 3**). Most of these eyes fortunately recovered.

Visual impairment and legal blindness

The primary cause of permanent vision loss is depicted in **Table 4**. Secondary glaucoma or complications related to glaucoma surgery were the most frequent cause of blindness or visual impairment (13/20, 59%). Permanent vision loss developed after a median 9.7 years (0-20.9 years) with a median of 4 episodes (range 1-20). Interestingly, the amount of uveitis episodes was not significantly different for VI/blind eyes compared with eyes with (sub-) normal vision.

Eight eyes were either blind at referral (n=2) or became blind after just one uveitis episode (n=6). Five of these had had a severe panuveitis resulting in a combination of complications such as hypotony (n=3), serous retinal detachment (n=2), CME (n=1) and optic disc edema (n=1). Three other eyes that lost their sight after just one uveitis episode, suffered from secondary glaucoma that aggressively progressed in the absence of inflammation.

Ocular hypertension and secondary glaucoma

Eighty-nine eyes (26%) developed ocular hypertension, which progressed to secondary glaucoma in 51 eyes (57% of eyes with ocular hypertension, 15% of all eyes). The

Table 2. Characteristics of eyes with HLA-B27-associated uveitis at presentation and during follow-up

	At presentation					During follow-up				
	(sub-)normal vision [a]		VI / blind [a]		p-value	(sub-)normal vision [a]		VI / blind [a]		p-value
	N	%	N	%		N	%	N	%	
Total number of eyes	**302**		**22**			**302**		**22**		
Episodes (median; range)	-		-		-	3 (1-27)		3.5 (1-20)		0.87
Eyes with 3+ cells or more (%)	42 [b]	14	5 [c]	24	0.21	144 [d]	52	12	57	0.65
Pan uveitis	26	8	2	18	0.13	58	19	9	41	**0.02**
Laterality										
Unilateral	139 [e]	48	13	59		53	18	8	36	
Alternating	120 [e]	41	4	18	**0.04**	176	58	7	32	**0.03**
Bilateral	33 [e]	11	5	23		73	24	7	32	
Duration >3 months	16 [c]	5	1	5	>0.99	50 [c]	17	10	46	**0.002**
Acute	195 [c]	65	15	68		21 [c]	7	3	14	
Recurrent	87 [c]	29	6	27		184 [c]	61	9	41	
Course										
Chronic	17 [c]	6	1	5	>0.99	87 [c]	29	8	36	0.09
Quiescent under therapy	n.a.		n.a.			9	3	2	9	
Fibrin	50	17	4	18	0.77	121	40	11	50	0.36
Keratic precipitates	6	32	8	36	0.67	186	62	17	77	0.14
Hypopyon	6	2	1	5	0.39	17	6	5	23	**0.01**
Corneal edema	26	9	1	5	>0.99	52	17	12	55	**<0.001**
Cystoid macular edema	16	5	1	5	>0.99	48	16	13	59	**<0.001**
Posterior synechiae	75	25	7	32	0.47	171	57	19	86	**0.01**
Hypotony [f]	2	1	2	10	**0.03**	6	2	12	55	**<0.001**
IOP>21mmHg [f]										
Total	15	6	9	43	**<0.001**	66	22	17	77	**<0.001**
Oc. Hypertension	-		-		-	31	10	2	9	>0.99
Glaucoma	-		-		-	35	12	15	68	**<0.001**
Cataract	4	1	1	5	0.30	61	20	13	59	**<0.001**
Band Keratopathy	-		-		-	2	1	3	14	**0.003**

Abbreviations: n.a.; not applicable, IOP: intraocular pressure, oc.: ocular.

[a] fourteen eyes from thirteen patients are not in either group: 3 eyes because of active inflammation at last follow-up which resulted in a vision ≤20/50, 2 eyes because visual impairment resulted directly from (pre-operative) cataract and post-operative vision is not known, and 9 eyes because vision loss is predominantly caused by an ocular comorbidity. [b] missing n=4, [c] missing n=1, [d] missing n=25, [e] missing n=10, [f] missing n=60

Table 3. Best Corrected Visual Acuity [a] of all eyes with HLA-B27associated uveitis

	BCVA at presentation		Worst BCVA		BCVA in quiescent state [b]	
	N	%	N	%	N	%
Total	335		338		281	
> 20/50	224	67	114	34	252	90
≤ 20/50 - >5/50	64	19	137	41	11	4
≤ 5/50 - HM	42	13	71	21	11	4
LP +	5	1	16	5	5	2

Abbreviations: BCVA: best corrected visual acuity, LP: light perception, HM hand movements.
[a] not including visual field abnormalities. [b] after last uveitis episode

characteristics of glaucoma patients are summarized in **Table 5**. Of these 51 eyes, 16 eyes (31%) developed severe visual field abnormalities such as a central scotoma (n=1), a double arcuate scotoma with >10° of central field left (n=7) or <10° of central field left (n=8). Perhaps not surprisingly, patients developing secondary glaucoma presented more often with an intraocular pressure of >21mmHg (21% vs 7%, p=0.004). Glaucomatous eyes had a more severe and chronic course (p<0.001), more often extension to panuveitis (p<0.001), and more frequently developed ocular complications. They also were more frequently treated with peri-ocular steroids, systemic steroids, DMARDs and biologicals and underwent more surgical interventions.

Table 4. Main cause of vision loss caused by HLA-B27 associated uveitis

Cause	N	%
Secondary glaucoma	12[a]	55
Macular atrophy after longstanding CME	3	14
Serous retinal detachment	2	9
Fulminant inflammation [b]	2	9
Hypotony (Ciliary body insufficiency)	2	9
Cornea decompensation	1	5
Total	**22**	

[a] In 2 out of 13 cases vision loss due to hypotony after glaucoma surgery
[b] In 2 patients vision loss developed after fulminant inflammation with several coinciding complications. It is therefore not possible to assign one primary cause.

The follow-up time was not different for patients with or without secondary glaucoma (10.7 vs 10.0 years, p=0.74) and eyes of patients that were referred before 2000 did not more often develop secondary glaucoma than eyes that were referred after that time (32/210 (15%) vs 19/126 (15%), p=0.97), debating the suggestion of changing treatments having a role in the development of secondary glaucoma.

Predictors for poor visual outcome

Possible predictors for blindness or visual impairment are presented in **Table 6** and **Figure 1**. Eighteen VI/blind eyes were used for survival analysis (see methods). Of these 18 eyes, 3 were visually impaired and 15 legally blind. A univariate Cox proportional hazard analysis showed multiple statistically significant predictors. These included ocular hypertension, hypotony, and panuveitis at presentation (p <0.001, p<0.001 and p=0.006 respectively), and the development of the following features or complications during follow-up: panuveitis (p=0.006), hypopyon (p=0.002), secondary glaucoma (p<0.001),

Table 5. Characteristics of HLA-B27-associated uveitis patients with or without secondary glaucoma

	No glaucoma		Glaucoma		
	N	%	N	%	p-value
Total number of patients	173		39		
VI/Blind in at least one eye	13	8	15	39	**<0.001**
Male gender	95	55	17	44	0.20
Follow-up years (median; range)	10.1 (0.5-44.7)		10.7 (1.2-44.3)		0.78
Age (mean; SD)	49.8 (14.5)		52.9 (15.9)		0.24
Age of onset (mean; SD) [a]	36.9 (14.0)		35.0 (16.6)		0.50
Age at presentation UMCU (mean; SD)	38.2 (12.8)		41.0 (16.7)		0.27
Follow-up outside of UMCU	138	48	33	65	**0.03**
Associated systemic disease	87	50	17	44	0.45
Therapy					
Peri-ocular steroids	85	49	33	85	**<0.001**
Systemic steroids	52	30	31	80	**<0.001**
DMARDs	28	16	19	49	**<0.001**
Biologicals	18	10	13	33	**<0.001**
Surgery	39	23	30	79	**<0.001**
Total number of eyes	287		51		
Episodes per eye (median; range)	8 (1-27)		5 (1-24)		**<0.001**
Duration >3 months [b]	41	14	22	43	**<0.001**
Chronic course [b]	69	24	30	59	**<0.001**
Laterality					
Unilateral	54	19	13	26	
Unilateral alternating	171	60	17	33	**0.001**
Bilateral	62	22	21	41	
Panuveitis	47	16	23	45	**<0.001**
Hypopyon	17	6	5	10	0.30
Fibrin	110	38	26	51	0.09
Cornea edema	47	16	22	43	**<0.001**
Hypotony	10	4	9	18	**<0.001**
Posterior synechiae	160	56	41	80	**0.001**
Cataract	47	16	32	63	**<0.001**
CME	39	14	23	45	**<0.001**

Abbreviations: CME: cystoid macular edema, DMARD: disease-modifying antirheumatic drug, SD: standard deviation, VI: visually impaired. [a] missing N=34, [b] missing N=1

hypotony due to cilliairy body dysfunction (p<0.001), CME (p<0.001), posterior synechiae (p=0.009). Multivariate analysis showed ocular hypertension, hypotony and panuveitis to be potential predictors for poor visual outcome at presentation, while secondary glaucoma and the development of hypotony were potential predictors during follow-up (see also **Table 6**).

DISCUSSION

In our study 9% of all patients with HLA-B27-associated uveitis suffered from irreversible visual impairment or blindness in at least one eye, as a direct result of uveitis. In 1% this vision loss was bilateral. Temporary vision loss up to 20/50 occurred in as much as two third of all patients at some point during follow-up. The personal, social and economic burden of vision loss in this relatively young population is indisputably high, with an increase of depression and self-reported impairment of performing daily activities, and the total annual costs of blindness caused by uveitis being roughly the same as the costs of blindness related to diabetes, despite the latter being much more common.[24,25]

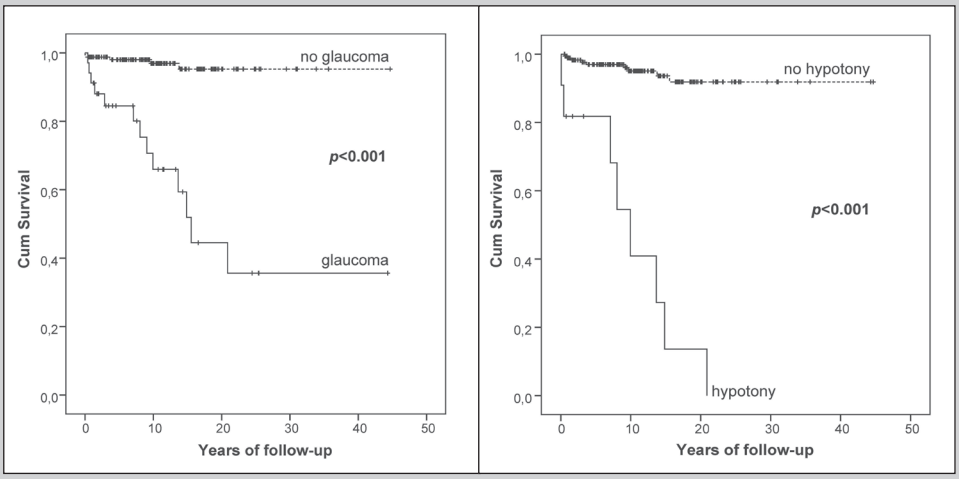


Figure 1. Kaplan-Meier estimate for developing severe vision loss (visually impairment or blindness) in patients with HLA-B27-associated uveitis with or without secondary glaucoma (left) or hypotony (right). P-values are calculated using the Log-rank test.

In contrast to uveitis in general, where CME (or macular atrophy due to chronicity of CME) is the most frequently reported cause of vision loss[26,27], we found secondary glaucoma to be the most common primary cause for irreversible vision loss. Although secondary glaucoma is an established complication of uveitis in general, it is typically linked to other types of uveitis, such as Fuchs heterochromic uveitis, Posner-Schlossman syndrome and juvenile iodiopathic arthritis associated uveitis.[28,29] The reported incidence of secondary glaucoma in HLA-B27-associated uveitis varies greatly with reported incidences between

Table 6 - Risk analysis for development of permanent vision loss in HLA-B27-associated uveitis

	Crude Hazard Ratio			Adjusted Hazard Ratio		
	HR	95% CI	p-val.	HR	95% CI	p-val.
General characteristics						
Male gender	0.86	0.34-2.18	0.74			
Age at first presentation	1.01	0.98-1.05	0.59			
Age at the end of follow-up	0.98	0.95-1.02	0.34			
Associated systemic disease	0.56	0.25-1.70	0.38			
Number of uveitis episodes	0.99	0.91-1.09	0.89			
At presentation						
BCVA ≤ 20/50	2.21	0.85-5.73	0.10			
≥3+ cells in anterior chamber	2.27	0.74-7.00	0.15			
Persistent duration (>3 months)	2.19	0.29-16.75	0.45			
Panuveitis	5.12	1.58-16.60	**0.006**	3.73	1.04-13.39	**0.04**
Hypopyon	5.18	0.65-39.57	0.12			
Keratic precipitates	1.37	0.53-3.53	0.52			
Fibrin	0.61	0.14-2.66	0.51			
Posterior synechiae	2.35	0.90-6.17	0.08			
IOP>21mmHg	6.95	2.55-18.92	**<0.001**	11.05	2.96-41.22	**<0.001**
Hypotony	17.31	3.83-78.72	**<0.001**	36.02	6.32-205.46	**<0.001**
Development during follow-up						
≥3+ cells in anterior chamber	1.82	0.64-5.18	0.26			
Persistent duration (>3 months)	2.50	0.97-6.46	0.06			
Panuveitis	3.68	1.45-9.31	**0.006**	1.04	0.35-3.09	0.95
Hypopyon	5.15	1.83-14.47	**0.002**	2.76	0.65-11.69	0.17
Fibrin	0.96	0.38-2.45	0.94			
Posterior synechiae	14.83	1.97-111.65	**0.009**	5.23	0.62-44.11	0.13
IOP>21mmHg						
Ocular hypertension	1.29	0.30-5.63	0.74			
Glaucoma	14.20	5.06-39.90	**<0.001**	5.16	1.30-20.53	**0.02**
Hypotony	21.26	8.14-55.48	**<0.001**	5.07	1.17-21.93	**0.03**
Cystoid macula edema	6.87	2.64-17.89	**<0.001**	1.26	0.34-4.73	0.73

Abbreviations: BCVA: best corrected visual acuity, CI: confidence interval, IOP: intraocular pressure

0-19%[10,15–17,20], which is in most reports somewhat lower that its HLA-B27 negative counterpart (2-31%)[10,16,17,20] The reason that glaucoma has such a high impact on outcome in this subgroup of uveitis is hitherto unknown, and should be further studied.

Despite the occurrence of secondary glaucoma in our study being in concordance with previous reports, there might be an overrepresentation of glaucoma in our tertiary referral center resulting in a potential skewing of the causes of blindness. Even so, the effect of secondary glaucoma on blindness in HLA-B27-associated uveitis might even still be underestimated, since an additional 14% of the eyes with secondary glaucoma had severe visual field defects just outside the criteria for blindness (e.g. a double arcuate scotoma with central vision of more than 10°), and are therefore at risk of becoming blind in the near future.

We found panuveitis, ocular hypertension and hypotony to be potential predictors at presentation for visual loss, and the development of secondary glaucoma and hypotony during follow-up. The reason that we found secondary glaucoma, and not ocular complications such as posterior synechiae or CME, to be significant predictors in multivariate analysis might be due to the relation, and potential co-linearity, with panuveitis. A previous study that identified potential predictors for poor visual outcome in HLA-B27-associated uveitis, studied vision loss during active inflammation as well as in quiescent disease.[19] Their potential predictors for (transient) visual impairment included posterior synechiae at presentation, corticosteroid sparing therapy, corticosteroid injections, and male gender, but not ocular hypertension. The differences between our studies might be explained by differences in follow-up time, which could result in an underestimation of the prevalence of complications and increased censoring of outcomes. Also the difference in definition of outcome (including transient vision loss) or the presence of blindness during active inflammation could contribute to the differences.
The choice of treatment, however, might influence the development of secondary glaucoma, as steroid therapy is related to the development of ocular hypertension. In case of severe inflammation, treatment with systemic or peri-ocular corticosteroids is the mainstay of treatment. At the same time, corticosteroids enhance the risk of ocular hypertension and glaucoma. It is unclear from our data whether development of secondary glaucoma is more related to chronicity of inflammation, or related to treatment with systemic or peri-ocular corticosteroids. The treatment strategies in our series are diverse and changed over time. Therefore, it is not possible to make conclusions about the influence of treatment on development of secondary glaucoma.

The number of uveitis attacks is intuitively related to an increased risk of vision loss, but from our results it seems that extension of inflammation (to panuveitis), rather than number of inflammatory episodes, is related to vision loss. Several patients already lost their sight after just one episode of uveitis. A substantial part of these patients presented with severe panuveitis causing several coinciding complications, such as hypotony, serous retinal detachment, CME and papillary edema. Although HLA-B27-associated uveitis typically affects the anterior part of the eye, involvement of the posterior part of the eye and presentation as severe panuveitis with multiple coinciding complications has been described by multiple studies.[15,17,18,21,30–33] It might therefore be considered as part of the spectrum of HLA-B27-associated ocular inflammations of which the clinician must be aware, considering its possibly devastating consequences for the patient's sight.
In our study, as well as in others, the presence of an HLA-B27-associated systemic disease was not related to an increased risk of blindness, despite a possible higher frequency

of uveitis attacks in some reports.[16,17,19] Half of our patients suffered from an associated systemic disease, which is in line with literature.[8,9,12,13,17,18] However, in theory, a potential negative influence of a systemic disease could be neutralized by a more intensive systemic treatment, as patients with an HLA-B27 related systemic disease were significantly more often treated with DMARDs or biologicals.

There are several limitations to this study that need to be taken into consideration while interpreting our results. Apart from the usual limitations of a retrospective study, including differences in follow-up time and the absence of patient assessment at regular intervals, which we corrected for as much as possible using survival analysis, the results from the multivariate analysis could be overestimated due to overfitting. The correction for paired sampling, by including only the eye with the worst visual outcome for risk analysis, may have resulted in an overestimation of the effect as well. However, since predictors for permanent vision loss are still missing, despite over 40 years of research since the association between HLA-B27 and uveitis was first identified, there is a high need for prognostic leads in this disease. We chose the best methods available with all the limitations that come with studying a rare outcome, despite a relatively large patient population in a tertiary referral center. Also, our median follow-up of over 10 years is one of the longest follow-up periods in all outcome related studies in this disease[13,15–20] contributing to the validity. Thus, keeping an overestimation of the effect in mind our results may still give a good indication which factors might predict permanent vision loss in HLA-B27 associated uveitis.

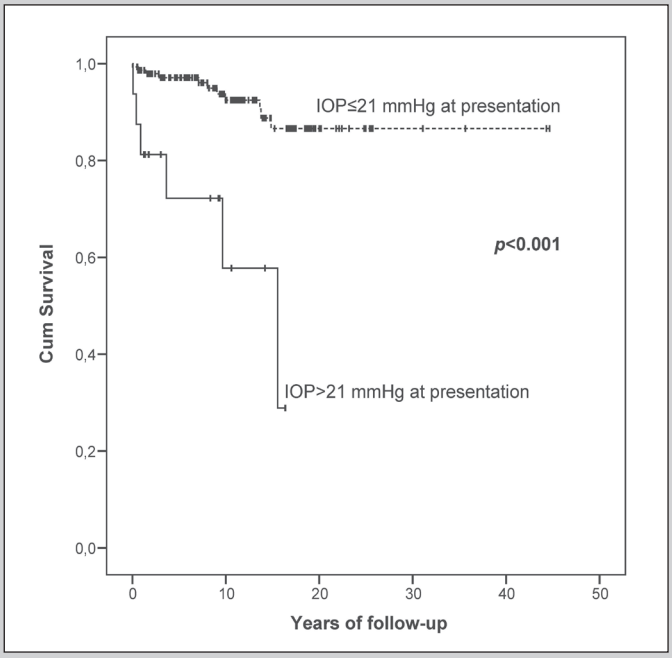
It is important to notice that visual impairment developed after a median of almost 10 years, with the last patient in our study developing blindness after 21 years. This means that even a 10 year follow-up, though adequate for risk analysis, might still be too short for the true estimation of the incidence of permanent vision loss caused by HLA-B27-associated uveitis.

In conclusion, our study shows that although the long-term visual prognosis of HLA-B27-associated uveitis is relatively good, some patients still suffer from irreversible significant vision loss after several years. The true incidence of vision loss in this disease is probably still underestimated. The most frequent cause of blindness in HLA-B27-associated uveitis is secondary glaucoma. Secondary glaucoma is in general associated with a more severe and complicated disease course, in which the role of steroid response is still unclear. These findings highlight the importance of proper control of intra-ocular pressure in HLA-B27-associated uveitis.

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Supplementary Figure 1. Kaplan-Meier estimate for developing severe vision loss (visually impairment or blindness) in patients with HLA-B27-associated uveitis with or without ocular hypertension at presentation. P values are calculated using the Log-rank test.

Supplementary table 1. Characteristics of all eyes affected with HLA-B27-associated uveitis

	At presentation				During follow-up			
	All Eyes	Permanent vision loss? [a] (sub-)normal vision	Blind / VI	p-value	All Eyes	Permanent vision loss? [a] (sub-)normal vision	Blind / VI	p-value
Total number of eyes (%)	**338**	**302** [a]	**22** [a]		**338**	**302** [a]	**22** [a]	
Episodes (median; range)	-	-	-	-	3 (0-27)	3 (1-27)	3.5 (1-20)	0.87
Eyes with 3+ cells or more	48/332 (15)	42/298 (14)	5/21 (24)	0.21	159 (51)	144/277 (52)	12 (57)	0.65
Panuveitis	32/337 (9)	26 (8)	2 (18)	0.13	70 (21)	58 (19)	9 (41)	**0.02**
Laterality								
Unilateral	159/327 (49)	139/292 (48)	13 (59)		67 (20)	53 (18)	8 (36)	
Alternating	129/327 (39)	120/292 (41)	4 (18)	**0.04**	188 (56)	176 (58)	7 (32)	**0.03**
Bilateral	39/327 (12)	33/292 (11)	5 (23)		83 (25)	73 (24)	7 (32)	
Duration >3 months	19/334 (6)	16/300 (5)	1 (5)	>0.99	63 (19)	50/301 (17)	10 (46)	**0.002**
Course								
Acute	217/333 (65)	195/299 (65)	15 (68)		26 (8)	21/301 (7)	3 (14)	
Recurrent	96/333 (29)	87/299 (29)	6 (27)	>0.99	202 (60)	184/301 (61)	9 (41)	0.09
Chronic	20/333 (6)	17/299 (6)	1 (5)		98 (29)	87/301 (29)	8 (36)	
Quiescent under therapy	n.a.	n.a.	n.a.	n.a.	11 (3)	9/301 (3)	2 (9)	
Fibrin	58/336 (17)	50/300 (17)	4 (18)	0.77	136 (40)	121 (40)	11 (50)	0.36
Keratic precipitates	109/335 (33)	96/300 (32)	8 (36)	0.67	211 (62)	186 (62)	17 (77)	0.14
Hypopyon	7/335 (2)	6/300 (2)	1 (5)	0.39	22 (7)	17 (6)	5 (23)	**0.01**
Cornea edema	29/336 (9)	26/301 (9)	1 (5)	>0.99	69 (20)	52 (17)	12 (55)	**<0.001**
Cystoid macula edema	17/335 (5)	16/300 (5)	1 (5)	>0.99	62 (18)	48 (16)	13 (59)	**<0.001**
Posterior synechiae	87/337 (26)	75 (25)	7 (32)	0.47	201 (59)	171 (57)	19 (86)	**0.01**
Hypotony	4/276 (1)	2/237 (1)	2 (10)	**0.03**	19 (6)	6 (2)	12 (55)	**<0.001**
IOP>21mmHG								
Total	25/276 (9)	15/242 (6)	9 (43)	**<0.001**	89 (26)	66 (22)	17 (77)	**<0.001**
Oc. Hypert.	-	-	-	-	38 (11)	31 (10)	2 (9)	>0.99
Glaucoma	-	-	-	-	51 (15)	35 (12)	15 (68)	**<0.001**
Cataract	6 (2)	4 (1)	1 (5)	0.30	79 (23)	61 (20)	13 (59)	**<0.001**
Band Keratopathy	-	-	-	-	6 (2)	2 (1)	3 (14)	**0.003**

Abbreviations: n.a.; not applicable, IOP: intraocular pressure. [a] fourteen eyes from thirteen patients are not in either group: 3 eyes because of active inflammation at last follow-up which resulted in a vision ≤20/50, 2 eyes because visual impairment resulted directly from (pre-operative) cataract and post-operative vision is not known, and 9 eyes because vision loss is predominantly caused by an ocular comorbidity.


4

Chapter 4

REDUCED NUMBER OF RELAPSES OF HUMAN LEUKOCYTE ANTIGEN-B27 ASSOCIATED UVEITIS DURING PREGNANCY

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Editor,

Human Leukocyte Antigen (HLA)-B27-associated anterior uveitis accounts for the vast majority of anterior uveitis cases.[1] Because of the relatively young patient population, combined with a recurrent nature, and the occasional need for therapeutic interventions with potential harmfull effects on pregnancies, we aimed to investigate the effect of pregnancy on the relapse rate of HLA-B27-associated anterior uveitis.

All women who had been treated at the University Medical Center Utrecht, and VU University Medical Center Amsterdam, for HLA-B27-associated anterior uveitis during the age of 18 and 42, were applied to for permission to retrospectively review their charts. This study was approved by the Institutional Ethics Board of both hospitals, and all aspects were performed in accordance with the Declaration of Helsinki. All patients signed written informed consent.
The relapse rate per year was calculated for three time-intervals per patient: during (full-term) pregnancy, the first 6 months post partum, and outside pregnancy. Follow-up started after the first documented episode of uveitis, to prevent inflating the relapse rate for a time-interval by starting it with a relapse. Miscarriages were removed from the time-intervals. Statistical analysis was performed using SPSS 21.0 (SPSS Inc., Chicago IL). To compare relapse rates between time-intervals, the Wilcoxon signed rank test was used. Conditional fixed-effects Poisson regression was used to correct for age at the time of an interval, and treatment with systemic immunosuppressive medication. A p-value of <0.05 was considered to be statistically significant.

Fifty-one patients were included, with a median follow up of 6.6 years (range 0.6-23.3), and a median of 5 uveitis episodes per patient (range 1-19). Eighteen patients had a total of 33 full-term pregnancies, and 6 miscarriages, between them. Five/18 women (28%) had a relapse during pregnancy. Seven/18 women (39%) had a relapse within 6 months after giving birth, of which the majority (5/7, 71%) occured between 10-25 weeks after childbirth.
During full-term pregnancy a significantly lower relapse rate was seen during pregnancy (median 0.00 (range 0.00-1.15, mean 0.17 (SD 0.42)), versus outside pregnancy: median 0.74 (range 0.00-1.92, mean 0.75 (SD 0.42), p=0.004). The relapse rate in the first 6 months postpartum was not different from the relapse rate outside pregnancy: median 0.00 (range 0.00-2.79, mean 0.56 (SD 0.86), p=0.27).

Controlling for age, and the use of systemic medication, the risk of recurrence during pregnancy was 20% of the risk outside of pregnancy (HR=0.20 (95%CI 0.07-0.55), p=0.002). To exclude unforeseen factors in the group of woman who were not pregnant during follow up, the multivariate analysis was repeated, excluding all women who had not been pregnant during the study period, which yielded similar results (see **Table 1**).

These findings are in line with previous studies in non-infectious uveitis (Chan et al. 2004, Chiam et al. 2013, Kump et al. 2006, Rabiah & Vitale 2003).[2–5] Chiam et al is the only study that described the course of HLA-B27-associated uveitis as a subgroup: they found a lower relapse rate during pregnancy, however, this did not reach statistical significance, probably due to a small patient population. We are now able to confirm these findings. In addition, we found that the relapse rate in female patients with HLA-B27-associated uveitis might increase slightly with their age.

Though our results should be interpreted keeping the retrospective nature, a setting in a tertiary referral center, and limited numbers in mind, the case cross-over design is well suited to study a transient factor, and the conditional fixed-effects Poisson regression analysis is a reliable analysis even when event frequencies are relatively low.[6] In conclusion, full-term pregnancy seems to reduce the relapse rate of HLA-B27-associated uveitis.

Table 1. The effect of pregnancy on relapse rate of uveitis, controlled for age, and systemic medication, using a fixed-effects Poisson regression

Group	Variable	HR (95% CI)	p-value
Complete cohort (n=52)	Pregnancy	0.20 (0.07-0.55)	**0.002**
	Older age, per 5 years	1.24 (1.04-1.48)	**0.02**
	Discontinued treatment with Systemic medication[a]	1.14 (0.75-1.74)	0.54
	Systemic medication[a]	1.26 (0.42-3.37)	0.68
Women with full-term pregnancy during follow-up only (n=18)	Pregnancy	0.21 (0.08-0.55)	**0.001**
	Older age per 5 years	1.22 (0.91-1.63)	0.18
	Systemic medication[a]	1.67 (0.76-3.65)	0.20

Abbreviations: 95% CI: 95% confidence interval, HR: hazard ratio

[a] Systemic medication include: systemic corticosteroids, disease modifying anti-rheumatic drugs (DMARDs) (leflunomide, mycofenolate mofetil, azathioprine, cyclosporine, cyclophosphamide, mycophenolate mofetil, tacrolimus, chlorambucil) and biologicals (adalimumab, etanercept, infliximab, rituximab, tocilizumab)

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5

Chapter 5

AQUEOUS HUMOR ANALYSIS IDENTIFIES HIGHER BRANCHED CHAIN AMINO ACID METABOLISM AS A MARKER FOR HLA-B27 ACUTE ANTERIOR UVEITIS AND DISEASE ACTIVITY

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ABSTRACT:

PURPOSE: The prognosis of HLA-B27 positive acute anterior uveitis is thought to be less favorable compared to HLA-B27-negative patients, suggesting distinct etiology of these clinically overlapping conditions. To advance our understanding of the biology of acute anterior uveitis, we characterized the metabolic profile of aqueous humor (AqH) of patients with HLA-B27 associated anterior uveitis (BAU) and idiopathic anterior uveitis (IAU).

METHODS: AqH samples from two independent cohorts totaling 30 BAU patients 16 IAU patients, and 20 cataract patients (CAT) were subjected to two individual rounds of Direct Infusion Mass Spectrometry (DIMS). Features predicted by DIMS that facilitated maximum separation between the disease groups in regression models were validated by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)-based quantification with appropriate standards.

RESULTS: Partial least square-discriminant analysis (PLS-DA) revealed metabolite profiles that were able to separate BAU from IAU patients. Pathway enrichment analysis, based on metabolites on which separation of the groups in the PLS-DA model was based, demonstrated the involvement of branched-chain amino acid biosynthesis, ascorbate and aldarate metabolism, the tricarboxylic acid (TCA) cycle, and glycolysis-diverting pathways (e.g. serine biosynthesis) across all investigated cohorts. Notably, the metabolite ketoleucine was elevated in BAU across all three runs and moderately - but robustly - correlated with anterior chamber cell count (correlation coefficient range = 0.41-0.81).

CONCLUSION: These results illustrate metabolic heterogeneity between HLA-B27-positive and HLA-B27-negative acute anterior uveitis, including an increase of branched-chain amino acid biosynthesis, that reflects disease activity in acute anterior uveitis.

INTRODUCTION

Acute anterior uveitis (AAU) is the most common form of uveitis. The presence or absence of the major histocompatibility complex class I allele ***HLA-B27*** in patients with AAU is essential to classify patients into HLA-B27-positive (10-88% of AAU, depending on geographic location and secondary or tertiary setting) or HLA-B27-negative (idiopathic) AAU.[1–3] Although HLA-B27-associated anterior uveitis (BAU) and HLA-B27-negative idiopathic acute anterior uveitis (IAU) share most clinical features, BAU generally has a younger age of onset, a more severe and recurrent disease course, and is linked to a less favorable visual outcome.[4,5] In addition, BAU often accompanies systemic rheumatic diseases, mostly ankylosing spondylitis.[6] The genetic association with the ***MHC (HLA-B27)*** and distinct disease evolution collectively hint towards a unique underlying biology for BAU over IAU. Yet to date, investigations of aqueous humor in BAU and IAU have mostly revealed shared molecular characteristics.[7,8]

Metabolomics refers to detection and quantification of a broad range of small metabolic products (i.e. metabolites) using mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy to map the complex biochemistry and cellular physiology of biological samples. This rapidly emerging ***omics*** field is commonly used to detect disease specific metabolic signatures to better understand pathophysiology or to aid in diagnosis and reveal prognostic biomarkers.[9] Related studies in non-infectious uveitis have already revealed changes in the metabolic composition of vitreous humor and plasma of various uveitis types.[10,11]

To shed more light on the role of HLA-B27 in disease we investigated metabolic profiles of BAU and IAU patients as close to the site of inflammation as possible: the aqueous humor (AqH). To aid in the identification of metabolites, we combined two rounds of untargeted metabolomics via ***Direct Infusion Mass Spectrometry*** (DIMS) to comprehensively map the metabolic patterns of AqH. Using unsupervised and supervised exploratory data mining we prioritized a set of features for validation by (semi-) targeted Liquid Chromatography – coupled tandem Mass Spectrometry (LC-MS/MS).[12,13]

METHODS

Patients and patient material

This study was approved by the Medical Ethical Committee of the University Medical Center Utrecht. All patients signed informed consent. The demographic characteristics of the cohorts are depicted in **Table 1**. In total, 70 samples were analyzed using DIMS

technology (see below) in two independent cohorts. Aqueous humor (AqH) samples from 30 HLA-B27 associated anterior uveitis (BAU) patients, 18 idiopathic anterior uveitis (IAU) patients, and 22 cataract patients (CAT) were collected between 2006 and 2014, and randomly assigned to a discovery (n=39), and a replication cohort (n=31). Specimens used for analysis were either the remainders from samples taken for diagnostic purpose to rule out an infectious cause, or were obtained during ocular surgery for disease complications (e.g. cataract or glaucoma surgery). Aqueous humor from cataract patients with no history of ocular inflammatory disease was obtained during cataract surgery. All AqH samples (± 20-75 µL) were stored at -80 degrees directly after sampling.

DIMS analysis

A diagram of the workflow used to establish metabolic profiles is shown under **Figure 1**. Per sample, a volume of 20 µL was mixed with 20 µL 0.6% formic acid in Milli-Q ultrapure water (Merck, Darmstadt, Germany) and 93.3 µL NSK-AB internal standard solution according to manufacturer's instructions (Cambridge Isotope Laboratories, Tewksbury, MA). After filtration over a 0.2 µm cut-off filter plate, the sample was analyzed by ***direct-infusion high-resolution mass spectrometry*** (***DI-HRMS***, hereafter called ***DIMS***) using an Advion TriVersa NanoMate (Ithaca, NY) with 5 µm ID chip-based infusion and a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). Mass spectrometry data were acquired in the scan range of m/z 70 to 600. The system was operated at a resolution of 140000 in both positive and negative mode (1.5 min each at 1.6 kV). To achieve high mass accuracy, mass calibration was performed before each experiment and internal lock masses were used. The mean intensity of three technical replicates was used for downstream computational analysis. For downstream analysis we only considered the negative electrospray ionization mode (ESI-) of the acquired dataset, because the ocular fluid samples were stored in polyethylene glycol (PEG) containing Eppendorf tubes, which suppressed signals for other metabolites in the positive mode. Raw data files were converted to mzXML format using MSConvert and processed using an in-house-developed untargeted metabolomics pipeline as described previously as well as the HDMB database (accurate mass, isotopic pattern).[14]

LC-MS/MS analysis

To validate the metabolites of interest identified by DIMS, we performed Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) in triplicate (technical replicates) on AqH samples with sufficient volume left after DIMS (n=19). To prepare the samples for LC-MS analysis we let 100 µL internal standards mixture in methanol evaporate to dryness in an Eppendorf vial, after which we added 50 µL of (cold) sample to the residue together

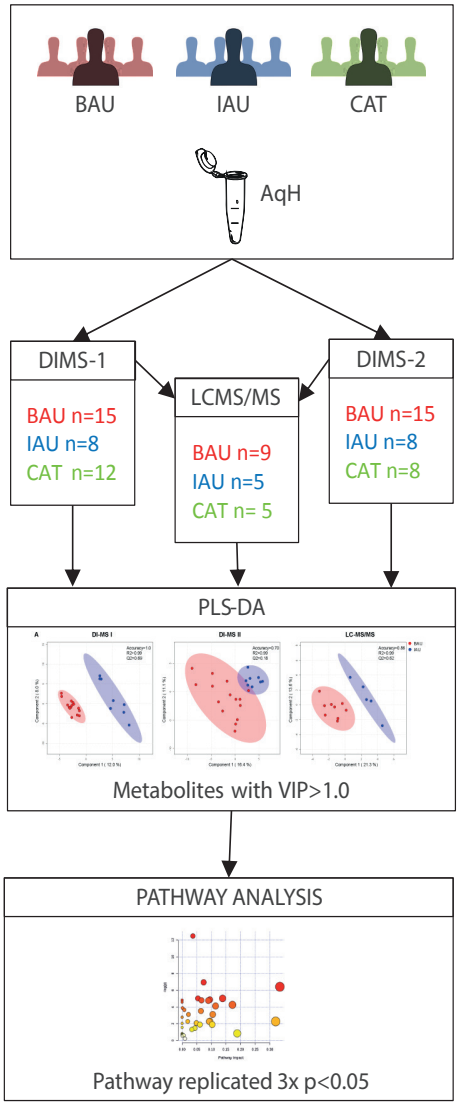


Figure 1. Flow diagram of methods used to identify meaningful metabolic differences between HLA-B27 associated acute anterior uveitis (BAU) and (non-infectious) idiopathic acute anterior uveitis IAU). Non-inflammatory cataract patients (CAT) were used as a reference but not taken in consideration for PLS-DA modelling. Aqueous humor (AqH) from patients and controls (first panel) is analyzed using Direct Infusion Mass Spectrometry in two independent cohorts (second panel). Relevant identified metabolites were validated by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). Metabolic profiles were analyzed by Partially Least Squares Discriminant Analysis (PLS-DA) for all three runs (DIMS I, II and LC-MS/MS) (third panel). Metabolites with a Variable Influence in the Projection (VIP) of>1.0 in this model were then entered into pathway enrichment analysis for each run (Metabolanalyst 3.0) (bottom panel). Pathways that were significantly indicated and replicated in all runs were considered significantly involved in AAU.

with 10 µL 4 M ice cold perchloric acid. After vortex mixing the mixture was put on ice for 5 minutes the vial was centrifuged at 13000 gouge (g) at 4°C and 48 µL was accurately transferred to a new vial. A volume of 2 µL 2 M KOH was added to neutralize the sample and precipitate the excess of perchloric acid. After keeping on ice for 5 min after vortex mixing the sample was centrifuged at 13000 g for 5 min at 4°C. The supernatant was transferred to a clean glass vial for LC-MS analysis. Analysis was performed using a Phenomenex Luna Omega PS C18 column (2.1x100 mm, 1.6 µm) positioned in an Acella UHPLC system operated at 30° and a flow rate of 150 µL min^{-1}. Upon sample injection a period of 3 min 100% solution A (6.5 mM ammoniumbicarbonate pH8) was followed by a 2.5 min linear gradient to 10% B (95% methanol + 5% 6.5 mM ammoniumbicarbonate pH8). After this the concentration B was linearly increased to 98% in 4.4 min and kept there for 4 min. Finally, the column was reconditioned at 100% A for 4 min prior to a next injection. An LTQ-Orbitrap XL was used for MS detection. Prior to analysis mass calibration was performed. The system was operated in data dependent analysis MS/MS mode at a resolution of 30000 in negative mode at a potential of 3.5 kV and a capillary temperature of 300°C. Peak identifications were performed in a search against the HDMB database (accurate mass, isotopic pattern) and a custom database (retention time, m/z, fragmentation pattern).

Data processing and statistical analysis

High quality peaks and putative metabolites that were detected in the majority of samples (the relative standard deviations of peak intensities and retention time were evaluated) were loaded into the Metaboanalyst 3.0 server. To improve data quality, samples with a high percentage of missing values were identified and removed: the data from the DIMS analyses were filtered by interquartile range (IQR) of intensity and were considered if they were detected in >70% of all samples. Data normalization was achieved by quantile normalization and auto-scaling of the dataset (plus log-transformation of the LC-MS/MS data to achieve normal distribution). We used exploratory data analysis, including principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), and hierarchical clustering with Euclidean distance and Ward's clustering method.[15,16] Based upon PCA analysis, we excluded 4 samples from the discovery cohort with strongly deviating coefficients for the first two components (IAU n=2, CAT n=2) (**Supplementary Figure 1**).

From the PLS-DA analysis the Variable Importance in Projection (VIP) value was obtained for each metabolite. A variable with a VIP Score >1 can be considered important in driving the projection used to summarize the PLS-DA model in which the groups are optimally separated.

The predicted metabolites with a VIP>1 were fed into ***the Integrated Pathway Analysis Module*** of the MetaboAnalyst server 3.0. Metabolic pathways were considered affected if metabolites functioning in their enzymatic reactions were found in altered abundance (replicated at $p<0.05$) across all three individual runs (DIMS 1+DIMS2+LCMS). A Mann Whitney U (MWU) test (SPSS v21 Inc., Chicago IL) was used to determine group differences between IAU and BAU and the direction of effect of individual metabolites derived from the pathway analysis.

Individual metabolites were tested for correlation with age or uveitis activity (anterior chamber cell count according to the SUN recommendations[17]) using Spearman's Rho test. Metabolite abundance between male and female patients was tested using a MWU test. Metabolites were considered to be influenced by age, sex or uveitis activity if a significant effect in the same direction was found in all three runs. In addition, Z-score transformed data of all three runs were accumulated ($Z = (Variable - Mean_{group})/ Standard\ Deviation_{group}$) for combined analysis. CAT samples were used as a reference only and not taken into consideration for statistical testing.

RESULTS

BAU and IAU show differences in metabolic AqH profiles

The demographic characteristics of the cohorts are depicted in **Table 1**. We performed two rounds of metabolic profiling of AqH of BAU, IAU, and CAT patients using DIMS which resulted in the identification of 147 metabolic compounds in the discovery cohort and 127 in the replication cohort (**Supplementary Table 1**).

Direct comparison of the metabolic profiles by principal component analysis revealed no consistent separate clusters for any of the groups which most likely can be attributed to the relatively large within-group variation (compared to between-group variation) (**Supplementary Figure 2**). To reveal more sophisticated differences between the two disease groups, we exploited supervised dimensionality reduction by discriminant analysis via PLS regression (partial least square-discriminant analysis; PLS-DA). PLS-DA generated excellent separation by low-dimensional projection of the disease groups with sufficient predictive accuracy for both DIMS runs as determined by internal cross validation of the projected models (**Figure 2A**). The metabolites considered to drive these differences (VIP score >1.0 of the PLS-DA) were mostly short-chain fatty acids (SCFAs) and branched-chain amino acids (BCAAs). Therefore, we subjected samples with sufficient volume left after DIMS to LC-MS/MS with standard compounds for (semi-) targeted validation directed at small fatty acids and organic acids. LC-MS/MS analysis yielded 29 metabolites that

Table 1. Cohorts

		BAU	IAU	p-value	CAT
Discovery cohort - DIMS 1	**N**	15	8		12
	Male gender; %	60%	13%	0.07*	25%
	Age; mean (SD)	44.6 (15.5)	55.0 (11.6)	0.13**	69.6 (7.9)
	Medication; n (%)				
	Topical steroids	13 (87%)	6 (75%)	0.59*	
	Periocular steroids <3m.	4 (27%)	1 (13%)	0.62*	
	Systemic steroids	3 (20%)	2 (25%)	1.00*	
	DMARDS or biologicals	1 (7%)	1 (13%)	1.00*	
	Anterior chamber cell count; n (%)				
	0	2 (13%)	4 (50%)		NA
	½+	3 (20%)	1 (13%)		NA
	1+	3 (20%)	1 (13%)		NA
	2+	5 (33%)	1 (13%)		NA
	3+	2 (13%)	0		NA
	4+	0	0		NA
	hypopyon	0	1 (13%)		NA
Replication cohort - DIMS 2	**N**	15	8		8
	Male gender; %	67%	50%	0.66*	50%
	Age; mean (SD)	40.5 (13.0)	48.8 (18.0)	0.22**	60.2 (8.9)
	Medication; n (%)				
	Topical steroids	13 (87%)	5 (63%)	0.30*	
	Periocular steroids <3m.	4 (27%)	1 (13%)	0.62*	
	Systemic steroids	1 (7%)	2 (25%)	0.27*	
	DMARDS or biologicals	2 (13%)	0	0.53*	
	Anterior chamber cell count[A]; n (%)				
	0	2 (14%)	3 (38%)		NA
	½+	5 (36%)	1 (13%)		NA
	1+	2 (14%)	1 (13%)		NA
	2+	2 (14%)	3 (38%)		NA
	3+	2 (14%)	0		NA
	4+	1 (7%)	0		NA
	hypopyon	0	0		NA
Discovery cohort – LC-MS/MS	**N**	9	5		5
	Male gender; %	67%	0%	**0.03***	0%
	Age; mean (SD)	35.2 (11.6)	55.7 (14.7)	**0.01****	60.7 (7.7)
	Medication; n (%)				
	Topical steroids	8 (89%)	4 (80%)	1.00*	
	Periocular steroids <3m.	2 (22%)	1 (20%)	1.00*	
	Systemic steroids	1 (11%)	1 (20%)	1.00*	
	DMARDS or biologicals	1 (11%)	0	1.00*	
	Anterior chamber cell count; n (%)				
	0	0	2 (40%)		NA
	½+	2 (22%)	1 (20%)		NA
	1+	2 (22%)	1 (20%)		NA
	2+	4 (44%)	1 (20%)		NA
	3+	1 (11%)	0		NA
	4+	0	0		NA
	Hypopyon	0	0		NA

*Fishers exact test, ** T-test, *** Mann Whitney U test. [A] anterior chamber cell count was unavailable from one patient in BAU group. **Abbreviations:** NA: not applicable

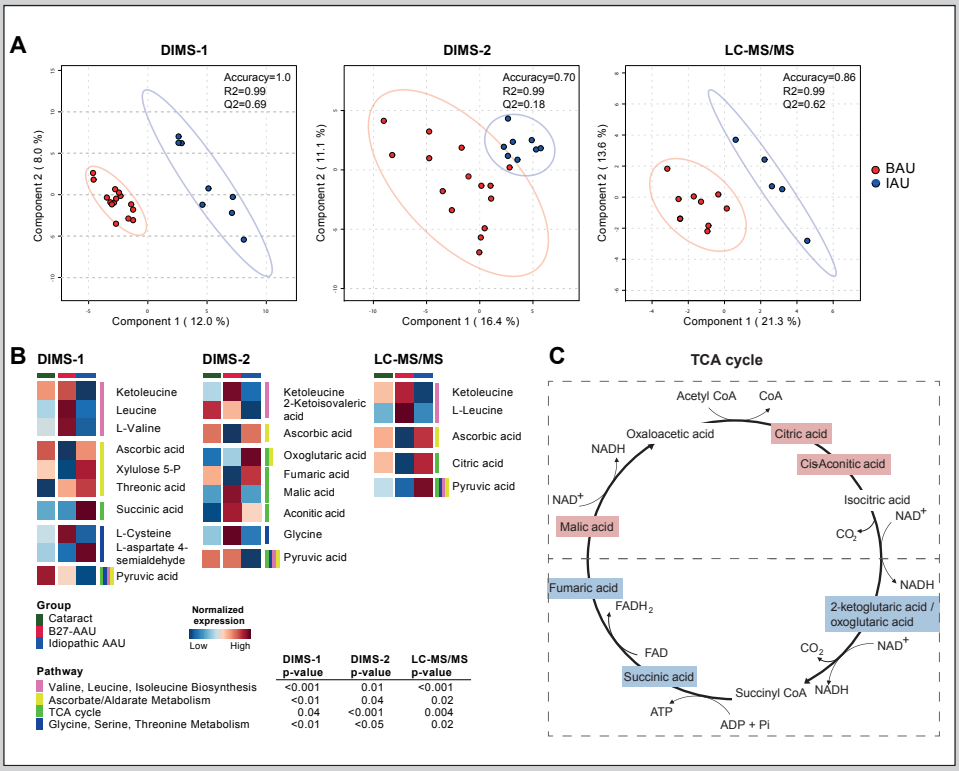


Figure 2. The metabolic composition of aqueous humor in patients with HLA-B27 associated acute anterior uveitis (BAU) is distinct from (non-infectious) idiopathic acute anterior uveitis (IAU). **A.** Partial least square-discriminant analysis (PLS-DA) of the intraocular metabolome separates BAU (in red) from IAU (in blue) in all three runs, indicating metabolic heterogeneity between these AAU entities. The explained variance of each component is shown in brackets. Ellipses display 95% confidence interval for the samples of the group. Accuracy values from internal cross-validation are given in the right corner of the graphs: R2 = fraction of y-variation modeled in the component (goodness of fit), Q2 = overall cross-validated R2 for the component (predictive ability). **B.** Replicated metabolic pathways in colors that were different between BAU and IAU in aqueous humor. Pathways are based on metabolites that had a VIP-value>1.0 in the PLS-DA (**Table 3**). For each metabolite, the mean peak intensity for DIMS and retention time for LC-MS/MS are shown for BAU and IAU, and cataract patients (CAT, green) as a reference. **C.** Metabolites in the TCA cycle that are significantly decreased or increased in the aqueous humor of BAU compared to IAU in ≥ 1 run. Blue: decreased in BAU vs IAU. Red: increased in BAU vs IAU.

were detected in >70% of all patients (**Supplementary Table 1**). In total, 18 annotated metabolites were considered detected in all three runs (DIMS-1, -2 and LC-MS/MS), and Leucine in two out of three runs (LC-MS/MS and the first DIMS) (**Table 2**). We compared the intensity levels of DIMS and LC-MS/MS for each of these 19 metabolites measured in the same samples, which demonstrated a positive correlation between DIMS and LC-MS/MS intensities for six metabolites (**Supplementary Figure 3**). Finally, of these six robustly

Table 2. The 20 replicated metabolites identified across all three mass spectrometry runs in aqueous humor of anterior uveitis patients. From the PLS-DA analysis the Variable Importance in Projection (VIP) value was obtained for each metabolite. A variable with a VIP Score >1 can be considered important in driving the projection used to summarize the PLS-DA model in which the groups are optimally separated.

		DIMS-1				DIMS-2				LC-MS/MS			
Putative metabolite	and / or	N	p-value	VIP	Ratio*	N	p-value	VIP	Ratio*	N	p-value	VIP	Ratio*
Ascorbic acid		23	0.12	**1.60**	0.44	23	**0.004**	**1.82**	0.15	12	**0.04**	**1.46**	0.39
Butyric acid (Butanoic acid)		23	0.08	0.92	1.52	23	0.48	0.89	0.48	9	0.12	**1.13**	0.65
Citric acid	Isocitric acid	23	1.00	0.26	1.27	23	**0.05**#	0.96	0.67	14	0.10	**1.45**	0.79
Fumaric acid	Maleic acid	23	0.25	0.55	0.67	23	**0.03**	**1.26**	0.55	9	**0.04**	0.56	0.42
Glutamine		23	0.65	0.85	1.06	23	**0.02**	0.71	0.74	14	0.13	0.96	5.85
Hexanoic acid (Caproic acid)		23	**0.002**	**2.09**	2.32	23	0.25	0.86	0.38	11	0.19	0.61	0.79
Hippuric acid		23	0.65	0.84	0.94	23	0.65	0.91	1.05	13	0.56	0.50	1.72
Indole		23	**0.001**	**1.74**	1.61	23	0.95	**1.24**	0.96	14	0.84	0.64	0.58
Ketoleucine (4-methyl-2-oxopentanoate)	(3-methyl-2-oxopentanoate)	23	0.09	**1.30**	1.23	23	0.22	**1.23**	1.11	14	**0.006**	**1.54**	4.66
Leucine	Isoleucine	23	0.09	**1.34**	1.54	-	-	-	-	14	**0.02**	**1.23**	5.39
Phenylalanine		23	0.11	0.33	0.60	23	1.00	**1.11**	1.02	14	0.95	0.51	0.95
Phenylpyruvic acid		23	0.12	0.40	1.08	23	0.40	0.85	0.96	14	0.32	0.87	1.33
Pivalic acid	(Iso-) Valeric acid	23	**0.006**	0.95	1.55	23	0.25	0.80	0.41	13	0.24	0.99	0.56
Pyruvic acid		23	0.05	**1.12**	3.15	23	0.20	**1.30**	1.37	14	0.16	**1.54**	0.70
Succinic acid (butanedioic acid)	Methylmalonic acid	23	**0.004**	**1.31**	0.41	23	0.12	0.95	0.79	10	0.73	0.61	0.59
Threonic acid	Erythronic acid	23	0.05	**1.26**	0.49	23	0.16	0.83	0.67	14	0.55	0.79	0.86
Tryptophan		23	0.18	0.71	0.91	23	0.27	0.42	0.67	14	0.84	**1.14**	0.98
Tyrosine		23	0.37	**1.42**	0.83	23	0.44	0.32	0.95	14	0.39	0.74	1.76
Uric acid		23	**0.01**	**1.04**	2.11	23	0.85	**1.35**	1.12	14	0.05	**1.66**	1.49

* ratio BAU/IAU. **Abbreviations:** DIMS: Direct Infusion Mass Spectrometry, LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry, VIP; variable importance in projection in partial least square-discriminant analysis (PLS-DA). Significant p-values ($p<0.05$, MWU test between BAU and IAU) or VIP values (VIP>1.0) are printed bold

identified metabolites, two also showed VIP>1.0 in all three runs: ketoleucine and uric acid. Ascorbic acid and pyruvic acid also had a VIP>1.0 in all three runs (**Table 2**), but these (putative) metabolites did not correlate between DIMS and LC-MS/MS (rho=0.15, p=0.58 and rho=-0.10, p=0.69 respectively).

Aqueous humor from BAU patients characterized by higher Valine/Leucine biosynthesis, lowered Ascorbate and changes in energy metabolism

Next, we considered all metabolites with a VIP>1.0 from each individual run (n= 53 for DIMS-1, n=52 for DIMS-2 and n=12 for LC-MS/MS) and subjected them to metabolic pathway analysis, with the aim to identify involved metabolic pathways consistently detected across all three runs. We detected four pathways in all cohorts (**Table 3**); the BCAA or Valine, Leucine, and Isoleucine biosynthesis (KEGG: ko00290, increased in BAU), the Ascorbate & Aldarate metabolism pathway (KEGG: ko00053, decreased in BAU), the Citric acid cycle (also known as Tricarboxylic acid (TCA) cycle or Krebs cycle, KEGG: ko00020) and the Glycine, Serine and Threonine Metabolism pathway (KEGG: ko00260). The relative abundance of the key metabolites of these pathways identified in BAU and IAU are illustrated in **Figure 2B-C**.

Discovery, Replication and Validation of Ketoleucine as a potential molecular marker for disease activity in anterior uveitis

We tested the individual metabolites that were replicated (ascorbic acid and ketoleucine) for an association with disease activity (anterior chamber cell count), age, sex or treatment. A negative correlation was evident for Ascorbic acid, for data obtained by DIMS-1 (rho=-0.46, p=0.03), with evidence for a similar trend in the other two runs (rho=-0.35, p=0.11 and rho=-0.32, p=0.31 for DIMS-2 and LC-MS/MS respectively). Ketoleucine correlated positively (rho= 0.41-0.81) with anterior chamber cell count – a hallmark sign of disease activity in anterior uveitis - in all three runs (**Figure 3**). This was most striking for the standard compound controlled LC-MS/MS (rho=0.81, p<0.001) and mainly driven by BAU patients (**Figure 3, right panel**).

DISCUSSION

Using multiple detection platforms and independent patient cohorts, we aimed to characterize the metabolic profile of aqueous humor of patients with HLA-B27 associated acute anterior uveitis (BAU) and idiopathic acute anterior uveitis (IAU) and observed that these clinically analogous conditions are accompanied by unique differences in metabolic profiles. These changes included metabolites present in branched-chain amino acid (BCAA) metabolism, ascorbate and aldarate metabolism, the tricarboxylic acid (TCA)

Table 3. Pathway enrichment analysis based on metabolites identified by PLS-DA for each mass spectrometry run *

	Pathway Name	Hits	p-value	Impact	Details
DIMS-1	**Phenylalanine, Tyrosine and Tryptophan biosynthesis**	6/27	**<0.001**	0.04	Indoleglycerol phosphate, Indole, L-Tyrosine, 6-deoxy-5-ketofructose 1-phosphate, L-Aspartate-semialdehyde, 4-hydroxyphenylpyruvic acid
	Valine, Leucine and Isoleucine biosynthesis	4/27	**<0.001**	0.07	Pyruvic acid, L-Leucine, L-Valine, Ketoleucine
	Arginine and Proline metabolism	6/77	**0.002**	0.33	Ornithine, L-Proline, Hydroxyproline, Creatine, Urea-1-Carboxylate, Pyruvic acid
	Phenylalanine metabolism	4/45	**<0.01**	0.05	Phenylacetic acid, Pyruvic acid, Succinic acid, L-Tyrosine
	Ascorbate and Aldarate metabolism	4/45	**<0.01**	0.14	Ascorbid acid, Pyruvic acid, Threonic acid, Xylulose 5-phosphate
	Lysine degradation	4/47	**<0.01**	0.09	Trimethyl-L-Lysine, Glutaric acid, Oxoadipic acid, Carnitine
	Aminoacyl-tRNA biosynthesis	5/75	**<0.01**	0.00	L-Cysteine, L-Valine, L-Leucine, L-Tyrosine, L-Proline
	Glycine, Serine and Threonine metabolism	4/48	**<0.01**	0.07	L-Aspartate-Semialdehyde, Creatine, L-Cysteine, Pyruvic acid
	Tyrosine metabolism	5/76	**<0.01**	0.09	L-Thyrosine, 4-Hydroxyphenylpyruvic acid, Vanillylmandelic acid, Pyruvic acid, Succinic acid
	Pantothenate and CoA biosynthesis	3/27	**0.01**	0.00	L-Cysteine, L-Valine, Pyruvic acid
	Cysteine and Methionine metabolism	4/56	**0.01**	0.17	L-Cysteine, L-Cystine, Pyruvic acid, L-Aspartate-semialdehyde
	Pentose phosphate pathway	3/32	**0.02**	0.11	Gluconic acid, Xylulose 5-phosphate, Pyruvic acid
	Propanoate metabolism	3/35	**0.02**	0.00	Succinic acid, L-Lactic acid, L-Valine
	Glutathione metabolism	3/38	**0.03**	0.01	L-Cysteine, Ornithine, Ascorbic acid
	Valine, leucine and isoleucine degradation	3/40	**0.03**	0.06	L-Leucine, L-Valine, Ketoleucine
	Citrate cycle (TCA cycle)	2/20	**0.04**	0.10	Succinic acid, Pyruvic acid
	Taurine and Hypotaurine metabolism	2/20	**0.04**	0.02	Pyruvic acid, L-Cysteine
DIMS-2	**Citrate cycle (TCA cycle)**	5/20	**<0.001**	0.29	Oxoglutaric acid, L-Malic acid, Aconitic acid, Pyruvic acid, Fumaric acid
	Phenylalanine, Tyrosine and Tryptophan biosynthesis	5/27	**<0.001**	0.26	Shikimic acid, Indole, L-Phenylalalnine, 3-dehydroquinate, 2-aminobenzoic acid
	Alanine, Aspartate and Glutamate metabolism	4/24	**<0.001**	0.00	N-Acetyl-L-Aspartic acid, Pyruvic acid, Oxoglutaric acid, Fumaric acid
	Pantothenate and CoA biosynthesis	4/27	**<0.001**	0.25	Pantothenic acid, Alpha-Ketoisovaleric acid, Pyruvic acid, Uracil

DIMS-2 (cont'd)	**Butanoate metabolism**	4/40	**<0.01**	0.13	4-hydroxybutyric acid, Pyruvic acid, Oxoglutaric acid, Fumaric acid
	Glyoxylate and Dicarboxylate metabolism	4/50	**<0.01**	0.03	Aconitic acid, Oxoglutaric acid, L-Malic acid, Pyruvic acid
	Valine, Leucine and Isoleucine biosynthesis	3/27	**0.01**	0.14	Pyruvic acid, alpha-ketoisovaleric acid, Ketoleucine
	β-Alanine metabolism	3/28	**0.01**	0.01	Malonic acid, Pantothenic acid, Uracil
	Vitamin B6 metabolism	3/32	**0.02**	0.12	Oxoglutaric acid, Pyridoxal, pyruvic acid
	Nitrogen metabolism	3/39	**0.03**	0.00	L-Phenylalalnine, 2-Aminobenzoic acid, Glycine
	Nicotinate and Nicotinamide metabolism	3/44	**0.04**	0.00	Pyruvic acid, Porpionic acid, Fumaric acid
	Phenylalanine metabolism	3/45	**0.04**	0.12	L-Phenylalalnine, Pyruvic acid, Fumaric acid
	Ascorbate and aldarate metabolism	3/45	**0.04**	0.13	Ascorbic acid, Pyrvic acid, oxoglutaric acid
	Arginine and proline metabolism	4/77	**0.04**	0.13	Proline, Creatine, Fumaric acid, Pyruvic acid
	Tryptophan metabolism	4/79	**0.04**	0.19	Indole, Aminobenzoic acid, 3-hydroxyanthranilic acid, Indoleacetic acid
	Glycine, Serine and Threonine metabolism	3/48	**<0.05**	0.19	Glycine, Creatine, Pyruvic acid
LC-MS/MS	**Valine, Leucine and Isoleucine biosynthesis**	3/27	**<0.001**	0.06	Pyruvic acid, L-Leucine, Ketoleucine
	Butanoate metabolism	3/40	**<0.001**	0.09	Pyruvic acid, Butyric acid, Diacetyl
	Citrate cycle (TCA cycle)	2/20	**0.004**	0.15	Citric acid, Pyruvic acid
	Valine, Leucine and Isoleucine degradation	2/40	**0.02**	0.06	L-Leucine, Ketoleucine
	Ascorbate and Aldarate metabolism	2/45	**0.02**	0.13	Ascorbate, Pyruvic acid
	Glycine, Serine and Threonine metabolism	2/48	**0.02**	0.00	Pyruvic acid, L-Tryptophan
	Glyoxylate and Dicarboxylate metabolism	2/50	**0.02**	0.00	Citric acid, Pyruvic acid

* Metabolites identified by partial least square-discriminant analysis (PLS-DA) with a variable importance in projection (VIP) score >1.0 were analyzed by the Integrated Pathway Analysis Module of the MetaboAnalyst server 3.0 which resulted in the pathways in this Table. The hits indicate the number of metabolites identified for each metabolic pathway (number of metabolites found in this study / number of metabolites known to be involved in the pathway). The impact score is a combination of the centrality of the metabolites within a certain pathway and pathway enrichment results and ranges from 0-1. The putative metabolites from DIMS or LC-MS/MS analyses that represent the "hits" for the pathway they are in are in "Details". **Bold + underscore;** pathway present in 3/3 analyses. **Bold;** pathway present in 2/3 analyses.

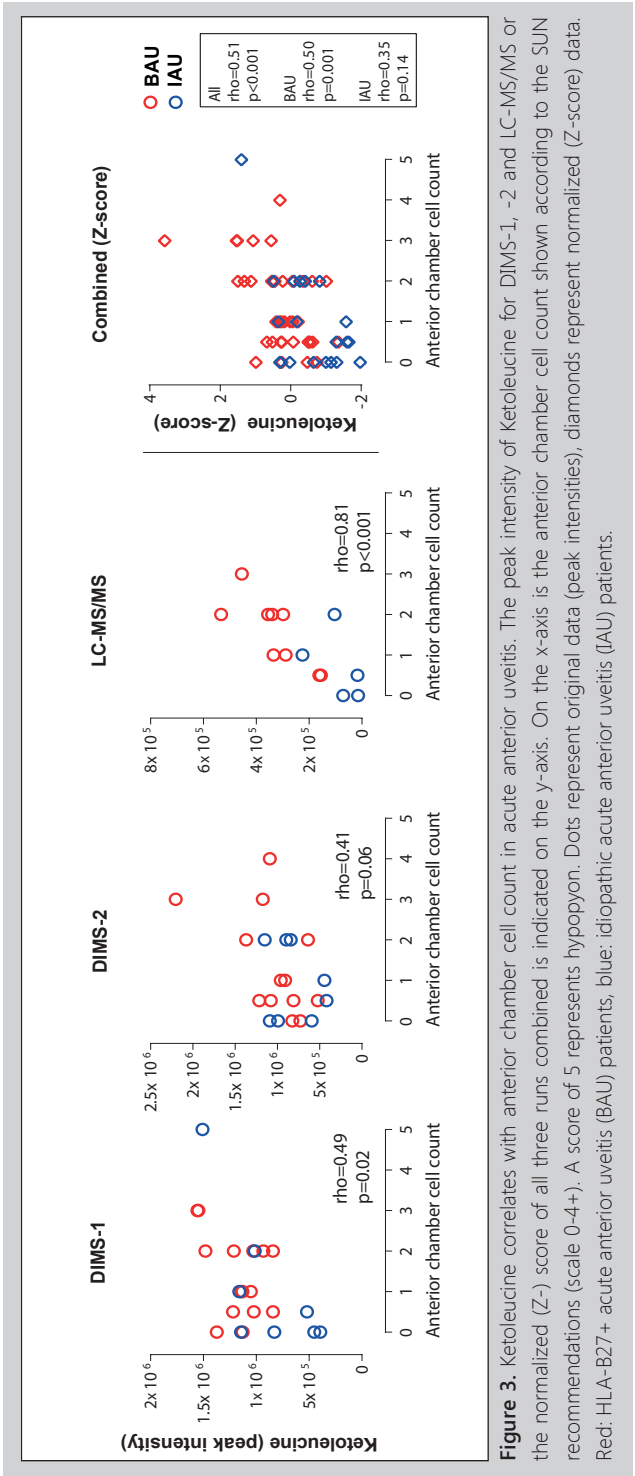


Figure 3. Ketoleucine correlates with anterior chamber cell count in acute anterior uveitis. The peak intensity of Ketoleucine for DIMS-1, -2 and LC-MS/MS or the normalized (Z-) score of all three runs combined is indicated on the y-axis. On the x-axis is the anterior chamber cell count shown according to the SUN recommendations (scale 0-4+). A score of 5 represents hypopyon. Dots represent original data (peak intensities), diamonds represent normalized (Z-score) data. Red: HLA-B27+ acute anterior uveitis (BAU) patients, blue: idiopathic acute anterior uveitis (IAU) patients.

cycle, and serine biosynthesis. Previous metabolic profiling of plasma of uveitis patients showed mainly involvement of amino acid, carbohydrate, and lipid metabolism, and in part reflect our observations in aqueous humor.[10]

In our study, several metabolites from the BCAA biosynthesis pathway were increased in BAU indicating a role for the BCAA biosynthesis pathway in BAU over IAU. Among the intermediates of this pathway, ketoleucine (a deaminated derivate of leucine) scored a VIP>1.0 in PLS-DA analysis of all three mass spectrometry runs and showed good correlation between DIMS and LC-MS intensities. Ketoleucine (also known as ***4-methyl-2-oxopentanoate***, ***α-ketoisocaproic acid***, or ***2-ketoisocaproate***) is interconverted from leucine by ***branched chain amino acid transaminase*** (BCAT)1 and BCAT2 as the first step in BCAA catabolism.[18] Interestingly, selective blocking of the BCAT1 activity in macrophages - a cell type considered central to initiating ***endotoxin-induced uveitis*** in rodents[19,20] - results in a dramatic attenuation of disease activity in inflammatory mouse models (e.g. collagen-induced arthritis) by redirecting the inflammatory macrophage transcriptome to a regulatory phenotype (so called M2 cells).[21]

About half of the BAU patients will eventually develop ankylosing spondylitis (AS), a rheumatic condition which shares a risk factor for disease development in HLA-B27 and is intimately linked to (subclinical) intestinal inflammation and ***gut microbiome dysbiosis***.[22,23] BCAA levels are also changed in plasma/serum of AS patients.[24,25] Interestingly, the gut microbiome contributes to altered levels of BCAAs[26] and fecal matter of AS patients show changes in BCAAs valine, leucine, and ketoleucine that correlate with erythrocyte sedimentation rate - a clinical sign of inflammation.[27] Note, the alterations in BCAA levels found in AS patients gut or serum were not observed in rheumatoid arthritis patients and are in line with our observations that BCAA metabolism may be specifically affected in HLA-B27-linked pathology.

Ketoleucine can be released from the cell, but is commonly reaminated into leucine. Leucine has been shown to be a potent activator of the pro-inflammatory mTOR signaling pathway and inhibiting mTOR is shown to be effective in treating non-infectious uveitis.[28–31] Alternatively, ketoleucine can be decarboxylated into downstream intermediates (e.g. acetyl-CoA) that fuel other metabolic pathways, such as the TCA cycle. The involvement of the TCA cycle was evident from increase in ***malic acid***, ***citric acid*** and ***aconitic acid*** in BAU compared with IAU, while subsequent intermediates derived from aconitic acid (***2-ketogultaric acid***, ***succinic acid***, ***fumaric acid***) were lower in BAU compared with IAU, forming a "break" between aconitic acid and 2-ketoglutaric acid (**Figure 2**). Curiously,

a very similar fragmentation of the TCA-cycle is a hallmark of the metabolic shift from resting (M0) to pro-inflammatory (M1) macrophages.[32,33] The local cell subset(s) driving this "TCA-signature" of BAU provide an exciting field of upcoming research.
Ascorbate & aldarate metabolism was decreased in BAU compared with IAU. ***Ascorbate*** (or ***ascorbic acid***, also known as ***vitamin C***) protects from inflammatory damage and attenuates inflammation as an antioxidant as well as through the inhibition of NF-κB signaling.[34,35] Note, intraocular ascorbate levels are known to decrease in the endotoxin-induced uveitis models of anterior uveitis described above.[36]

Collectively, these findings point towards a more severe eye inflammation in BAU compared to IAU which raises the question whether the metabolic differences detected reflect distinct pathology or merely differences in disease activity between the two conditions. Indeed, we did observe a positive correlation between ketoleucine and anterior chamber cell count (and perhaps there exists an inverse relation between ascorbate and anterior chamber cell count as well). However, the correlation between ketoleucine and disease activity was predominantly observed in BAU and considering the BCAA link with AS, differences in this BCAAs derivate may very well be related to HLA-B27 pathology as well as disease activity. Regardless, the severity of the inflammatory activity in the anterior segment significantly guides clinical decision making in patients with anterior uveitis. Objective quantification of this process has therefore been a key research goal for years in ophthalmology. To overcome inter-observer variability of manual counting of infiltrating cells (anterior chamber cell counting) by ophthalmologists, anterior segment optical coherence tomography and laser flare photometry have been developed as more objective imaging methods to assess disease activity.[37,38] Here, we provide rationale that evaluation of metabolic markers could complement monitoring disease activity in BAU patients. For example, we envision that elucidating the Raman signatures of BCAAs such as ketoleucine may catalyze the development of noninvasive monitoring of these metabolites in the anterior chamber using Raman spectroscopy.[39]

The outcomes of this study need to be considered in light of the following limitations: Metabolomics may provide a powerful tool to interrogate the metabolic machinery underlying eye disease, but the use of untargeted mass spectrometry needs careful design of strategies that maximize signal-to-noise ratio, minimize unambiguous annotation, and ensure robustness of the identified peaks.[40–42] DIMS is able to perform mass spectrum acquisition without prior chromatographic separation which results in fast measurements on low sample volumes, which makes it a useful tool for untargeted, hypothesis generating approaches.[43,44] However, metabolites with a similar mass share peaks and this may lead to

ambiguous annotation.[13] To overcome this challenge, we followed a top-down untargeted design to prioritize spectra of peaks for detailed and standard controlled technical and biological replication, here standard controlled LC-MS/MS for short-chain fatty acids. This analysis confirmed the increased intensity of 4-methyl-2-oxopentanoate (i.e., ketoleucine, over 3-methyl-2-oxopentanoate) in B27-AAU. However, other overlapping peaks might have hampered detection of correlation between DIMS and LC-MS/MS data for a number of metabolites.

Furthermore, the identified pathways are based on PLS-DA models of the metabolic profiles. PLS-DA is a standard multivariate classification algorithm widely used within the metabolomics field because of its sufficient handling of collinearity and noise while at the same time conveniently summarizing the classification potential of individual metabolites into a variable importance on projection (VIP) score that aids in prioritizing metabolites for further analysis. However, regression models including PLS-DA are prone to overfitting.[45] To minimize the technical and statistical limitations of PLS-DA models, we followed a study design based on independent detection platforms and independent patient cohorts.[46] The need for such stringent criteria is clearly demonstrated by the number of validated metabolites across all three runs – an approach that is not commonly exploited in ocular fluid studies.

Finally, the patient cohorts were not matched in sex, age, treatment or uveitis activity because ocular fluid collection is restricted to AqH taps for diagnostic purposes or surgery. This resulted in a predominance of males and younger patients in the BAU group, which is inherent to the two diseases. We found, however, no significant and consistent association between age or sex and the metabolites that were replicated (ascorbic acid and ketoleucine). Also, additional factors affecting metabolic status such as diet or smoking were not taken into account. We believe however that independent biological replication using two patient cohorts has substantially decreased bias from such unaccounted factors.

In summary, our results show that HLA-B27 associated uveitis patients have distinct metabolic profiles in their aqueous humor compared to the HLA-B27 negative counterpart, which is characterized by changes in branched chain amino acid (BCAA) synthesis and the TCA cycle, in particular the metabolic compound ketoleucine and substantiates the potential involvement of the gut microbiome in HLA-B27 associated uveitis.

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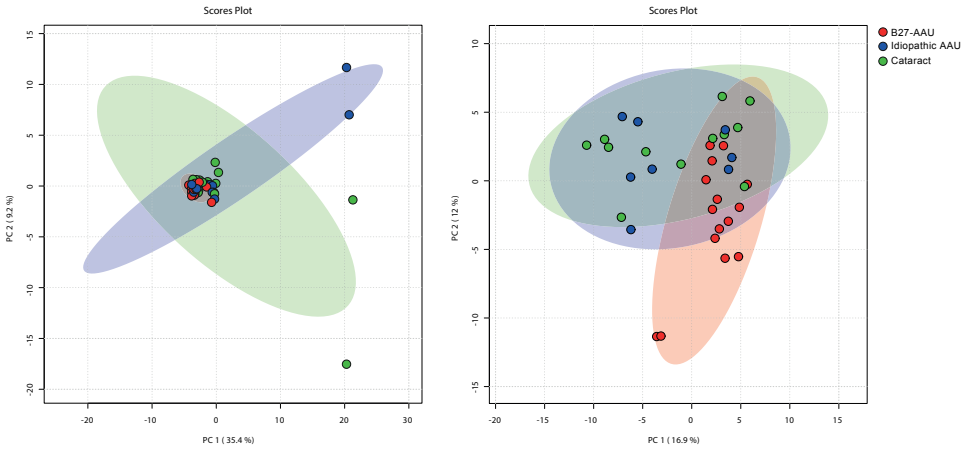
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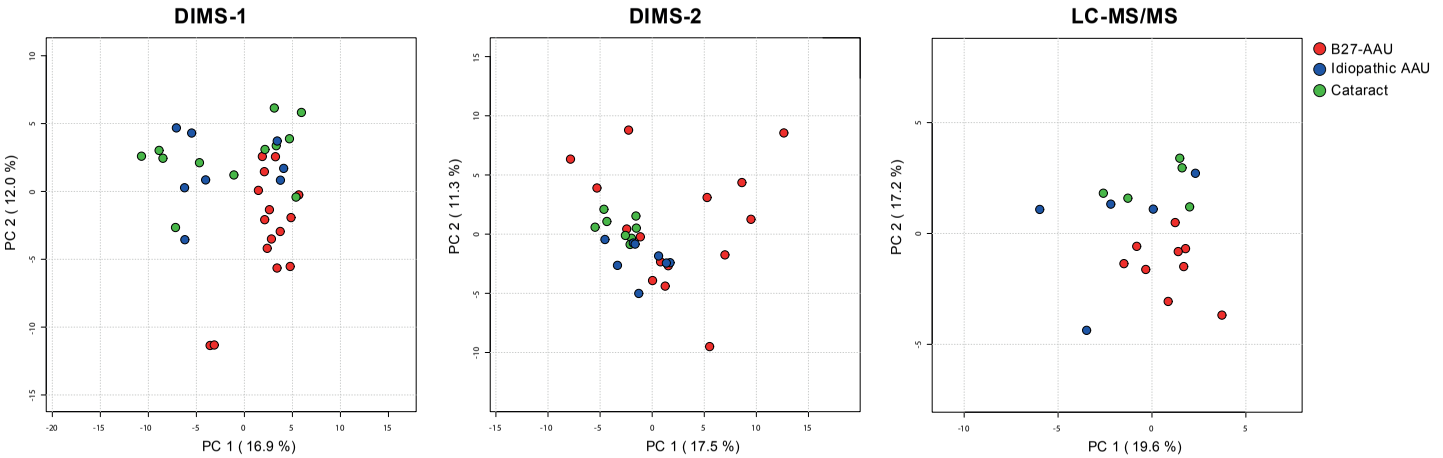
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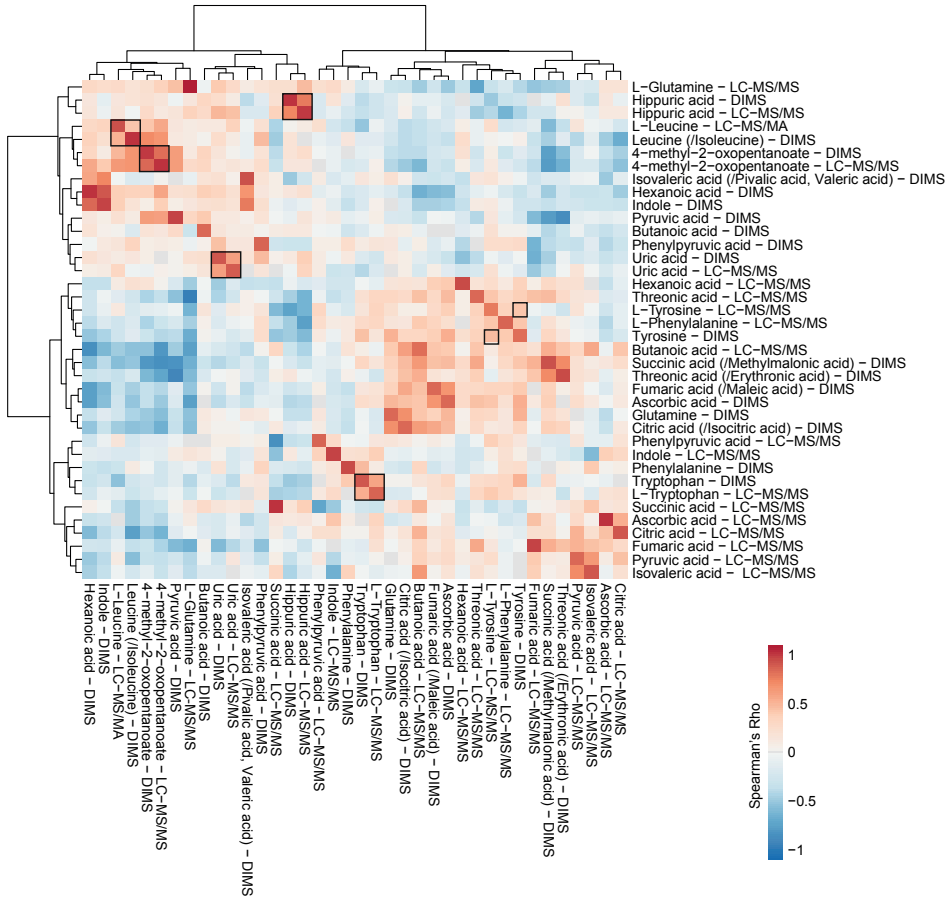
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Supplementary Figure 1. Principal component analysis (PCA) of aqueous humor metabolome by DIMS-I identify four outliers. PCA plots summarize the metabolic composition for each aqueous humor sample in comparison to the other samples. PCA represents is a low-dimensional (i.e. 2 dimensions) overview of multivariate data. This overview may reveal the relationships between samples, groups of samples, and may identify outliers. Samples close to each other have a similar intraocular metabolome, whereas those far from each other are dissimilar with respect to the composition of metabolites in aqueous humor. Left panel: principal component analysis (PCA) of 147 annotated peaks in Direct Infusion Mass Spectrometry (DIMS) analysis from the discovery cohort of all patients, including cataract patients. Four samples (idiopathic AAU n=2, cataract n=2) can be seen with strong deviating coefficients for the first two components which are considered technical outliers and excluded from further analysis. Right panel: PCA of same data after exclusion of the four outliers shows a distinct, but also overlapping, metabolic signature for HLA-B27 associated acute anterior uveitis.



Supplementary Figure 2. Principal component analysis of metabolite profile of DIMS-I, DIMS-II, and LC-MS/MS. The samples from HLA-B27 associated anterior uveitis patients are indicated in red samples from the idiopathic acute anterior uveitis patients are indicated in blue and non-inflammatory cataract patients are indicated in green. The explained variances of the principal components (PC) are shown in brackets. Direct comparison of the metabolic profiles by principal component analysis revealed no consistent separate clusters for any of the groups which most likely can be attributed to the relatively large within-group variation (compared to between-group variation). (DIMS-1/LC-MS/MS; ankylosing spondylitis n=2, psoriasis n=1. DIMS-2; ankylosing spondylitis n=4).
Abbreviations: B27-AAU: HLA-B27 associated acute anterior uveitis, Idiopathic-AAU: idiopathic acute anterior uveitis, DIMS: Direct Infusion Mass Spectrometry, LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry.



Supplementary Figure 3. Correlation of metabolites identified by DIMS and LC-MS/MS in aqueous humor samples of the same patients. Colors represent Spearman's rho from blue (rho=-1.0) to red (rho=1.0). Nineteen metabolites were identified in DIMS-1 and LC-MS/MS in the same patient-samples. Of these, six correlated significantly (p< , marked by boxes in the heatmap). Clustering was performed with the ClustVis server using Eucledian distance and Ward linkage. Dendrograms for columns and rows indicate the identified clusters.

Supplementary Table 1. The identified (putative) metabolites using DIMS and LC-MS/MS in aqueous humor samples of HLA-B27 positive and negative (idiopathic) acute anterior uveitis patients.

Putative metabolite	And/or	DIMS-1		DIMS-2		LC-MS/MS	
		p-value	VIP	p-value	VIP	p-value	VIP
1.3-Dehydroquinic acid		0.56	0.77	0.18	0.83	-	-
2-Deoxytetronic acid		0.33	0.40	**0.02**	**1.07**	-	-
2-Hydroxyadipic acid		0.80	**1.06**	0.44	0.42	-	-
2-Ketoisovaleric acid		0.22	0.42	0.85	**1.56**	-	-
2-Propylglutaric acid	Suberic acid	-	-	0.90	**1.14**	-	-
3-4-Dihydroxycyclohexa-1-5-diene-1-carboxylic acid		0.65	**1.06**	0.52	0.83	-	-
3-4-dihydroxy-L-Phenylalanine		-	-	-	-	0.78	**1.05**
3-Dehydroshikimic acid		0.75	0.26	0.48	**1.25**	-	-
3-Hydroxydodecanedioic acid		0.90	0.58	-	-	-	-
3-Methyladipic acid	Pimelicacid	**<0.05**	**1.32**	0.90	0.66	-	-
3-OH-Anthranilic acid		0.18	0.42	0.61	**1.08**	-	-
4-Hydroxybenzoic acid	3-Hydroxybenzoic acid	0.22	0.50	0.90	0.57	-	-
4-Hydroxyphenylacetic acid		**0.01**	0.46	0.05	0.73	-	-
4-Hydroxyphenylpyruvic acid		**<0.05**	**1.27**	0.44	0.72	-	-
5-Hydroxyhexanoic acid		0.06	0.74	0.70	**1.38**	-	-
5-Hydroxyindoleacetic acid		0.08	**1.31**	0.90	0.86	-	-
5-Hydroxymethyl-2-furoic acid		0.75	0.27	0.75	0.62	-	-
5-Hydroxytryptophan		0.07	0.19	-	-	-	-
6-deoxy-5-ketofructose1-P		0.07	**1.90**	-	-	-	-
7-Hydroxyoctanoic acid		**0.003**	0.93	0.70	0.94	-	-
Acetoacetic acid		0.25	0.96	0.85	0.80	-	-
Acetylglycine		0.37	0.58	0.40	0.46	-	-
Aconitic acid		0.56	0.79	0.25	**1.23**	-	-
Adipic acid (Hexanedioic acid)	3-Methylglutaric acid	0.22	0.78	0.08	0.76	-	-
Alanine	Sarcosine	0.33	0.25	-	-	-	-
Allantoin		**0.01**	**1.18**	0.14	**1.29**	-	-
Alpha-amino-beta-carboxy-muconic epsilon-semialdehyde		**0.02**	**1.31**	-	-	-	-

Alpha-Ketoglutaric acid	Oxoglutaric acid	0.80	0.73	**0.008**	**1.61**	-	-
Alpha-ketooctanoic acid		**0.01**	**1.81**	0.30	0.85	-	-
Aminoadipic acid		0.16	0.80	0.70	0.54	-	-
Aminolevulinic acid		0.65	0.50	0.70	0.98	-	-
Aminophenol		0.95	**1.09**	0.18	**1.23**	-	-
Anthranilic acid		**0.005**	0.38	0.80	**1.54**	-	-
Arabinose		0.18	0.78	0.52	**1.09**	-	-
Arginine		0.44	0.67	0.90	0.80	-	-
Ascorbic acid (Ascorbate)		0.12	**1.60**	**0.004**	**1.82**	**0.04**	**1.46**
Aspartic acid		0.33	0.99	0.37	0.83	-	-
Azelaic acid		**0.001**	**1.05**	0.48	**1.01**	-	-
Benzoic acid		-	-	1.0.	0.87	0.10	**1.16**
Butyric acid (Butanoic acid)		0.08	0.92	0.48	0.89	0.12	**1.13**
Butyrylglycine		**0.01**	**1.88**	-	-	-	-
Capric acid (Decanoic acid)		-	-	-	-	0.55	**1.04**
Caproic acid (Hexanoic acid)		**0.002**	**2.09**	0.25	0.86	0.19	0.61
Carnitine		0.07	**1.42**	0.61	0.76	-	-
Catechol		-	-	0.90	0.91	-	-
Chorismic acid		-	-	0.52	0.77	-	-
Citric acid	Isocitric acid	1.0.	0.26	**<0.05**	0.96	0.10	**1.45**
Citrulline		0.30	0.69	-	-	-	-
Cortisol		-	-	-	-	0.14	0.69
Creatine		0.16	**1.28**	**0.02**	**>1.00**	-	-
Creatinine		0.75	0.29	-	-	-	-
Cysteine		0.05	**1.02**	-	-	-	-
Cysteinyl Glycine		0.14	0.74	-	-	-	-
Cystine		0.18	**1.20**	1.0.	0.62	-	-
Diacetyl		-	-	-	-	0.74	**1.18**
Dihydroxybutyric acid		0.33	0.75	0.90	0.67	-	-
Furoic acid		0.52	0.80	>0.05	**1.05**	-	-
Gamma-Aminobutyric acid (GABA)	Dimethylglycine	**0.001**	0.82	0.52	0.76	-	-

Galactitol		0.90	0.71	0.61	0.86	-	-
Galactose-1 phosphate	6P-Glucose or Fructose-6P or Fructose-1P	0.95	0.78	0.08	0.86	-	-
Gentisic acid		0.95	0.93	0.37	0.84	-	-
Gluconic acid	Galactonic acid	0.37	**1.62**	0.85	0.81	-	-
Glutaconic acid		0.12	**1.12**	**0.02**	**1.28**	-	-
Glutamic acid		0.33	0.69	0.95	0.96	-	-
Glutamine		0.65	0.85	**0.02**	0.71	0.13	0.96
Glutaric acid	Ethylmalonic acid or Methylsuccinic acid	0.12	**1.18**	0.14	0.98	-	-
Glyceraldehyde-3P		0.09	0.88	0.06	**1.09**	-	-
Glyceric acid		0.70	0.56	0.95	0.81	-	-
Glycerol		**0.02**	**1.05**	-	-	-	-
Glycine		0.08	0.79	0.09	**1.06**	-	-
Glycolic acid		0.80	0.40	0.56	0.96	-	-
Glycylproline (Gly-Pro)		**0.002**	**1.97**	-	-	-	-
Heptadecanoic acid		-	-	**0.01**	**1.36**	-	-
Hexanoylglycine		0.56	0.24	0.65	0.97	-	-
Hexenedioic acid	Methylglutaconic acid	0.80	0.73	0.44	**1.16**	-	-
Hexose	Galactose / Glucose /Fructose	0.75	0.45	0.80	0.63	-	-
Hippuric acid		0.65	0.84	0.65	0.91	0.56	0.50
Histidine		0.75	0.40	-	-	-	-
Homovanillic acid	4-Hydroxyphenyllactic acid	0.22	0.52	0.80	0.82	-	-
Hydroxybutyric acid	Hydroxyisobutyric acid	0.18	0.86	0.65	**1.12**	-	-
Hydroxydecenoic acid		0.08	0.48	-	-	-	-
Hydroxyglutaric acid		0.37	0.76	**0.02**	**1.10**	-	-
Hydroxyhippuric acid		**<0.05**	0.67	0.07	0.74	-	-
Hydroxyisovaleric acid		**0.02**	**1.24**	0.95	0.80	-	-
Hydroxyproline	Propionylglycine	0.11	**1.35**	0.90	0.91	-	-
Hydroxypyruvic acid		-	-	-	-	0.06	0.82
Hydroxysebacic acid		0.56	**1.01**	0.11	0.20	-	-
Hydroxysuberic acid		**0.02**	**1.63**	0.08	0.85	-	-
Indole		**0.001**	**1.74**	0.95	**1.24**	0.84	0.64

Indole-3-acetic acid		**0.001**	0.91	0.70	**1.00**	-	-
Indole-3-glycerol phosphate	(3-Indolyl)-glycerol phosphate	0.12	**1.02**	-	-	-	-
Isovalerylglycine	2-Methylbutyrylglycine	0.80	0.92	**0.04**	**1.19**	-	-
Ketoleucine (4-methyl-2-oxopentanoate)	3-methyl-2-oxopentanoate	0.09	**1.30**	0.22	**1.23**	**0.006**	**1.54**
Lactic acid	Glyceraldehyde	0.06	**1.25**	-	-	-	-
L-aspartate 4-semialdehyde		**0.02**	**1.37**	-	-	-	-
Leucine	Isoleucine	0.09	**1.34**	-	-	**0.02**	**1.23**
Linoleic acid		-	-	0.20	**1.12**	-	-
Lysine		0.27	0.58	-	-	-	-
LysoPC(16:0)	LysoPE(19:0)	-	-	0.56	**1.23**	-	-
LysoPE(21:0)	LysoPC(18:0)	-	-	0.16	**1.26**	-	-
LysoPC(18:1)	LysoPE(21:1)	-	-	0.09	**1.35**	-	-
Fumaric acid	Maleic acid	0.25	0.55	**0.03**	**1.26**	**0.04**	0.56
Malic acid		**<0.05**	0.70	0.11	**1.10**	-	-
Malonic acid		0.37	0.57	0.05	**1.14**	-	-
Malonylcarnitine		0.40	0.36	-	-	-	-
Methionine		0.09	0.57	-	-	-	-
Methioninesulfoxide		**0.01**	0.74	0.61	0.48	-	-
Methoxytyrosine	Methyldopa	-	-	0.27	**1.23**	-	-
Methylcitric acid		0.12	**1.41**	**0.01**	**1.25**	-	-
Methylhistidine		0.07	**1.03**	-	-	-	-
Mevalonic acid		0.12	0.67	0.44	0.99	-	-
N-Acetylaspartic acid		0.06	0.91	0.09	**1.14**	-	-
N-Acetylhexosamine		0.65	0.29	0.56	0.75	-	-
N-Formylkynurenine		-	-	0.85	**1.01**	-	-
N-Nitrosopyrrolidine		0.75	0.79	-	-	-	-
Oacetylserine		0.65	0.50	0.70	0.98	-	-
Octanoic acid		-	-	-	-	0.46	0.51
Octenedioic acid		0.95	0.35	0.25	0.69	-	-
Oleic acid		0.27	0.74	-	-	-	-
Ornithine		**0.01**	**1.75**	-	-	-	-

Oxaloacetic acid		0.75	0.46	-	-	-	-
Oxoadipic acid		0.07	**1.25**	0.37	0.91	-	-
Palmitic acid (hexadecanoic acid)		0.30	0.56	0.22	**1.25**	-	-
Pantothenic acid		0.44	0.35	0.90	**1.01**	-	-
Phenol		0.25	0.59	-	-	-	-
Phenylacetic acid		0.09	**>1.00**	0.11	0.71	-	-
Phenylalanine		0.11	0.33	1.0.	**1.11**	0.95	0.51
Phenyllactic acid		0.40	<1.00	0.75	0.81	-	-
Phenylpyruvic acid		0.12	0.40	0.40	0.85	0.32	0.87
Phosphoethanolamine		0.61	0.52	0.11	**1.06**	-	-
Phosphoric acid		0.22	0.37	0.95	0.70	-	-
Proline		**0.007**	**1.48**	0.85	**1.06**	-	-
Propionic acid		0.70	0.64	0.33	**1.03**	-	-
Protocatechuate		0.52	0.75	0.61	0.87	-	-
Pyridoxal		0.14	0.29	0.61	**1.32**	-	-
Pyridoxamine		**<0.05**	**1.62**	-	-	-	-
Pyroglutamic acid		0.22	0.71	0.37	0.73	-	-
Pyruvic acid		>0.05	**1.12**	0.20	**1.30**	0.16	**1.54**
Quinic acid		0.07	0.43	0.25	0.56	-	-
Quinolinic acid		-	-	0.33	0.81	-	-
Ribulose5PRibose5P		0.70	0.56	0.95	0.89	-	-
Sebacic acid		0.18	0.87	0.44	**1.06**	-	-
Sedoheptulose		0.48	**1.02**	0.07	**1.61**	-	-
Serine		0.56	0.81	**0.01**	0.98	-	-
Shikimic acid		0.44	0.90	0.08	**1.38**	-	-
Stearic acid		0.85	0.78	0.33	0.82	-	-
Suberylglycine		**0.03**	**1.47**	-	-	-	-
Succinic acid	Methylmalonic acid	**0.004**	**1.31**	0.12	0.95	0.73	0.61
Succinylacetone		**0.02**	0.93	**0.04**	0.67	-	-
Sulfuric acid		0.20	0.37	-	-	-	-
Taurine		-		0.27	0.88	-	-

Threonic acid	**Erythronic acid**	0.05	**1.26**	0.16	0.83	0.55	0.79
Tiglylglycine	**3-Methylcrotonylglycine**	**0.005**	**2.12**	-	-	-	-
Trimethyllysine		0.09	**1.45**	-	-	-	-
Tryptamine						0.28	0.67
Tryptophan		0.18	0.71	0.27	0.42	0.84	**1.14**
Tyramine						0.56	0.39
Tyrosine		0.37	**1.42**	0.44	0.32	0.39	0.74
Uracil		-	-	0.08	**1.47**	-	-
Urea-1-carboxylate		**0.008**	**1.98**	-	-	-	-
Uric acid		**0.01**	**1.04**	0.85	**1.35**	>0.05	**1.66**
Uridine		0.18	0.93	0.44	0.73	-	-
Urocanic acid		0.56	0.93	0.70	**1.33**	-	-
(Iso-) Valeric acid	**Pivalic acid**	**0.006**	0.95	0.25	0.80	0.24	<1.00
Valine	**Betaine**	0.06	**1.92**	0.65	0.73	-	-
Vanillic acid		0.95	0.43	0.48	0.67	-	-
Vanilmandelic acid		0.08	**1.08**	-	-	-	-
Xanthine		0.85	0.34	0.48	0.95	-	-
Xylulose-5P		0.11	**1.14**	0.07	0.71	-	-

Direct-infusion high-resolution mass spectrometry (DIMS), Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS); From the PLS-DA analysis the Variable Importance in Projection (VIP) value was obtained for each metabolite. A variable with a VIP Score >1 can be considered important in driving the projection used to summarize the PLS-DA model in which the groups are optimally separated.


6

Chapter 6

SYSTEMIC INFLAMMATORY IMMUNE SIGNATURES IN A PATIENT WITH CRB1 LINKED RETINAL DYSTROPHY

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ABSTRACT

PURPOSE: In this report we describe, for the first time, the activation of the peripheral immune compartment in a patient with a ***CRB1*** linked retinal degenerative disease, masquerading as intermediate uveitis.

METHODS: To monitor the immune system during systemic immunosuppressive treatment, given for the initial diagnosis of intermediate uveitis, blood samples were taken before and during therapy, for analysis of peripheral blood mononuclear cell-subsets and circulating immune mediators, by multi-color flow cytometry and multiplex immunoassay.

RESULTS: The levels of various pro-inflammatory immune mediators (including MIF, TSLP, CCL2/MCP-1, CXCL9, CXCL10, IFN-β, IL-6, IL-17, IL-21, IL-22, and IL-23) were elevated in serum at the first time point, and decreased under immunosuppressive treatment. In parallel, the frequency of activated (CD86+) CD1c+ myeloid dendritic cells in blood was proportional to the central foveal thickness measured by optical coherence tomography.

CONCLUSION: These observations challenge the current view on the distinct pathophysiology of retinal degenerative and retinal inflammatory conditions in this patient with CRB1 linked retinal dystrophy.

INTRODUCTION

Retinal dystrophies are a heterogeneous group of severe inherited retinal diseases characterized by progressive deteriorating of photoreceptors that are visually debilitating and potentially blinding. One of more than 200 genes linked to retinal dystrophies is the retinal gene ***Crumbs homologue 1 (CRB1)*** that encodes a protein critical to canonical retinal development.[1] Over 150 genetic mutations in ***CRB1*** have been reported in patients with retinal dystrophies. The clinical manifestation of retinal dystrophies linked to mutations in sequence of the ***CRB1*** gene may vary considerably, and includes conditions such as retinitis pigmentosa, Leber congenital amaurosis, and cone-rod dystrophy.[2] Although the ***CRB1***-related disease spectrum hallmarks fundus specific patterns and retinal dysfunction typical to 'degenerative' conditions, recently, Hettinga et al.[3] described several patients with among others ***CRB1*** related retinal dystrophy masquerading as intraocular inflammation. Here, we performed longitudinal immunophenotyping in the blood of one of the patients from this series, and report on hitherto unknown activation of various pro-inflammatory pathways and immune cells in blood.

CASE DESCRIPTION

A fourteen year old girl, without medical history, was referred to our tertiary hospital with visual complaints including blurred vision and floaters. At presentation the best corrected visual acuity (BCVA) was 20/32 and 20/25 (Snellen equivalent) for the right and left eye respectively. Ophthalmological examination revealed inflammation of the vitreous, cystoid macular edema (CME) and multifocal chorioditis-like lesions, indicative for intraocular inflammation (**Figure 1A-B**). Screening by a pediatric rheumatologist and clinical immunologist did not detect any underlying systemic disease. Diagnostic screening included extensive laboratory testing (including full blood count, CRP, blood sedimentation rate, ACE, ANA, HLA typing, antibody titers for various infectious agents, liver and kidney function tests), urine screening for proteins, tuberculin skin test for tuberculosis, X-ray and CT imaging of the lungs, and MRI imaging of the brain. The patient was initially diagnosed with idiopathic intermediate uveitis, which was supported by a second opinion at another tertiary referral center. Consequently, she was treated with several local and systemic anti-inflammatory and immune-modulatory agents, including systemic corticosteroids, methotrexate, mycofenolate mofetil, tumor necrosis factor (TNF)-α inhibitors, and eventually tocilizumab. All therapies were equally ineffective in controlling disease. During tocilizumab therapy (the period described in this study), BCVA of both eyes increased slightly from 20/50 to 20/40 (Snellen equivalent) for the right eye, and from 20/66 to 20/33 for the left eye. However, though improvements in visual acuity were seen, the overall clinical picture did not show any permanent improvement, and

visual acuity continued to decrease. Subsequent genetic testing revealed a heterozygous mutation in the ***CRB1*** gene and the diagnosis of retinal dystrophy was established.

METHODS

During this period we monitored the circulating immune compartment of this patient. After informed consent, blood was drawn before the start of tocilizumab therapy (timepoint I), and after 2, 5, and 6 months (timepoints II-IV respectively). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation and analyzed by multi-color flow cytometry (Fortessa, BD Biosciences). PBMCs were directly stained with fluorochrome conjugated antibodies to analyze myeloid dendritic cells (anti-CD3-PerCP-CY5.5, anti-CD19 -PerCP-CY5.5, and anti-CD56- PerCP-CY5.5, anti-HLA-DR-PB, anti-CD16-BV500, anti-CD14-APC-H7, anti-CD141-PE, anti-CD303-FITC, anti-CD86-PE-Cy7, anti-CD1c-APC (BD Biosciences)). Acquired data were analyzed using FlowJo software (FlowJo, LLC). Forty-six immune mediators were simultaneously measured in undiluted serum using our in-house developed and validated (ISO9001 certified) multiplex immunoassay based on Luminex technology.[4]

Table 1. Cytokine analysis in serum of a patient with retinal dystrophy [a]

Immune mediator	Timepoint I (pg/mL)	Timepoint II (pg/mL)	Timepoint III (pg/mL)	Timepoint IV (pg/mL)
IL-1β	110.4	112.9	<	<
IL-6	39.5	64.9	4.0	4.2
IL-13	552.2	569.3	46.1	35.8
IL-17	815.6	865.0	<	<
IL-21	57060.4	67640.9	353.6	256.9
IL-22	337.2	356.7	<	<
IL-23	53.0	7.8	<	<
IL-29	345.6	286.5	<	<
TNF-α	7.1	11.5	<	<
TNF-β	53.8	46.0	<	<
IFN-α	16.9	15.8	<	<
IFN-β[b]	857.1	1339.7	10.9	6.1
INF-γ	134.7	118.9	<	<
MIF	15529.3	17133.9	144.3	213.3
TSLP	193.9	248.6	0.9	0.5
CCL2 / MCP-1	702.9	691.5	35.0	45.5
CCL25 / TECK	1232.5	1347.3	107.0	111.7
CXCL8 / IL-8	111.4	109.4	11.1	9.9
CXCL9 / MIG	5077.2	6155.2	31.9	25.1
CXCL10 / IP-10	3387.9	3352.5	155.6	147.4
CXCL13 / BLC	779.1	908.3	26.5	30.2

[a] Selection of serum cytokines showing largest decrease or direct relation to other cytokine pathways. [b] U/mL. Values below detection limit are indicated with '<'.

RESULTS

The levels of various pro-inflammatory immune mediators (including Macrophage Migration Inhibitory Factor (MIF), Thymic Stromal Lymphopoietin (TSLP), chemokine CC ligand-2 (CCL2) (also known as monocyte chemoattractant protein-1, MCP-1), CXCL9 and CXCL10, interferon-beta (IFN-β) and Interleukin (IL)-6, IL-17, IL-21, IL-22, IL-23) were elevated in serum at the first time point and decreased in subsequent time points (see also **Table 1**). In parallel, the frequency of activated (as indicated by CD86+) CD1c+ myeloid dendritic cells in blood was proportional to the central foveal thickness measured by optical coherence tomography (**Figure 1C**).

DISCUSSION

Retinal dystrophies comprise a group of progressive retinal disorders frequently caused by mutations in the ***CRB1*** gene.[2] Studies of the CRB1 gene revealed its critical role in retinal development and homeostasis and an extensive line of evidence demonstrates that genetic disruption of the function of this retinal gene results in the death of photoreceptors and progressive deterioration of retinal function with associated vision loss.[1] Consequently, efforts for better understanding the CRB1 linked degenerative disease are mostly focused on studying the molecular implications of genetic mutations on retinal dysfunction. However, endogenous or environmental factors outside the retina may also play a significant role in modulating the phenotype in patients, complicating its diagnosis, and potentially alter its clinical evolution.

Indeed, recent studies have revealed circumstantial evidence that suggests a contributing role of inflammatory pathways in retinal dystrophies: Patients with RD may manifest with inflammation of the vitreous, and show elevated levels of pro-inflammatory cytokines and chemokines in ocular fluids. Curiously, as described for ocular inflammatory diseases, patients with retinal dystrophies may show peripheral immune responses toward retinal antigens – indicating co-occurring and possible harmful autoimmune mechanisms directed against the retina.[5–7]

In this study, we further substantiate this concept and describe additional hitherto unknown inflammatory changes that can be detected in the blood of such a patient. Most strikingly, cytokines such as IL-17, and IL-23, which are hallmark cytokines for T-helper 17 cells, a subset of T cells that are considered to play a central role in autoimmune uveitis[8], were elevated in blood, and decreased after immunosuppressive/modulatory treatment that included tocilizumab. In addition, activated CD1c+ myeloid dendritic cell frequency was proportional to the degree of foveal thickness, which makes it tempting to suggest a role for this cell subset in the development of CME in this patient. Interestingly, this immune

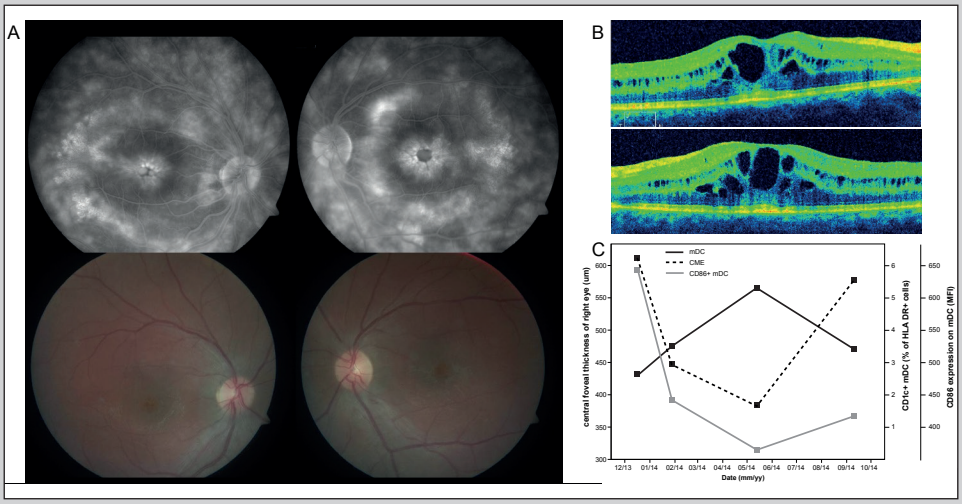


Figure 1. Cystoid macular edema at several time points during immune-monitoring. **A.** Upper left and right: fluorescein angiography of the right and left eye of an patient with retinal dystrophy at presentation revealing extensive fluorescein leakage in the macular area. Lower left en right: fundoscopy color images of the right and left eye of the same patient at presentation. **B.** Optical Coherence Tomography images of the macula of the right (upper image) and left (lower image) eye at presentation revealing CME. **C.** Dynamics of foveal thickness (measured by OCT), the frequency of CD1c+ mDC cells in blood and their activation status (measured by expression of activation marker CD86) through time. **Abbreviations;** CME: Cystoid Macular Edema, mDC: myeloid dendritic cell, MFI: mean fluorescence intensity, OCT: Optical Coherence Tomography.

cell was recently linked to disease activity in non-infectious uveitis commonly associated with macular edema.[9,10] These findings support a role for several inflammatory factors in the pathogenesis of retinal dystrophies. The inflammatory signatures may perhaps be the result of a (secondary) immune response directed against the damaged ocular tissues. The exact mechanism remains poorly understood, however, pro-inflammatory cytokines and chemokines produced by activated retina resident microglia,[11–14] Müller cells, and the retinal pigment epithelium (RPE) have been implicated.[15] Whether the activation of microglia is induced by photoreceptor cell death, or actually causes this photoreceptor cell death remains to be determined[12], however, accumulating evidence supports the concept that activated microglia play an active role in photoreceptor cell death through phagocytosis and the production of pro-inflammatory mediators.[13,14]

Although the influence of inflammation on the natural course of disease remains to be elucidated, the here described case reveals that various inflammatory signatures are well observed at the clinical and molecular levels, which is a thought-provoking observation that makes it tempting to speculate on its implications for the care of this subgroup of patients. Considering it is a primary ocular genetic defect, anti-inflammatory therapy

is currently not indicated for retinal dystrophy, because it will not resolve the cause of disease. However, the emerging role of inflammation in retinal dystrophies may provide opportunities for influencing the natural course and secondary complications of this group of diseases. Although we are at the dawn of gene therapy to correct the genetic defect in retinal dystrophies, currently, gene therapy is not yet widely available. In contrast, various potent immunomodulatory agents are available that could potentially be used to influence the inflammatory responses to prevent secondary complications (macular edema) to delay vision loss and improve quality of life. Research in this area, which is currently lacking, is likely to receive more attention in the near future.[16,17] In the past, treatment with intravitreal corticosteroid has been applied for treatment of secondary macular edema in Retinitis Pigmentosa patients with mixed results.[18,19]. It is important to note that the temporary improvements in visual function in the case described here may also be the reflection of the natural disease course, and further studies are necessary to further elucidate the contribution of inflammation on the clinical course of CRB1 linked retinal dystrophies.

In summary, these observations substantiate that in our patient with ***CRB1*** linked retinal dystrophy masquerading as intraocular inflammation, the disease is accompanied by molecular activation of inflammatory cytokine pathways and immune cells in the blood. As such, these results challenge the current view on the distinct pathophysiology of retinal degenerative and retinal inflammatory conditions in this patient.

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7

Chapter 7

A DISEASE-ASSOCIATED microRNA CLUSTER LINKS INFLAMMATORY PATHWAYS AND AN ALTERED COMPOSITION OF LEUKOCYTE SUBSETS TO NON-INFECTIOUS UVEITIS

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ABSTRACT

PURPOSE: The cause of non-infectious uveitis (NIU) is poorly understood, but is considered to be mediated by a complex interplay between genetic, environmental and – relatively unexplored – epigenetic factors. MicroRNAs (miRNAs) are non-coding small RNAs that are important epigenetic regulators implicated in pathological signaling. Therefore, we mapped the circulating miRNA-ome of NIU and implement miRNA perturbations within the broader context of the immune system.

METHODS: We designed a strategy to robustly identify changes in the miRNA profiles of two 2 independent cohorts totaling 54 untreated patients with active and eye-restricted disease and 26 age-matched controls. High-resolution miRNA-ome data were obtained by TaqMan OpenArray technology and subsequent RT-qPCR. Flow cytometry data, and proteomic data spanning the cellular immune system were used to map the uveitis-miRNA signature to changes in the composition of specific leukocyte subsets in blood.

RESULTS: Using stringent selection criteria, we identified and independently validated a miRNA cluster that is associated with NIU. Pathway enrichment analysis for genes targeted by this cluster revealed significant enrichment for the PI3K/Akt, MAPK, FOXO and VEGF signaling pathways, and photoreceptor development. In addition, unsupervised multi-domain analyses linked the presence of the uveitis-associated miRNA cluster to a different composition of leukocyte subsets, more specifically CD16+CD11c+HLA-DR- cells.

CONCLUSION: Together, this study identifies a unique miRNA cluster associated with non-infectious uveitis that was related to changes in leukocyte subsets demonstrating systemic changes in epigenetic regulation underlying non-infectious uveitis.

INTRODUCTION

Non-infectious uveitis (NIU) comprises a heterogeneous group of recurrent or chronic sight-threatening intraocular inflammations that eventually lead to permanent visual impairment or blindness in up to 19% of patients.[1–4] Since NIU affects more than 1 out of every 1000 individuals and usually demands treatment for decades the clinical and economic impact of NIU is enormous.[5–7]

NIU is well acknowledged as an immune-mediated disease. This is based upon (genetic) association with numerous immune-related molecules, the frequent occurrence of uveitis in relation to systemic inflammatory conditions, and the beneficial response to immunosuppressive therapy.[8–12] Despite our growing understanding that susceptibility to NIU is in part genetic, and epigenetic modulation contributes to various ocular conditions, studies investigating the epigenetic landscape of uveitis are scarce.[13–15]

One of the mechanisms through which epigenetic regulation of gene expression takes place is through microRNAs.[16] MicroRNAs (miRNAs) are small RNAs abundantly found in almost all biological tissues. MiRNAs interfere with the translation of messenger RNA (mRNA) of more than halve of the protein-coding genome and consequently orchestrate complex biological circuits including immunity.[17–20] Although miRNA-binding to mRNA generally has a modest effect on protein expression, changes in the expression of miRNAs can have dramatic and widespread impact on cellular signaling.[21] As a result, a large number of profiling studies have been conducted to find changes in the levels of miRNAs that could potentially be used to diagnose or monitor disease or provide novel therapeutic targets for treatment.[22,23] But despite their potential miRNA profiling studies are particularly prone to low reproducibility and have hitherto delivered little clinical utility.[24,25] This can in part be attributed to experimental design (e.g. validation via independent cohorts). In addition, functional understanding of the implications of changes in miRNA levels are usually confined to knockdown and/or overexpression studies of only one or a few targets in single (non-primary) cell types.[26,27]

To overcome the aforementioned limitations, we designed a strategy to robustly identify differences in the miRNA profile of the serum of patients with NIU.

MATERIALS AND METHODS

Patients and patient material

We collected blood from a total of 54 adult patients with one of three archetypical types of non-infectious uveitis: HLA-B27-associated Acute Anterior Uveitis (AU), Idiopathic Intermediate Uveitis (IU) or Birdshot Uveitis (BU). Patients were seen at the outbound

patient clinic of the department of Ophthalmology of the University Medical Center Utrecht between July 2014 and December 2016. Patients were divided between a discovery cohort (AU n=9, IU n=9, BU n=10) and a replication cohort (AU n=10, IU n=6, BU n=10). All patients had active uveitis (new onset or relapse) at the time of sampling. None of the patients had a related systemic auto-inflammatory or autoimmune disease, nor did they receive systemic immunomodulatory treatment in the last 3 months, other than a low dose of oral prednisolone (≤10mg, n=1). Uveitis was classified and graded in accordance with the Standardization of Uveitis Nomenclature (SUN) classification.[28] For detailed information on the clinical work-up see **Supplementary Methods**. Twenty-six age and gender matched anonymous blood donors with no history of ocular inflammatory disease (UMC Utrecht) served as unaffected controls (HC). This study was conducted in compliance with the Helsinki principles. Ethical approval was requested and obtained from the Medical Ethical Research Committee in Utrecht and all patients signed written informed consent before participation.

Table 1. Characteristics of the discovery and replication cohorts investigated in this study

Discovery cohort	**AU**	**IU**	**BU**	**HC**	**p-value**
N	9	9	10	16	n.a.
Male / Female	3/6	2/7	4/6	6/10	0.90*
Age in years; mean ± SD	47.7 ± 17.0	39.3 ± 14.0	52.9 ± 13.2	41.4 ± 9.8	0.09**
Disease duration in years; median (range)	5.8 (0.1-39.3)	3.7 (0.2-20.0)	1.3 (0.2-15.1)	n.a.	0.14***
Replication cohort	**AU**	**IU**	**BU**	**HC**	**p-value**
N	10	6	10	10	n.a.
Male / Female	2/8	3/3	6/4	4/6	0.39*
Age in years; mean ± SD	45.9 ± 16.1	31.7 ± 10.5	45.8 ± 12.2	41.5 ± 14.0	0.19**
Disease duration in years; median (range)	8.1 (0.2-22.3)	4.9 (0.4-14.1)	0.9 (0.2-33.2)	n.a.	0.41***

Abbreviations: BU: Birdshot uveitis, AU: HLA-B27 associated anterior uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, n.a.: not applicable. * Fisher's exact test, ** ANOVA, *** Kruskal-Wallis.

MicroRNA profiling - OpenArray

Total RNA was extracted from 200 µL serum using Exiqon's miRCURY™ RNA Isolation Kit for biofluids (Exiqon), according to the manufacturer's instructions. RNA extraction was performed for all serum samples on the same day and the order of samples randomized according to previous recommendations.[29] Because there are currently no universally applicable endogenous serum control miRNAs and to facilitate reproducibility (standardized controls) we used non-human miRNA (ath-MiR-159a as a spike-in control for normalization.[30] More details on RNA isolation are provided in the **Supplementary Methods**.

We screened for 758 miRNAs in the serum of the discovery cohort using the ***TaqMan*** OpenArray platform (Thermo Fisher) according to the manufacturer's instructions (see also **Supplementary Methods**). The resulting expression levels, given in Cycle threshold (Crt-) values of all miRNAs, were normalized by subtraction of the mean Crt-value of the spike-in, resulting in a deltaCrt ($\Delta Crt = Crt_{\text{mean target}} - Crt_{\text{mean miR-159a}}$). Differences in miRNA expression levels between patients and controls were assessed comparing these ΔCrt values of patients to healthy controls, using the comparative threshold cycle method.[31] In short: expression levels are presented as the Fold Change ($FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Crt = \Delta Crt_{\text{patient}} - \Delta Crt_{\text{reference}}$) compared to the healthy control that represented the median of the spike-in - and was set to 1(FC). MiRNAs were selected for validation if they were well expressed (mean Crt<27, amplification score of >1.24), in >90% of all samples, with FC of ≥ 2 or <0.5, and a ***p***-value of <0.05.

Validation of miRNAs - TaqMan single RT-qPCR

We performed ***TaqMan*** single quantitative reverse transcription PCR (RT-qPCR) (see also **Supplementary Methods**) for ten miRNAs that passed the selection criteria, on the same samples from the discovery cohort. The Ct values from the TaqMan assay were compared with the Crt values from the OpenArray platform. MiRNAs were considered technically validated when Spearman's Rho was >0.5 and ***p***<0.05. All validated miRNAs were tested in a second, independent replication cohort (n=36) for biological validation.

Statistical Approaches

The ***OpenArray*** output was analyzed using the ***Thermo Fisher Cloud*** software, which follows an independent samples ***t*** test to compare ΔΔC(r)t data with a ***p***-value threshold below 0.05 (2-tailed). To facilitate discovery of potentially meaningful mediators, we applied correction for multiple testing only to the combined data set (discovery + replication cohort), which was obtained following a more robust three-staged strategy of discovery, validation, and replication using independent technologies and cohorts. In the combined cohorts, non-parametric tests (Mann Whitney U test, Kruskal-Wallis with post-hoc Dunn's with adjusted p values, Spearman's rho) were used to compare groups or test for correlations. Selected individual miRNAs were subjected to ***receiver operating characteristic*** (ROC) curves using the FC values. Details on the association between miRNAs (ΔΔCt) and clinical characteristics are described in **Supplementary Methods.** To correct for multiple testing, a ***p***-value of <0.0055 (0.05/9 clinical parameters) was considered significant. The FC values (or Singular Value Decomposition (SVD) imputed values, max 10%) for miRNAs that met the amplification and expression criteria were subjected to unsupervised hierarchical clustering (Euclidian distance, Ward's linkage

method). Clustering was performed with ClustVis or MetaboAnalyst.[32,33] Statistical analyses were performed in SPSS version 21.0 (SPSS Inc., Chicago IL), GraphPad Prism (GraphPad, La Jolla, CA, USA), MetaboAnalyst Server v3.0, and R v3.3.2.

Target analysis

Predicted and reported mRNA targets of the validated miRNAs were mapped using miRGATE[34] and MirTargetLink[35]. Pathway enrichment analysis was performed for Gene Ontology (GO) biological processes[36], Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[37] and Pathway Ontology (PW) pathways[38] on all gene targets that were targeted by at least two miRNAs, using ToppGene Suite (BMI CCHMC, Cincinnati, OH)[39]. To visualize shared pathways, unscaled FDR corrected ***p***-values for pathways related to inflammation and eye biology were outlined in a heatmap generated by Clustvis software.[32]

Leukocyte cell subset microRNA and Proteome analyses

Global MicroRNA expression data for 9 primary leukocyte populations were derived from four non-coding RNA microarray data sets available via the Gene Expression Omnibus (see **Supplementary Methods**). Normalized miRNA profiles (n=825) for these leukocyte subsets were subjected to hierarchical clustering or principle component analysis to interrogate global miRNA expression profiles (MetaboAnalyst 3.0).[33] Mass-spectrometry-based proteomics data for the 9 primary leukocyte subsets (162 samples) was used to obtain the cell-specific protein expression data.[40] For detailed information see **Supplementary Methods**.

Flow cytometry analysis of lymphoid and myeloid populations

Peripheral blood mononuclear cells (PBMCs) from 30 patients and 15 healthy controls were obtained by ficol gradient centrifugation and stored at -80C. Thawed PBMCs were stained with antibodies listed in **Supplementary Table 1**, randomized for measurement by BD LSR Fortessa™ Cell analyzer (BD Bioscience). Samples were grouped according to low miR-233-3p versus high miR-233-3p levels (cut-off < FC 1.29 ≥ to median of unaffected controls, **Supplementary Table 2**). To minimize technical variability inherent to manual gating, we performed unsupervised hierarchical clustering using Citrus[41] on 50,000 random events from pre-gated (**Supplementary Figure 1**) viable single cells exported from Flow Jo Software for each sample with a 1% minimum cluster size to reduce granularity and arcsin hyperbolic transformation value at 200. In short, Citrus applies an unsupervised clustering algorithm to map the hierarchy of phenotypically related cell clusters which results in a so called "tree". This is followed by a supervised classification model to highlight stratifying clusters for pre-defined conditions (high versus

low miRNA levels). Cells were clustered based on the expression of lineage (CD3/CD56/CD19), CD14, CD1c, HLA-DR, CD123, CD11c, CD141, and CD303 proteins. Since we investigated groups with unequal numbers of samples, we explored the abundance of cell populations using the Significance Analysis of Microarrays (SAM) with significance inferred for false discovery rate <1%.

RESULTS

High-throughput miRNA profiling reveals 10 differentially expressed miRNAs

We devised a three-staged strategy with stringent selection criteria to identify differently expressed miRNAs in serum of non-infectious uveitis (NIU) patients (**Supplementary Figure 2**). The demographic data of the NIU patients and controls from the discovery cohort and the replication cohort are shown in **Table 1**. First, we screened for 758 circulating miRNAs in 28 patients and 16 healthy controls (discovery cohort) using a high-throughput real-time PCR platform (*OpenArray™*).

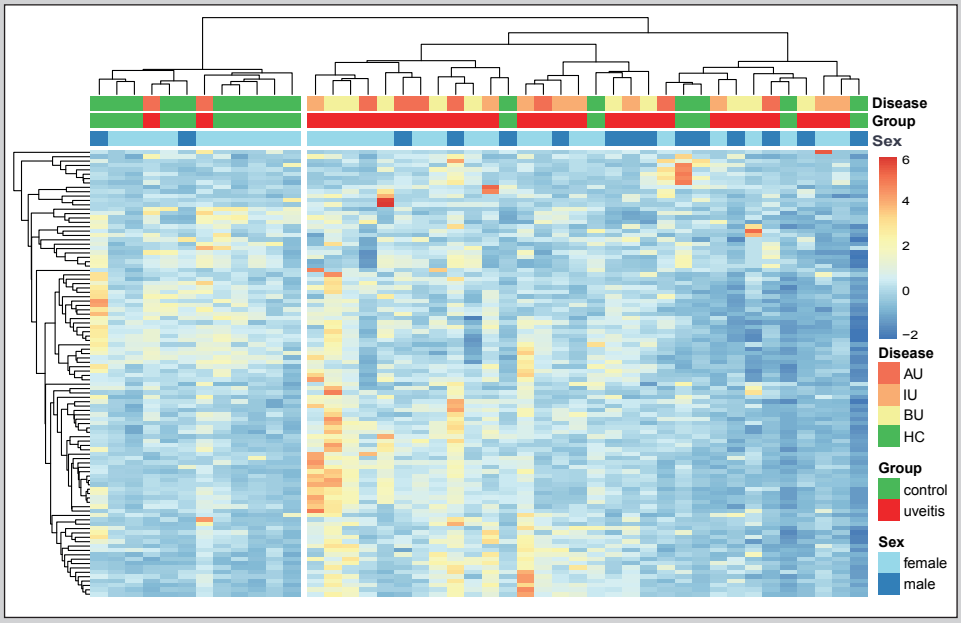


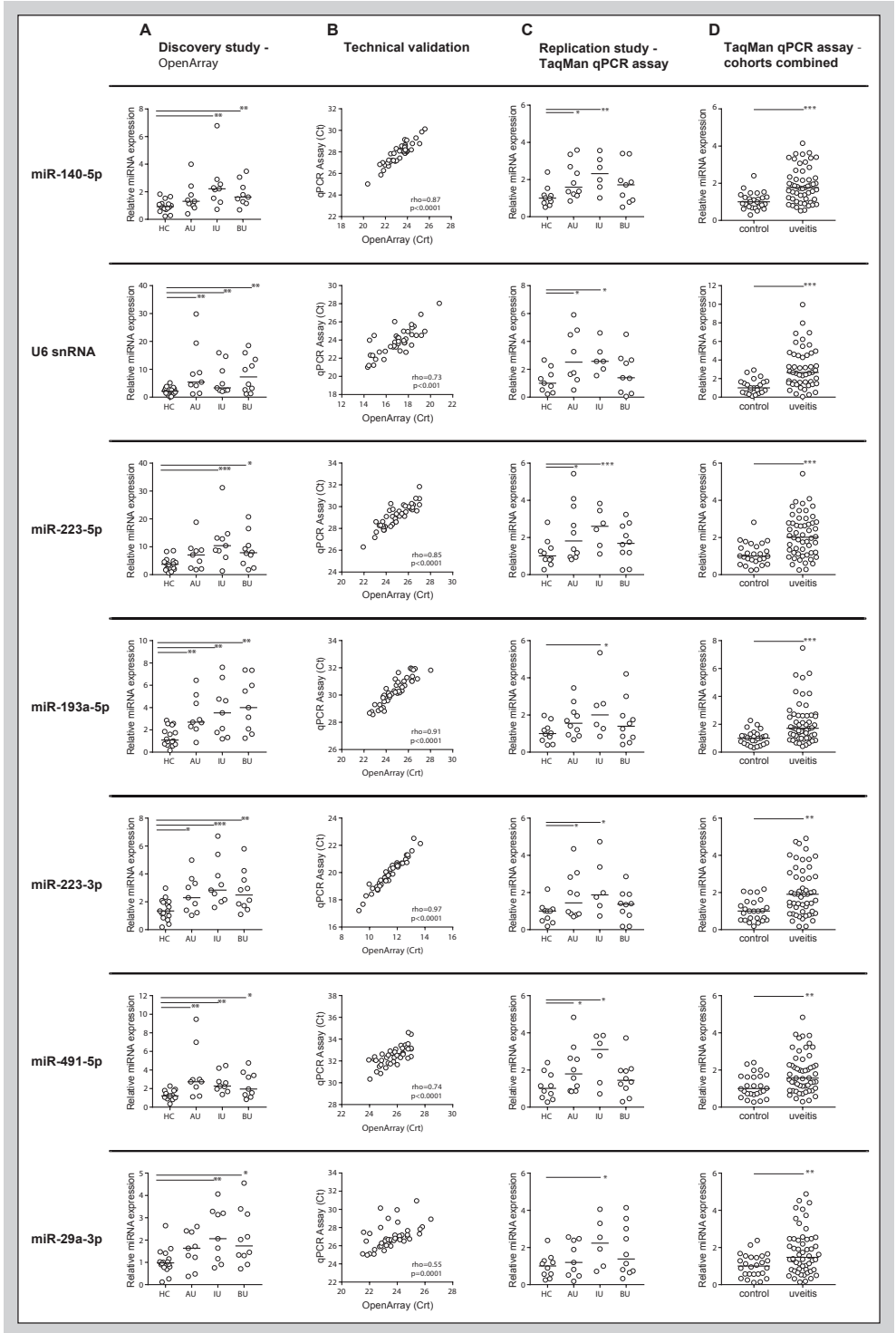
Figure 1. Unsupervised hierarchical clustering of the serum miRNA-ome of the discovery cohort. Heatmap of unsupervised hierarchical clustering based on 102 detected miRNAs that met quality control thresholds from the discovery cohort (screening study). Depicted are the transformed fold change (FC) values (see methods) from patients with HLA-B27 associated uveitis (AU), Idiopathic Intermediate uveitis (IU), Birdshot uveitis (BU) and healthy controls (HC). Unit variance scaling is applied to rows. Heatmap colors represent the fold changes in a color-coded way: blue (low) to red (high). Clustering was performed with ClustVis using correlation distance and ward linkage and is depicted as dendrograms for columns and rows.

A total of 102 miRNAs passed quality control (**Supplementary Table 3**). We could distinguish patients from controls based on the overall miRNA profile (**Figure 1**), which exhibited mostly increased levels for the majority of detected miRNAs (**Supplementary Figure 3**). To aid in the elimination of background variation and improve reproducibility of our findings, we maintained a stringent two-fold change cut-off and $p<0.05$ for significance. This approach yielded six miRNAs (miR-140-5p, miR-491-5p, miR-223-3p, miR-223-5p, miR-193a-5p and miR-29a-3p) and a small nuclear RNA (U6 snRNA) that were upregulated in one or more patient groups compared to healthy controls (**Supplementary Table 4 & Supplementary Figure 3**). Between the uveitis groups, three miRNAs (miR-127-3p, miR-375, miR-409-3p) were differentially expressed (**Supplementary Table 4 & Supplementary Figure 3**), resulting in a total of 10 miRNAs that were selected for replication. High-throughput miRNA profiling technologies are inherently prone to detect certain miRNAs over others.[42,43] We performed several investigations that showed no apparent detection bias and thus we consider the detected miRNA profile a genuine representative sample of the blood borne miRNA-ome of non-infectious uveitis (see **Supplementary Figure 4**).

Replication of seven serum miRNAs in an independent cohort

We next technically validated the miRNAs from the discovery cohort using RT-qPCR. The expression levels for 9/10 miRNAs demonstrated strong correlation (rho 0.55-0.97, all $p \leq 0.0001$) between these technologies and were thus considered technically validated (**Figure 2 & Supplementary Table 4**). Next, we investigated the levels of these 9 miRNAs in an independent cohort of 26 patients and 10 controls (**Table 1**). Biological replication was achieved for six miRNAs (miR-140-5p, miR-193a-5p, miR-223-3p, miR-223-5p, miR-29a-3p, miR-491-5p) and U6 snRNA in at least one of the uveitis disease groups versus

Figure 2. Discovery, validation and replication of seven miRNAs increased in serum of patients with non-infectious uveitis. From left to right: **A.** Results from OpenArray in Discovery cohort (n=44). On the y-axis are the relative expression levels (i.e. the transformed fold change (FC) values (see methods)) shown for the patients compared to the mean expression of healthy controls. P-values are from independent T test. **B.** Correlation between expression levels for selected miRNAs obtained by OpenArray and TaqMan single RT qPCR assay. Spearman's Rho is used to test for correlation. **C.** Results from TaqMan single RT qPCR assay in the replication cohort (n=36). On the y-axis are the relative expression levels (i.e. the transformed fold change (FC) values (see methods)) shown for the patients compared to the mean expression of healthy controls. P-values are from independent samples T test. **D.** Results from TaqMan single RT-qPCR assay of both cohorts combined (n=80) and stratified for uveitis patients and healthy controls. P-values are from Mann Whitney U (uveitis vs control). **Abbreviations:** HC: Healthy Control, AU: HLA-B27-associated anterior uveitis, IU: Idiopathic Intermediate Uveitis, miR: microRNA.
* p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001


A
Discovery study - OpenArray
B
Technical validation
C
Replication study - TaqMan qPCR assay
D
TaqMan qPCR assay - cohorts combined
miR-140-5p
U6 snRNA
miR-223-5p
miR-193a-5p
miR-223-3p
miR-491-5p
miR-29a-3p
Relative miRNA expression
qPCR Assay (Ct)
OpenArray (Crt)
HC
AU
IU
BU
control
uveitis
rho=0.87 p<0.0001
rho=0.73 p<0.001
rho=0.85 p<0.0001
rho=0.91 p<0.0001
rho=0.97 p<0.0001
rho=0.74 p<0.0001
rho=0.55 p<0.0001

healthy controls (**Figure 2 & Supplementary Table 4**). All were significantly higher expressed in uveitis patients compared to the controls (**Figure 2D**). Since an increase in the levels of the replicated miRNAs was observed for all uveitis subtypes we combined the *Taqman* RT-qPCR data from both cohorts to investigate the discriminative power of the uveitis associated cluster. The analysis of the area under the receiver operating characteristic curve revealed that these miRNAs displayed relatively good specificity and sensitivity (**Supplementary Table 2**). We observed no statistically significant correlations between the levels of selected miRNAs and age, gender, lymphocyte and leukocyte count, development of ocular complications, or need for treatment with systemic medication. Interestingly, we did find an inverse correlation between disease duration and the serum levels of U6 snRNA, which was seemingly driven by AU patients (**Supplementary Figure 5**).

Uveitis miRNA-cluster targets inflammatory and ocular biology pathways

To aid in the understanding of the widespread downstream effects of the identified miRNAs in non-infectious uveitis, we narrowed down relevant disease pathways by selecting genes targeted by at least two of the uveitis-miRNAs using *miRTargetLink*.[35] Together, 6 miRNAs shared 37 gene targets (**Figure 3A**). Pathway enrichment analysis (see **Methods**) for these overlapping gene targets revealed significant (FDR corrected $p<0.05$) enrichment for inflammatory and ocular biology pathways (**Figure 3B**).

The non-infectious uveitis specific miRNA cluster is associated with altered frequencies of CD16$^+$ leukocytes

The expression levels of the seven miRNAs were strongly correlated with each other and therefore further considered as a single uveitis-associated miRNA cluster (**Figure 3C and Supplementary Figure 6**). We hypothesized that the uveitis-associated miRNA cluster can reflect specific changes in leukocyte populations.[44–47] To investigate this, we first used non-coding RNA array data (825 overlapping miRNAs) across 113 samples of nine primary leukocyte populations - covering most cell populations of the immune system. Consistent with literature, the derived miRNA-ome was highly cell-type specific (**Figure 4A**). Principle component analysis clearly distinguished a lymphoid cluster (T cells, B cells, NK cells) and two myeloid clusters (**Figure 4B**). Among the major miRNAs contributing to the clustering of these populations were miR-223-3p and miR-29a-3p (**Figure 4B and Supplementary Figure 7**). The expression of the other uveitis-associated miRNAs also varied considerably between the populations (**Supplementary Figure 8**). Using correlation, we mapped the uveitis miRNAs to remote clusters of expression across the leukocyte populations (**Supplementary Figure 9**). To verify whether the distinguished

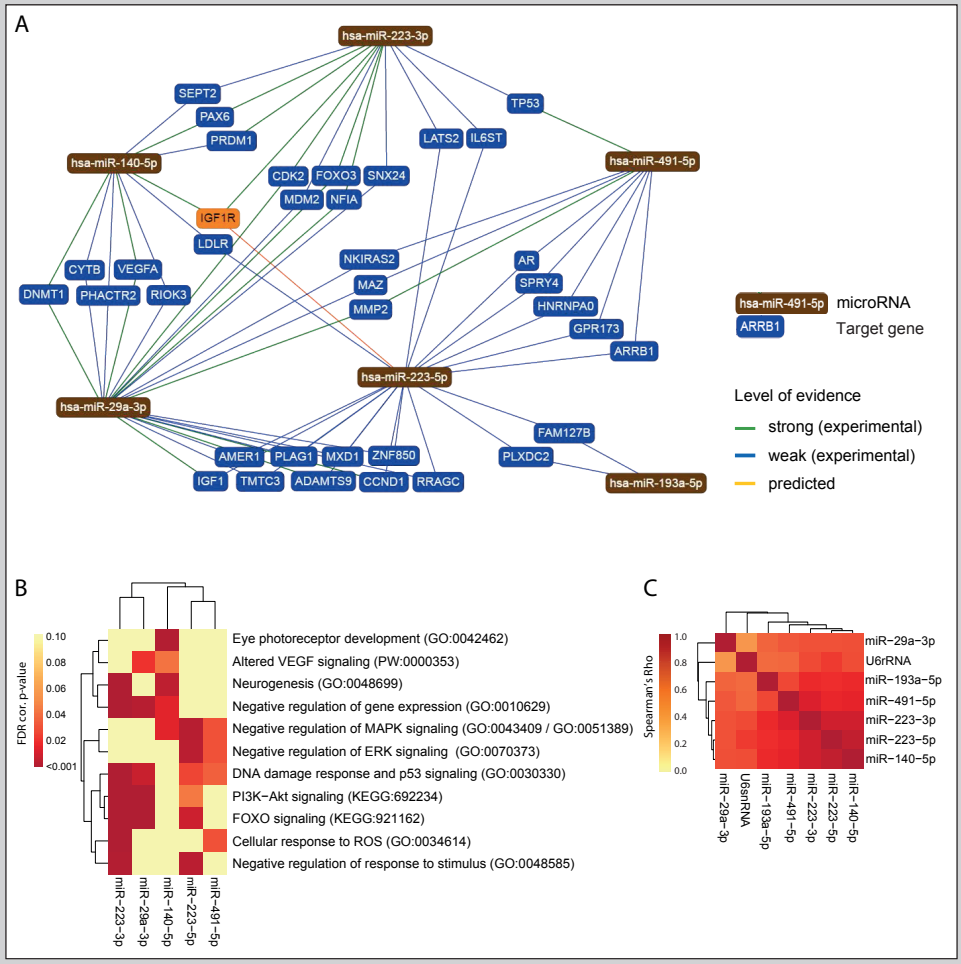


Figure 3. miRNA-Target analysis of the validated serum microRNAs in non-infectious uveitis**. A.** miRNA-target network of established overlapping mRNA targets of the uveitis-associated miRNAs. MiRNAs are depicted in brown boxes and the mRNA-targets are depicted in blue (targeted by two miRNAs) or orange (targeted by three miRNAs) boxes. Lines indicate level of evidence: strong (green), weak (blue) or predicted (yellow). Figure generated with MirTargetLink **B.** Heatmap of unscaled FDR corrected p-values for miRNA-target pathways related to inflammation and eye biology. Pathway analysis (ToppGene suite[39]) was performed on mRNA targets that were shared between at least 2 miRNAs. Clustering was performed using Euclidean distance and Ward linkage **C.** Heatmap of unsupervised hierarchical clustering of the correlation of expression (TaqMan RT-qPCR for the discovery and replication cohorts combined) for validated serum cluster. Heatmap colors represent Spearman's Rho (calculated on ddCT). **Abbreviations:** DNA: DeoxyriboNucleic Acid, ERK: Extracellular Regulated Kinase, FoxO: Forkhead Box O, MAPK: Mitogen-Activated Protein Kinase, miR: microRNA, PI3K: phosphatidylinositol 3-kinase, ROS: Reactive Oxygen Species, VEGF: Vascular Endothelial Growth Factor.

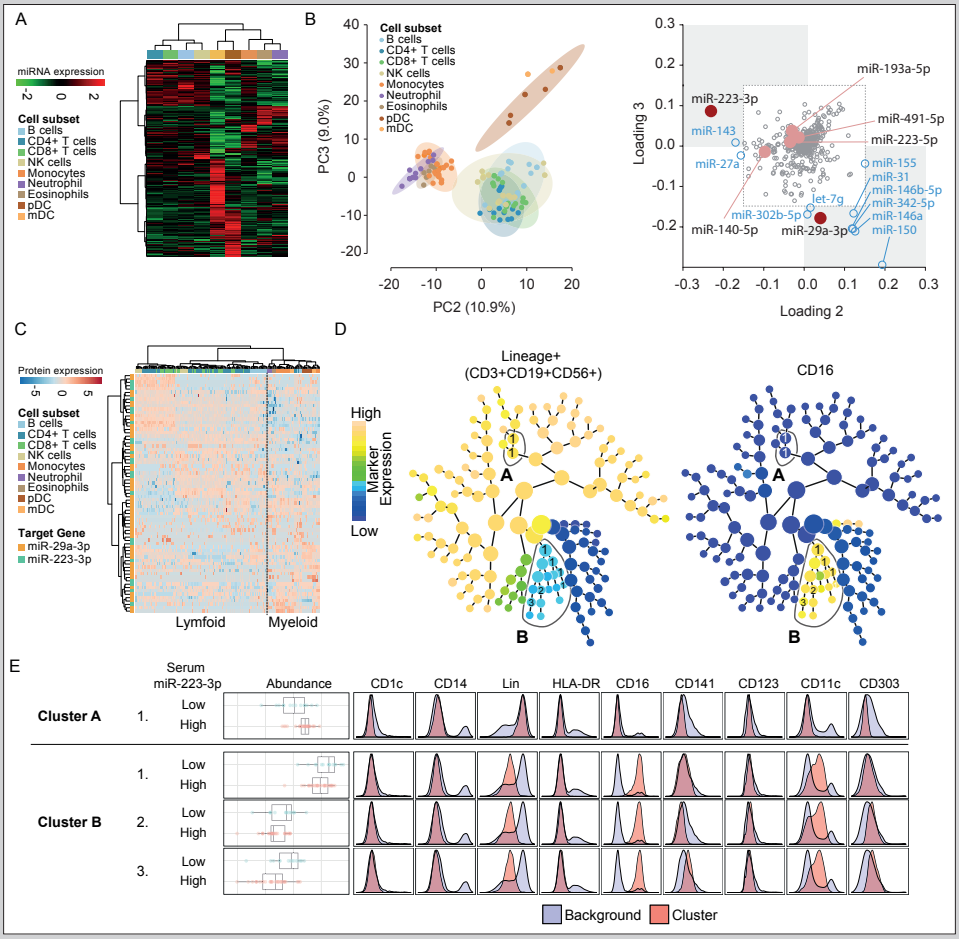


Figure 4. Meta-analysis of the leukocyte miRNA-ome links the uveitis-associated cluster to leukocyte composition in blood. **A.** Meta-analysis of the cellular miRNA-ome (n=825) of nine primary leukocyte populations (n=113). Indicated is the average relative cellular miRNA expression for each investigated cell subset. Ward's method of hierarchical clustering of miRNA data was performed with Pearson correlation distance. **B.** Principle component analysis (PCA) of the cellular miRNA-ome of the leukocyte subsets. Three populations are clearly distinguishable by the second and third principal components. Loading plot of PCA shows that miR-29a-3p and miR-223-3p (dark red) are among the top miRNAs (>1.5 loading score, light blue) that contribute to these clusters. The relative position of the other uveitis-associated miRNAs is indicated. **C.** Unsupervised hierarchical clustering of proteomic data of validated target genes (n=74, Fig. S9 and Supplementary Table 4) for miR-29a-3p and miR-223-3p in nine leukocyte cell subsets (see methods) distinguishes lymphocyte and myeloid populations. **D.** Citrus tree of identified common cell populations (>1% of sample) in blood that distinguish individuals with relatively high levels of the uveitis-associated miRNA cluster from individuals with relatively lower levels in serum. Contours indicate clusters (A and B) with a significantly different abundance in individuals with relatively higher miRNA levels (Significance Analysis of Microarrays (SAM), by FDR of 1%). Expression of CD16 and lineage (CD3/CD19/CD56) marker are indicated in color scale. High-dimensional phenotype for each cluster is outlined in **Supplementary Figure 10**.

(Legend Figure 4 continued) **E.** Histogram plots of the relative expression of each cell surface marker by the identified clusters (in red) compared to the overall expression of all other clusters (blue). The relative abundance of the identified clusters in individuals with high/low levels of the uveitis miRNA cluster are indicated.

expression of the miR-29a-3p and miR-223-3p across the leukocyte subsets reflects meaningful down-stream biological differences, we mined mass-spectrometry based proteomic data of the investigated cell subsets (n=162) for the expression of validated target genes (**Supplementary Table 5 & Supplementary Figure 10**). Unsupervised hierarchical clustering of the miRNA-target proteome of miR-29a-3p and miR-223-3p clearly discerned the myeloid and lymphoid cell subsets (**Figure 4C**).

Finally, we wished to explore whether the miRNA cluster tagged changes in specific leukocyte subsets in uveitis patients. To this aim, we exploited available flow cytometry data from 30 NIU patients and 15 controls from this study(see **Supplementary Methods** and **Supplementary Table 2**). Unbiased computational mining of the flow cytometry data by Citrus identified two cell clusters that displayed significantly changed cell abundance according to the expression of the miRNA signature. These included a cluster of lymphocytes (***Cluster A*** in **Figure 4D-E** and **Supplementary Figure 11**) and a cluster of populations characterized by high CD16 expression, dim expression of lineage marker (lin^{dim}) and CD11c, and low levels of HLA-DR and the monocyte marker CD14 (Cluster B1-3, **Supplementary Figure 11**), reminiscent of CD16+ $CD56^{dim}$ Natural Killer (NK) cells.[48] This CD16+ cluster also contained populations with low, but distinguished expression of CD141 and CD303, most likely residual granulocyte populations (Cluster B2 and B3, **Figure 4D-E**).[49,50] These observations link a disease-associated circulating miRNA cluster with marked changes in the leukocyte repertoire in the blood of patients with non-infectious uveitis.

DISCUSSION

Using a high dimensional and multi-level approach we identified and independently validated the presence of a uveitis-specific miRNA cluster. This uveitis cluster comprised six miRNAs and a small nuclear RNA that suggest the involvement of several inflammatory signaling cascades (PI3K/Akt, MAPK, FOXO and VEGF signaling) involved in several eye diseases. In addition, our work shows improved power to validate (70% of selected miRNAs were replicated) as well as to understand the potential role of circulating miRNAs in pathological conditions without the necessity to have large cohorts.

Non-infectious Uveitis (NIU) denotes a collective of clinically heterogeneous intra-ocular inflammatory diseases that share immune characteristics with – and commonly form an underappreciated feature of – systemic (auto) inflammatory conditions.[10,51–53] This led us to deliberately study two cohorts (discovery and replication cohort) of treatment-free patients with eye-restricted and active disease. This allowed us to better address the impact of intra-ocular inflammation on miRNAs in the circulation. To the best of our knowledge, this resulted in the first miRNA investigation of NIU patients without underlying (or potentially confounding) systemic disease.[54–56]

The main aim of the study was to explore the circulating miRNA profile of NIU to better understand its enigmatic etiology and lay the groundwork for emerging non-coding RNA-based therapeutics to reverse NIU.[57] Yet, a major obstacle for bringing small RNAs into consideration for therapeutic targeting is the notoriously low concordance and reproducibility between available technologies for detecting differential expression, which consequently results in conflicting reports on candidate identification. Although recommendations for quality control[58] have improved reproducibility, we also invested additional efforts in assembling an independent replication cohort to be able to identify a robust set of uveitis-associated miRNAs.

The quest for understanding the downstream effects of serum miRNA perturbations is a highly ambiguous one. Individual miRNAs regulate numerous genes while a single gene can be regulated by a panel of miRNAs.[17,59] Yet, most biological processes are tuned by the concerted action of multiple miRNAs targeting the same pathways.[18,19,60] Given the close correlation of miRNA levels, as well as overlapping predicted and experimentally validated pathways, it is tempting to speculate that the identified miRNA signature acts as a pathological ensemble driving - or responding to - eye inflammation. Functional experiments with appropriate multi-miRNA knock-out and conditional (over)expression models will be necessary to pin-point the functional consequences of our observations.
Several recent key studies highlight the importance of our observations. MiR-223-3p is upregulated in animal models of uveitis and is able to drive inflammation via T cells and myeloid dendritic cells (mDCs), two cell types that are implicated in the biology of uveitis.[10,61–65] Myeloid-derived mir-223 is critical to innate immunity during gut inflammation and skewed serum levels of this miRNA may hint towards a dysbiotic microbiome.[66–68] This is particularly interesting since commensal microbiota can trigger autoimmune uveitis in mice.[69] Consequently, miR-223-3p has been suggested as a good candidate for miRNA targeting in the treatment of inflammatory diseases such as NIU.[70]

We explored if the levels of the uveitis-associated signature were correlated with changes in specific blood cell populations of these same patients. We focused on leukocytes because previous studies have highlighted a predominant function of leukocytes in uveitis.[10] We realize that the flow cytometry panels used here may lack the resolution needed for an in-depth analysis of all leukocyte populations in blood (outside the scope of this study), yet, we were able to highlight several interesting changes; we identified a decreased frequency of a cell population distinguished by high CD16 expression. Based upon other cell surface makers ($CD11c^{+}HLA\text{-}DR^{-}CD14^{-}lin^{dim}$), this cluster most likely represents the cytotoxic $CD16^{+}CD56^{dim}$ NK cells, a cell type that plays a critical role in the first line of defense against infected, cancerous and auto-reactive cells. Also, this is the major NK subset in blood that typically express killer immuno-globulin-like receptors (KIRs), a receptor that is implicated in the biology of non-infectious uveitis.[48,71]
It is tempting to speculate that the increased frequency of the lymphocyte cluster represents $CD4^{+}$ T cells. We observed that the uveitis-associated miR-140-5p and mir-29a-3p are typically higher expressed in lymphocyte populations, including $CD4^{+}$ T cells. MiR-140-5p, which had the highest discriminative power between uveitis patients and controls, is aberrantly expressed and implicated in pathogenic T cell function in multiple sclerosis[72,73] , a disease linked to IU.[74–76] It is important to emphasize, however, that the functions of miR-140-5p (and miR-29a-3p) most likely expand beyond T cell function. For example, we also noted associated pathways relevant for ocular biology, including VEGF signaling[77–79], photoreceptor development and retina homeostasis.[80,81] These ocular biology pathways may attempt to control damage to ocular tissues.

U6 snRNA is a small nuclear RNA (snRNA) that is normally localized in the nucleus, but blood micro-vesicles or exosomes – which vastly increase in number in the circulation during inflammation[82] – are also particularly enriched for U6 nuclear RNA.[83,84] Curiously, U6 snRNA is widely used for normalization of miRNA expression studies, while we here show that the levels of this miRNA are robustly upregulated in the serum of uveitis patients.[85,86] This is also increasingly reported for other conditions.[87–90]

Our results have to be interpreted with the following study limitations in mind. First, although we believe that our stringent selection criteria contributed to the robust identification and replication of uveitis-associated miRNAs, it consequently also limited sample size and power to detect more subtle differences. It seems reasonable to speculate that this may have prevented the detection of a relation between the miRNAs levels and heterogeneous clinical end-points, and hampered the validation of differences between uveitis-subtypes. Overall, we would like to emphasize that the study of miRNAs in serum is

by no means exhaustive, and recapitulate that the identified miRNAs most likely function in clusters of functionally related mediators that orchestrate molecular route function into complex behavior of the immune system in uveitis. As such, the here identified miRNAs signature serves as an important starting point to further functionally dissect the epigenetic regulation by these miRNAs and their interaction within the cellular immune system of patients with NIU.

In conclusion, our data demonstrate systemic changes in epigenetic regulation that link serum miRNA levels to changes in leukocyte populations underlying inflammatory eye disease.

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SUPPLEMENTARY METHODS

Patients and patient material

We collected blood from a total of 54 adult patients with one of three archetypical types of noninfectious uveitis in the absence of systemic inflammatory disease: HLA-B27-associated Acute Anterior Uveitis (AU), Idiopathic Intermediate Uveitis (IU), or Birdshot Uveitis (BU). Patients were seen at the outbound patient clinic of the department of Ophthalmology of the University Medical Center Utrecht (UMCU) between July 2014 and December 2016. Patients were divided between two cohorts: a discovery cohort (AU n=9, IU n=9, BU n=10) and a replication cohort (AU n=10, IU n=6, BU n=10). All patients had active uveitis (new onset or relapse of disease) at the time of sampling. Patients were excluded if they had a systemic auto-inflammatory or autoimmune disease, or if they had received systemic immunomodulatory treatment in the last 3 months, other than a low dose of oral prednisolone (≤10mg) (only one (BU) patient had a recurrence of uveitis while receiving 10mg prednisolone. Other patients did not receive any systemic immunomodulatory treatment at time of sampling). To exclude ankylosing spondylitis all patients with clinical complaints were evaluated by a rheumatologist. Patients were excluded if there were signs of inflammation in the sacroiliac joints on X-Ray.

Uveitis was classified and graded in accordance with the Standardization of Uveitis Nomenclature (SUN) classification.[1] Each patient underwent a full ophthalmological examination by an experienced ophthalmologist, routine laboratory screening, and a chest X-Ray. Laboratory screening included erythrocyte sedimentation rate, renal and liver function tests, angiotensin converting enzyme (ACE), and screening for various infectious agents in the serum including syphilis, Lyme disease, and an Interferon-Gamma Release Assay (IGRA) tuberculosis test. HLA typing (HLA-B27 or HLA-A29) was performed in all patients to confirm AU or BU. Common viral causes were excluded by PCR and Goldmann-Witmer coefficients assessment of aqueous humor.

We compared the three uveitis groups with each other as well as with 26 age and gender matched seemingly healthy donors that had no history of ocular inflammatory disease. These healthy controls (HC) were acquired through an anonymous blood donating facility within the UMC Utrecht.This study was conducted in compliance with the Helsinki principles. Ethical approval was requested and obtained from the Medical Ethical Research Committee in Utrecht and all patients signed written informed consent before participation.

RNA isolation of serum miRNAs

After blood withdrawal, the clot activator tubes were laid to rest at a bench at room temperature for 30 minutes and spun down for 10 minutes at 2000g. Serum was obtained, snap frozen, and stored at -80°C immediately after acquisition until later use. We extracted RNA from 200 µL serum using Exiqon's miRCURY™ RNA Isolation Kit for biofluids (Exiqon), according to the manufacturer's instructions. In accordance with previous recommendations, RNA extraction was done for all samples on the same day and the order of samples was randomized to minimize potential confounding effects of the extraction method.[2]

RNA samples were spiked with ath-MiR-159a that served as a control miRNA and was used for technical normalization. Screening for miRNAs in the serum of the discovery cohort was performed using Taqman real-time PCR on the OpenArray platform (Thermo Fisher) according to the manufacturer's instructions. This method allows for the simultaneous analysis of 758 miRNAs split into two pools: pool A and B. Reverse transcription (RT) was performed on 2.5uL RNA using miRNA multiplex RT primers (v2.1 for Pool A or v3.0 for Pool B) and the TaqMan miRNA reverse transcription kit (Thermo Fisher). RT products were preamplified using the Megaplex™ PreAmp Primers, in the presence of the TaqMan PreAmp Master Mix (Thermo Fisher) using the following thermal cycler conditions: 10min, 95°C; 2min, 55°C; 2min, 72°C and 16 cycles of 15 sec, 95°C and 4min, 60°C followed by a single cycle 0f 10min, 96°C. The pre-amplified products were diluted 1:40 in 0.1xTE buffer pH 8.0 and subsequently 1:2 with TaqMan OpenArray Real-Time PCR Master Mix. The miRNA profiling was performed on the QuantStudio 12 K Flex Real-Time PCR System (Thermo Fisher).

Data were analyzed using ***Thermo Fisher Cloud*** software, v1.0 (www.thermofisher.com). The expression levels, given in Cycle threshold (Crt-) values of all miRNAs, were normalized by subtraction of the mean Crt-value of the spike-in miRNA ath-MiR-159a, resulting in a deltaCrt ($\Delta Crt = Crt_{\text{mean target}} - Crt_{\text{mean miR-159a}}$). Differences in miRNA expression levels between patients and controls were assessed comparing these ΔCrt values of patients to healthy controls, using the comparative threshold cycle method.[3] In short: expression levels are presented as the Fold Change ($FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Crt = \Delta Crt_{patient} - \Delta Crt_{reference}$) compared to the healthy control that represented the median of the spike-in - and was set to 1(FC). Individual samples with very low expression (Crt>27) were set to 27 and miRNAs that were detected at a mean Crt>27 were excluded altogether, under the assumption that not enough miRNA would be present for valid analysis above this threshold. Individual samples that had a FC>50 were excluded as outliers.

MiRNAs were selected for validation if they were well expressed (amplification score of >1.24), in >90% of all samples, with FC of ≥ 2 or <0.5, and a ***p***-value of <0.05.

Validation of miRNAs - TaqMan single RT-qPCR

To validate the obtained results from the profiling study we performed ***TaqMan*** single quantitative reverse transcription PCR (RT-qPCR) for twelve miRNAs identified on the OpenArray platform that passed the selection criteria, on the same samples. MiRNA-specific RT-qPCR assays were purchased from Thermo Fisher for hsa-miR-140-5p (ID: 001187), hsa-miR-223-3p (ID: 002295), hsa-miR-223-5p (ID: 002098), hsa-miR-29a-3p (ID: 002112), hsa-miR-491-5p (ID: 001630), U6 snRNA (ID: 001973), hsa-miR-193a-5p (ID: 002281), hsa-miR-127-3p (ID: 000452), hsa-miR-375-3p (ID: 000564), hsa-miR-409-3p (ID: 002332), HSA-MIR-1274B (ID: 002884), hsa-miR-345-5p (ID: 002186). The cDNA was synthesized from 2.5uL total RNA using individual miRNA-specific stem-loop primers according to manufacturer's protocol in the presence of 3.3 U/µl MultiScribe RT enzyme (Thermo Fischer). miRNA levels were quantified in duplicate, with TaqMan Fast Advance Master Mix and specific primers of the miRNA assay, using the Quantstudio 12 K Flex Real-Time PCR system. The Ct values from the TaqMan assay were compared with the Crt values from the OpenArray platform. miRNAs were considered technically validated when Spearman's Rho was >0.5 and ***p***<0.05. All validated miRNAs were tested in a second, independent replication cohort (n=36) for biological validation. RT-qPCR data were obtained as described above, with the exception that baseline threshold cycles (Ct) were used and that normalization was performed to the median of ΔCt values from the healthy control group per miRNA (discovery and replication cohort were separately normalized to attain ΔΔCt values).

Correlation of miRNAs with clinical parameters

We tested for association between miRNAs (ΔΔCt) and gender, age, disease duration, and the number of uveitis attacks (AU), leukocyte and lymphocyte count measured at the day of sampling (available for 16 patients (AU n=7, IU n=1, BU n=8)), development of glaucoma, cystoid macular edema (CME), or use of systemic immunosuppressive treatment after sampling. We maintained a minimum of 1 year follow-up after sampling (14 out of 54 patients had <1 year of follow-up after sampling and where excluded from this particular analysis). The median time since sampling was 2.2 years (range 1.1-2.8) for the 40 patients that fulfilled these criteria (from both cohorts). To correct for multiple testing, a ***p***-value of <0.0055 (0.05/9 clinical parameters) was considered significant.

Statistics

The miRNA data measured on the ***OpenArray*** were analyzed using the ***Thermo Fisher Cloud*** software, which follows an independent samples ***t*** test to compare ΔΔC(r)t data with a ***p***-value threshold below 0.05 (2-tailed). To facilitate discovery of potentially meaningful mediators by high-throughput technology in a relatively limited sample size, we applied correction for multiple testing only to the combined data set (discovery + replication cohort), which was obtained following a more robust three-staged strategy of discovery, validation, and replication using independent technologies and cohorts. In the combined cohorts, non-parametric tests (Mann Whitney U test, or Kruskal-Wallis with post-hoc Dunn's with correction for multiple testing) were used to compare group differences. To test for correlations Spearman's rho test was used. To demonstrate differentiating power of individual miRNAs ***receiver operating characteristic*** (ROC) curves were generated using the FC values. The FC values (or Singular Value Decomposition (SVD) imputed values, max 10%) for miRNAs that met the amplification and expression criteria were subjected to unsupervised hierarchical clustering (Euclidian distance measure and the Ward's linkage method). Clustering was performed in the ClustVis server (http://biit.cs.ut.ee/clustvis/) or the MetaboAnalyst server (http://www.metaboanalyst.ca/).[4,5] Statistical analyses were performed in SPSS version 21.0 (SPSS Inc., Chicago IL), GraphPad Prism (GraphPad, La Jolla, CA, USA), MetaboAnalyst Server v3.0, and R v3.3.2.

Target analysis

Predicted and reported mRNA targets of the validated miRNAs were mapped using miRGATE[6] and MirTargetLink[7]. We screened for overlap in gene targets between the miRNAs using MirTargetLink. To facilitate the interpretation of a list or gene targets and aid in understanding of the potential function of the miRNAs, we performed pathway enrichment analysis for Gene Ontology (GO) biological processes[8], Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[9] and Pathway Ontology (PW) pathways[10] on all potential targets that had overlap with at least one other miRNA, using ToppGene Suite (BMI CCHMC, Cincinnati, OH)[11]. To visualize shared pathways, unscaled FDR corrected ***p***-values for pathways related to inflammation and eye biology were outlined in a heatmap generated by Clustvis software.[4]

Leukocyte cell subset microRNA and Proteome analyses

Global MicroRNA expression data for 9 primary leukocyte populations were derived from four non-coding RNA microarray data sets available via the Gene Expression Omnibus public repository of the NCBI (accession no. GSE19183, GSE28487, GSE28489, GSE98830). Raw data scans (.CEL files) were read into R (R version 3.3.2). Samples were

pre-processed with Affy package version 3.3.1 for Affymetrix Multispecies miRNA-1 Array and Limma package version 3.3.3 for Agilent-021827 Human miRNA Microarray. A total of 825 overlapping (hsa-tagged) human micro-RNAs for 114 samples were pooled and quantile-normalized. A principle component analysis was performed to interrogate miRNA expression profiles for each of sample in MetaboAnalyst 3.0[5] with the exception of the first component to reduce technical variation and batch effect.[12] Validated gene targets of miR-29a-3p (n=80) and miR-223-3p (n=40) with strong experimental evidence were derived from interaction networks generated with mirTargetLink[7]. Mass-spectrometry-based proteomics data for each of 9 primary leukocyte subsets (total 162 samples) was used to obtain the cell-specific protein expression data (intensity data present in >50% samples available for 74 of the proteins of target genes of miR-29a-3p and miR-223-3p).[13] Selected proteome data for functionally distinct subsets (e.g. T regulatory or Th17 cells) were pooled within a major leukocyte population (e.g. CD4+) to match overall proteome of aforementioned non-coding RNA profiling studies. Unsupervised hierarchical clustering was performed on quantile normalized, log-transformed, auto-scaled proteome data using the MetaboAnalyst 3.0 server[5] and default elements enlarged for graphical purposes.

Flow cytometry analysis of lymphoid and myeloid populations

Peripheral blood mononuclear cells (PBMCs) from 30 patients and 15 healthy controls from patients in this study were obtained by ficol gradient centrifugation and stored at -80C. Thawed PBMCs were stained with antibodies listed in **Supplementary Table 1** and measured by BD LSR Fortessa™ Cell analyzer (BD Bioscience). Next, we compared all individuals with low miR-233-3p versus high miR-233-3p levels (cut-off < FC 1.29 ≥ to median of unaffected controls – see **Supplementary Table 2**).

We performed unsupervised hierarchical clustering using Citrus[14] on 50,000 random events from pre-gated (**Supplementary Figure 1**) viable single cells exported from Flow Jo Software from each patient and control sample with a 1% minimum cluster size to reduce granularity and arcsin hyperbolic transformation value at 200. Citrus applies an unsupervised clustering algorithm (Euclidean distance with Ward's linkage) to map the hierarchy of phenotypically related cell clusters followed by a supervised classification model to highlight stratifying clusters for pre-defined conditions (miRNA levels). Cells were clustered based on the expression of lineage (CD3/CD56/CD19), CD14, CD1c, HLA-DR, CD123, CD11c, CD141, and CD303 proteins. Since we investigated groups with unequal numbers of samples, we explored the abundance of cell populations using the Significance Analysis of Microarrays (SAM) with significance inferred for false discovery rate <1%.

Supplementary Table 1. Antibody panel used for Flow Cytometry staining of Peripheral Blood Mononuclear Cells

Chanel	Marker	Clone	Company
FITC	CD123	7G3	BD Pharmingen
APC	CD1c	AD5-8E7	Miltenyi
AF700	CD3 /CD19/CD56	UCHT1 / HIB19 / B159	BioLegend / eBioscience / BD Pharmingen
eFluor780	CD14	61D3	eBioscience
BV421	HLA-DR	L243	BioLegend
BV510	CD16	3G8	BD Horizon
BV711	CD141	1A4	BD Horizon
PE	CD169	7-239	eBioscience
PE-Cy7	CD303	201A	BioLegend
PE-CF594	CD11c	B-ly6	BD Horizon

Supplementary Table 2. Receiver Operating Characteristic analysis on the seven differentially expressed serum miRNAs

miRNA	AUC	95%CI	Cutoff (FC)	Sensitivity	Specificity
U6 rRNA	0.82	0.73-0.91	1.52	0.78	0.73
miR-140-5p	0.79	0.69-0.88	1.48	0.65	0.85
miR-193a-5p	0.78	0.68-0.87	1.31	0.67	0.81
miR-223-5p	0.78	0.66-0.87	1.71	0.61	0.85
miR-223-3p	0.74	0.63-0.85	1.29	0.69	0.73
miR-491-5p	0.70	0.57-0.81	1.16	0.70	0.65
miR-29a-3p	0.69	0.58-0.79	1.29	0.61	0.69

Abbreviations: 95% CI: 95% confidence interval, AUC: area under the curve, FC: Fold Change, miR: microRNA

Supplementary Table 3. 102 identified[a] microRNAs in the serum of non-infectious uveitis patients

			HC		AU		IU		BU	
Assay ID	miRNA - Assay ID	miRNA - miRBase ID	Mean ΔCrt (SE)	FC (95% CI)	Mean ΔCrt (SE)	FC (95% CI)	Mean ΔCrt (SE)	FC (95% CI)	Mean ΔCrt (SE)	FC (95% CI)
000379	hsa-let-7c	hsa-let-7c-5p	13.80 (0.14)	1.00 (0.81-1.23)	13.82 (0.15)	0.99 (0.79-1.25)	13.98 (0.20)	0.89 (0.64-1.23)	13.41 (0.22)	1.31 (0.93-1.87)
000387	hsa-miR-10a	hsa-miR-10a-5p	13.34 (0.22)	1.00 (0.72-1.39)	13.37 (0.25)	0.98 (0.66-1.46)	13.84 (0.20)	0.71 (0.51-0.98)	13.29 (0.25)	1.04 (0.69-1.56)
000390	hsa-miR-15b	hsa-miR-15b-5p	8.66 (0.23)	1.00 (0.71-1.40)	8.46 (0.14)	1.14 (0.91-1.43)	7.97 (0.18)	1.61 (1.20-2.16)	8.40 (0.26)	1.20 (0.79-1.81)
000391	hsa-miR-16	hsa-miR-16-5p	4.79 (0.15)	1.00 (0.80-1.25)	4.21 (0.23)	1.49 (1.03-2.16)	4.27 (0.31)	1.43 (0.87-2.36)	3.97 (0.33)	1.77 (1.05-2.96)
000395	hsa-miR-19a	hsa-miR-19a-3p	9.55 (0.21)	1.00 (0.73-1.37)	9.13 (0.23)	1.33 (0.92-1.93)	9.05 (0.25)	1.41 (0.95-2.10)	8.95 (0.20)	1.51 (1.10-2.07)
000396	hsa-miR-19b	hsa-miR-19b-3p	4.21 (0.17)	1.00 (0.77-1.29)	3.82 (0.17)	1.31 (1.00-1.72)	3.91 (0.24)	1.24 (0.85-1.80)	3.68 (0.21)	1.45 (1.05-2.00)
000397	hsa-miR-21	hsa-miR-21-5p	7.03 (0.17)	1.00 (0.78-1.28)	6.68 (0.20)	1.27 (0.93-1.75)	6.74 (0.14)	1.23 (0.99-1.52)	6.70 (0.12)	1.26 (1.04-1.53)
000402	hsa-miR-24	hsa-miR-24-3p	4.43 (0.23)	1.00 (0.72-1.40)	3.98 (0.16)	1.37 (1.06-1.75)	3.87 (0.22)	1.48 (1.04-2.09)	3.99 (0.19)	1.36 (1.01-1.82)
000403	hsa-miR-25	hsa-miR-25-3p	7.53 (0.27)	1.00 (0.68-1.48)	6.90 (0.17)	1.54 (1.18-2.01)	7.12 (0.29)	1.33 (0.84-2.10)	6.61 (0.28)	1.89 (1.23-2.92)
000405	hsa-miR-26a	hsa-miR-26a-5p	6.56 (0.23)	1.00 (0.71-1.41)	6.43 (0.17)	1.09 (0.83-1.43)	6.28 (0.18)	1.22 (0.91-1.63)	6.68 (0.25)	0.92 (0.62-1.37)
000407	hsa-miR-26b	hsa-miR-26b-5p	7.50 (0.23)	1.00 (0.72-1.39)	7.38 (0.14)	1.09 (0.87-1.36)	7.06 (0.28)	1.36 (0.87-2.14)	7.28 (0.24)	1.17 (0.81-1.69)
000408	hsa-miR-27a	hsa-miR-27a-3p	9.14 (0.26)	1.00 (0.68-1.46)	8.65 (0.14)	1.40 (1.12-1.76)	8.55 (0.26)	1.50 (0.99-2.28)	8.76 (0.26)	1.30 (0.86-1.95)
000409	hsa-miR-27b	hsa-miR-27b-3p	10.01 (0.22)	1.00 (0.72-1.39)	9.72 (0.11)	1.22 (1.03-1.46)	9.54 (0.21)	1.39 (1.00-1.94)	9.95 (0.16)	1.04 (0.81-1.34)
000411	hsa-miR-28	hsa-miR-28-5p	9.97 (0.30)	1.00 (0.65-1.55)	10.01 (0.18)	0.97 (0.72-1.30)	9.88 (0.19)	1.06 (0.78-1.43)	10.30 (0.34)	0.79 (0.46-1.36)
000413	hsa-miR-29b	hsa-miR-29b-3p	13.33 (0.20)	1.00 (0.75-1.34)	13.03 (0.20)	1.23 (0.89-1.70)	12.78 (0.35)	1.46 (0.83-2.60)	13.17 (0.30)	1.12 (0.70-1.79)
000417	hsa-miR-30a-5p	hsa-miR-30a-5p	7.30 (0.19)	1.00 (0.76-1.32)	6.49 (0.19)	1.75 (1.30-2.37)	6.72 (0.18)	1.49 (1.12-2.00)	6.75 (0.28)	1.46 (0.94-2.27)
000419	hsa-miR-30c	hsa-miR-30c-5p	5.27 (0.22)	1.00 (0.73-1.38)	5.11 (0.12)	1.12 (0.92-1.36)	5.35 (0.11)	0.95 (0.79-1.14)	5.39 (0.28)	0.92 (0.59-1.44)
000420	hsa-miR-30d	hsa-miR-30d-5p	9.77 (0.23)	1.00 (0.72-1.39)	9.06 (0.20)	1.64 (1.19-2.24)	9.21 (0.23)	1.47 (1.01-2.13)	9.09 (0.31)	1.60 (0.98-2.62)
000422	hsa-miR-30e-3p	hsa-miR-30e-3p	12.93 (0.30)	1.00 (0.64-1.55)	12.62 (0.16)	1.24 (0.96-1.61)	12.10 (0.29)	1.78 (1.12-2.82)	12.50 (0.20)	1.35 (0.99-1.85)
000431	hsa-miR-92a	hsa-miR-92a-3p	3.15 (0.21)	1.00 (0.73-1.36)	2.56 (0.19)	1.51 (1.12-2.03)	3.08 (0.19)	1.05 (0.77-1.43)	2.58 (0.30)	1.49 (0.93-2.39)
000436	hsa-miR-99b	hsa-miR-99b-5p	9.59 (0.30)	1.00 (0.64-1.57)	9.17 (0.18)	1.34 (1.01-1.78)	9.42 (0.21)	1.12 (0.81-1.56)	9.43 (0.26)	1.12 (0.75-1.68)
000442	hsa-miR-106b	hsa-miR-106b-5p	7.10 (0.23)	1.00 (0.71-1.41)	6.83 (0.16)	1.21 (0.94-1.56)	6.59 (0.31)	1.43 (0.87-2.34)	6.71 (0.27)	1.31 (0.86-1.98)
000449	hsa-miR-125b	hsa-miR-125b-5p	10.82 (0.25)	1.00 (0.70-1.44)	10.60 (0.33)	1.17 (0.68-1.98)	10.52 (0.20)	1.23 (0.89-1.69)	10.41 (0.28)	1.33 (0.86-2.05)
000451	hsa-miR-126#	hsa-miR-126-5p	9.40 (0.18)	1.00 (0.77-1.30)	9.15 (0.16)	1.19 (0.92-1.53)	9.45 (0.17)	0.97 (0.73-1.28)	9.25 (0.22)	1.11 (0.79-1.56)
000452	hsa-miR-127	hsa-miR-127-3p	10.78 (0.31)	1.00 (0.63-1.59)	10.09 (0.29)	1.61 (1.01-2.57)	11.13 (0.38)	0.79 (0.43-1.44)	11.12 (0.26)	0.79 (0.53-1.18)
000454	hsa-miR-130a	hsa-miR-130a-3p	7.61 (0.22)	1.00 (0.72-1.39)	7.41 (0.22)	1.15 (0.81-1.63)	7.75 (0.27)	0.91 (0.59-1.38)	7.47 (0.20)	1.10 (0.80-1.51)

000456	hsa-miR-130b	hsa-miR-130b-3p	10.46 (0.24)	1.00 (0.71-1.42)	9.62 (0.21)	1.79 (1.27-2.53)	10.07 (0.18)	1.31 (0.97-1.75)	9.94 (0.25)	1.43 (0.97-2.10)
000457	hsa-miR-132	hsa-miR-132-3p	9.54 (0.21)	1.00 (0.73-1.37)	8.80 (0.23)	1.68 (1.15-2.44)	8.84 (0.18)	1.62 (1.23-2.15)	8.91 (0.19)	1.55 (1.15-2.09)
000464	hsa-miR-142-3p	hsa-miR-142-3p	7.10 (0.17)	1.00 (0.78-1.29)	7.15 (0.18)	0.97 (0.73-1.28)	7.02 (0.22)	1.06 (0.75-1.50)	7.36 (0.21)	0.84 (0.60-1.16)
000468	hsa-miR-146a	hsa-miR-146a-5p	7.18 (0.20)	1.00 (0.75-1.34)	7.02 (0.20)	1.12 (0.82-1.54)	6.81 (0.14)	1.29 (1.03-1.63)	7.03 (0.37)	1.11 (0.62-1.99)
000470	hsa-miR-148a	hsa-miR-148a-3p	9.72 (0.26)	1.00 (0.67-1.49)	9.21 (0.30)	1.43 (0.88-2.32)	8.84 (0.31)	1.84 (1.10-3.08)	9.36 (0.18)	1.29 (0.97-1.72)
000471	hsa-miR-148b	hsa-miR-148b-3p	12.78 (0.22)	1.00 (0.72-1.38)	12.40 (0.30)	1.30 (0.80-2.10)	12.72 (0.30)	1.04 (0.64-1.70)	12.59 (0.34)	1.15 (0.67-1.96)
000473	hsa-miR-150	hsa-miR-150-5p	5.55 (0.19)	1.00 (0.75-1.33)	5.48 (0.16)	1.05 (0.81-1.36)	5.37 (0.35)	1.13 (0.64-2.00)	5.31 (0.27)	1.18 (0.77-1.80)
000475	hsa-miR-152	hsa-miR-152-3p	11.10 (0.33)	1.00 (0.61-1.64)	10.58 (0.21)	1.43 (1.03-1.99)	10.91 (0.24)	1.14 (0.78-1.66)	10.84 (0.30)	1.19 (0.74-1.92)
000480	hsa-miR-181a	hsa-miR-181a-5p	10.22 (0.27)	1.00 (0.67-1.49)	10.07 (0.21)	1.12 (0.80-1.56)	9.93 (0.23)	1.22 (0.85-1.76)	10.18 (0.32)	1.03 (0.63-1.70)
000482	hsa-miR-181c	hsa-miR-181c-5p	13.90 (0.12)	1.00 (0.84-1.19)	13.78 (0.18)	1.08 (0.82-1.43)	13.92 (0.16)	0.98 (0.77-1.26)	13.83 (0.11)	1.05 (0.87-1.26)
000491	hsa-miR-192	hsa-miR-192-5p	8.60 (0.19)	1.00 (0.75-1.33)	8.33 (0.26)	1.21 (0.81-1.82)	8.32 (0.19)	1.22 (0.90-1.66)	8.15 (0.16)	1.37 (1.07-1.76)
000494	hsa-miR-195	hsa-miR-195-5p	9.15 (0.17)	1.00 (0.78-1.29)	8.64 (0.29)	1.42 (0.90-2.25)	8.82 (0.33)	1.26 (0.74-2.15)	8.38 (0.30)	1.71 (1.07-2.74)
000507	hsa-miR-203	hsa-miR-203a-3p	13.00 (0.27)	1.00 (0.68-1.48)	12.73 (0.39)	1.21 (0.63-2.29)	12.92 (0.52)	1.05 (0.46-2.43)	13.08 (0.21)	0.95 (0.66-1.35)
000508	hsa-miR-204	hsa-miR-204-5p	13.69 (0.18)	1.00 (0.76-1.31)	13.29 (0.18)	1.33 (1.00-1.76)	13.53 (0.20)	1.12 (0.82-1.54)	13.32 (0.24)	1.30 (0.89-1.90)
000515	hsa-miR-212	hsa-miR-212-3p	12.11 (0.20)	1.00 (0.75-1.34)	11.96 (0.28)	1.11 (0.71-1.73)	12.18 (0.28)	0.95 (0.61-1.49)	12.01 (0.32)	1.07 (0.65-1.76)
000524	hsa-miR-221	hsa-miR-221-3p	6.48 (0.26)	1.00 (0.68-1.47)	6.23 (0.17)	1.19 (0.90-1.56)	6.43 (0.14)	1.04 (0.83-1.29)	6.52 (0.24)	0.97 (0.67-1.41)
000528	hsa-miR-301	hsa-miR-301a-3p	10.77 (0.26)	1.00 (0.69-1.46)	10.52 (0.18)	1.20 (0.89-1.60)	10.40 (0.23)	1.30 (0.89-1.88)	10.51 (0.19)	1.20 (0.89-1.63)
000539	hsa-miR-324-5p	hsa-miR-324-5p	9.61 (0.26)	1.00 (0.68-1.47)	8.94 (0.17)	1.59 (1.20-2.10)	9.25 (0.24)	1.28 (0.87-1.87)	9.34 (0.29)	1.21 (0.77-1.89)
000543	hsa-miR-328	hsa-miR-328-3p	7.05 (0.23)	1.00 (0.71-1.41)	6.68 (0.13)	1.29 (1.05-1.59)	7.08 (0.22)	0.98 (0.69-1.40)	7.01 (0.22)	1.03 (0.73-1.45)
000545	hsa-miR-331	hsa-miR-331-3p	7.21 (0.27)	1.00 (0.67-1.50)	6.91 (0.14)	1.22 (0.99-1.52)	7.21 (0.16)	1.00 (0.78-1.27)	7.23 (0.26)	0.98 (0.65-1.48)
000546	hsa-miR-335	hsa-miR-335-5p	10.26 (0.33)	1.00 (0.61-1.63)	9.82 (0.18)	1.36 (1.02-1.81)	10.40 (0.30)	0.91 (0.56-1.46)	10.38 (0.25)	0.92 (0.62-1.36)
000554	hsa-miR-361	hsa-miR-361-5p	11.07 (0.27)	1.00 (0.68-1.48)	10.42 (0.30)	1.57 (0.96-2.55)	10.52 (0.32)	1.45 (0.87-2.43)	10.51 (0.28)	1.47 (0.94-2.31)
000563	hsa-miR-374	hsa-miR-374a-5p	8.53 (0.29)	1.00 (0.65-1.53)	8.45 (0.23)	1.06 (0.74-1.52)	8.24 (0.23)	1.22 (0.85-1.76)	8.61 (0.21)	0.95 (0.68-1.32)
000564	hsa-miR-375	hsa-miR-375	10.95 (0.39)	1.00 (0.56-1.78)	10.37 (0.28)	1.50 (0.96-2.33)	10.86 (0.42)	1.06 (0.55-2.07)	11.42 (0.33)	0.72 (0.43-1.21)
000565	hsa-miR-376a	hsa-miR-376a-3p	9.67 (0.40)	1.00 (0.56-1.79)	9.64 (0.21)	1.02 (0.73-1.42)	9.66 (0.45)	1.00 (0.49-2.05)	10.23 (0.66)	0.67 (0.24-1.90)
000580	hsa-miR-20a	hsa-miR-20a-5p	3.51 (0.20)	1.00 (0.74-1.35)	3.22 (0.19)	1.22 (0.90-1.65)	3.42 (0.28)	1.07 (0.69-1.66)	3.02 (0.24)	1.40 (0.96-2.04)
000602	hsa-miR-30b	hsa-miR-30b-5p	5.05 (0.22)	1.00 (0.72-1.39)	4.98 (0.11)	1.05 (0.88-1.25)	5.04 (0.12)	1.00 (0.83-1.21)	5.26 (0.28)	0.87 (0.56-1.34)
001014	hsa-miR-20b	hsa-miR-20b-5p	10.99 (0.17)	1.00 (0.78-1.28)	10.69 (0.30)	1.23 (0.76-1.99)	10.67 (0.32)	1.25 (0.75-2.08)	10.53 (0.27)	1.38 (0.91-2.09)
001090	hsa-miR-93	hsa-miR-93-5p	9.14 (0.16)	1.00 (0.79-1.27)	8.69 (0.19)	1.37 (1.01-1.87)	8.45 (0.25)	1.61 (1.08-2.41)	8.39 (0.25)	1.68 (1.13-2.51)
001097	hsa-miR-146b	hsa-miR-146b-5p	9.62 (0.14)	1.00 (0.82-1.23)	9.39 (0.14)	1.17 (0.94-1.46)	9.36 (0.20)	1.20 (0.87-1.64)	9.39 (0.21)	1.17 (0.84-1.64)

001141	hsa-miR-451	hsa-miR-451a	3.69 (0.21)	1.00 (0.74-1.35)	3.17 (0.24)	1.43 (0.97-2.11)	3.43 (0.38)	1.20 (0.65-2.20)	2.82 (0.37)	1.82 (1.02-3.26)
001187	hsa-miR-140	hsa-miR-140-5p	10.85 (0.19)	1.00 (0.76-1.32)	10.26 (0.31)	1.51 (0.91-2.49)	9.56 (0.30)	2.44 (1.51-3.95)	9.90 (0.24)	1.92 (1.31-2.83)
001319	hsa-miR-374-5p	hsa-miR-374b-5p	9.77 (0.32)	1.00 (0.62-1.61)	9.79 (0.27)	0.99 (0.64-1.52)	9.61 (0.25)	1.12 (0.75-1.68)	10.02 (0.30)	0.84 (0.53-1.35)
001515	hsa-miR-660	hsa-miR-660-5p	10.52 (0.22)	1.00 (0.72-1.39)	9.90 (0.26)	1.54 (1.02-2.33)	9.83 (0.25)	1.61 (1.08-2.41)	9.81 (0.16)	1.64 (1.27-2.11)
001518	hsa-miR-532	hsa-miR-532-5p	10.31 (0.13)	1.00 (0.83-1.20)	9.92 (0.19)	1.32 (0.97-1.79)	10.10 (0.19)	1.16 (0.85-1.59)	9.70 (0.34)	1.53 (0.90-2.63)
001551	hsa-miR-597	hsa-miR-597-5p	13.75 (0.12)	1.00 (0.83-1.20)	13.64 (0.24)	1.08 (0.74-1.57)	14.26 (0.11)	0.70 (0.59-0.84)	13.67 (0.14)	1.05 (0.83-1.33)
001597	hsa-miR-645	hsa-miR-645	14.19 (0.15)	1.00 (0.80-1.25)	14.07 (0.17)	1.09 (0.83-1.43)	14.20 (0.30)	0.99 (0.62-1.60)	14.18 (0.20)	1.01 (0.73-1.39)
001610	hsa-miR-411	hsa-miR-411-5p	12.96 (0.28)	1.00 (0.66-1.52)	12.98 (0.29)	0.99 (0.62-1.56)	13.20 (0.33)	0.85 (0.50-1.45)	13.77 (0.16)	0.57 (0.44-0.74)
001630	hsa-miR-491	hsa-miR-491-5p	13.44 (0.17)	1.00 (0.78-1.28)	12.19 (0.34)	2.39 (1.39-4.10)	12.42 (0.19)	2.04 (1.51-2.75)	12.65 (0.30)	1.73 (1.08-2.78)
001821	hsa-miR-484	hsa-miR-484	4.69 (0.14)	1.00 (0.81-1.24)	4.15 (0.13)	1.45 (1.17-1.79)	4.59 (0.11)	1.07 (0.90-1.27)	4.29 (0.24)	1.32 (0.91-1.91)
001973	U6 rRNA	U6 snRNA	5.21 (0.36)	1.00 (0.58-1.71)	3.32 (0.52)	3.73 (1.63-8.51)	3.57 (0.40)	3.12 (1.64-5.92)	3.38 (0.47)	3.56 (1.70-7.47)
001984	hsa-miR-590-5p	hsa-miR-590-5p	10.86 (0.45)	1.00 (0.52-1.94)	9.45 (0.80)	2.66 (0.74-9.55)	10.53 (0.40)	1.26 (0.67-2.37)	10.09 (0.28)	1.70 (1.10-2.64)
001986	hsa-miR-766	hsa-miR-766-3p	12.59 (0.24)	1.00 (0.70-1.43)	12.08 (0.32)	1.42 (0.85-2.37)	12.54 (0.13)	1.03 (0.84-1.28)	12.68 (0.41)	0.94 (0.48-1.83)
002083	hsa-miR-502-3p	hsa-miR-502-3p	13.95 (0.12)	1.00 (0.83-1.20)	13.92 (0.16)	1.02 (0.78-1.33)	14.37 (0.09)	0.75 (0.65-0.86)	14.09 (0.10)	0.91 (0.77-1.06)
002098	hsa-miR-223#	hsa-miR-223-5p	14.51 (0.25)	1.00 (0.69-1.46)	13.71 (0.39)	1.74 (0.94-3.23)	12.79 (0.30)	3.29 (2.05-5.30)	13.36 (0.36)	2.22 (1.27-3.89)
002112	hsa-miR-29a	hsa-miR-29a-3p	11.21 (0.24)	1.00 (0.70-1.42)	10.65 (0.34)	1.48 (0.86-2.53)	10.07 (0.31)	2.20 (1.35-3.61)	10.20 (0.27)	2.01 (1.31-3.08)
002122	hsa-miR-376c	hsa-miR-376c-3p	12.35 (0.27)	1.00 (0.67-1.49)	11.69 (0.29)	1.58 (0.99-2.52)	12.19 (0.43)	1.12 (0.54-2.31)	12.66 (0.28)	0.81 (0.52-1.25)
002161	hsa-miR-324-3p	hsa-miR-324-3p	13.54 (0.17)	1.00 (0.78-1.28)	12.84 (0.19)	1.63 (1.19-2.22)	12.61 (0.33)	1.91 (1.12-3.26)	12.85 (0.34)	1.62 (0.94-2.77)
002169	hsa-miR-106a	hsa-miR-106a-5p	4.80 (0.16)	1.00 (0.79-1.27)	4.46 (0.16)	1.26 (0.98-1.63)	4.71 (0.20)	1.06 (0.77-1.46)	4.40 (0.22)	1.32 (0.93-1.86)
002186	hsa-miR-345	hsa-miR-345-5p	13.51 (0.14)	1.00 (0.82-1.22)	12.70 (0.18)	1.75 (1.31-2.34)	12.62 (0.30)	1.85 (1.15-2.99)	12.65 (0.29)	1.81 (1.15-2.84)
002187	hsa-miR-942	hsa-miR-942-5p	12.83 (0.13)	1.00 (0.83-1.21)	12.17 (0.16)	1.59 (1.23-2.05)	12.73 (0.40)	1.07 (0.56-2.03)	12.28 (0.24)	1.47 (1.01-2.15)
002228	hsa-miR-126	hsa-miR-126-3p	6.97 (0.18)	1.00 (0.77-1.30)	6.89 (0.21)	1.06 (0.76-1.48)	6.84 (0.15)	1.09 (0.86-1.40)	7.12 (0.26)	0.90 (0.60-1.34)
002245	hsa-miR-122	hsa-miR-122-5p	8.25 (0.31)	1.00 (0.63-1.58)	7.96 (0.48)	1.22 (0.56-2.65)	8.22 (0.23)	1.02 (0.70-1.49)	7.39 (0.27)	1.82 (1.15-2.87)
002249	hsa-miR-143	hsa-miR-143-3p	11.96 (0.22)	1.00 (0.72-1.38)	11.51 (0.35)	1.37 (0.79-2.38)	10.99 (0.31)	1.96 (1.19-3.23)	11.11 (0.24)	1.80 (1.24-2.62)
002253	hsa-miR-101	hsa-miR-101-3p	12.80 (0.19)	1.00 (0.76-1.32)	12.90 (0.17)	0.93 (0.71-1.22)	12.58 (0.29)	1.16 (0.73-1.85)	12.36 (0.25)	1.36 (0.92-2.01)
002258	hsa-miR-340	hsa-miR-340-5p	13.97 (0.11)	1.00 (0.85-1.17)	13.85 (0.16)	1.09 (0.84-1.41)	14.09 (0.12)	0.92 (0.76-1.12)	14.03 (0.11)	0.96 (0.80-1.16)
002259	hsa-miR-340#	hsa-miR-340-3p	15.21 (0.16)	1.00 (0.79-1.27)	15.38 (0.16)	0.88 (0.68-1.16)	15.47 (0.19)	0.83 (0.62-1.12)	15.15 (0.18)	1.04 (0.78-1.38)
002271	hsa-miR-185	hsa-miR-185-5p	12.06 (0.19)	1.00 (0.76-1.31)	11.53 (0.38)	1.44 (0.79-2.63)	11.57 (0.33)	1.41 (0.84-2.37)	11.45 (0.24)	1.53 (1.05- 2.22)
002277	hsa-miR-320	hsa-miR-320a	6.52 (0.13)	1.00 (0.82-1.21)	5.77 (0.21)	1.68 (1.20-2.35)	6.41 (0.19)	1.08 (0.79-1.48)	6.09 (0.26)	1.35 (0.89-2.03)
002281	hsa-miR-193a-5p	hsa-miR-193a-5p	12.74 (0.25)	1.00 (0.69-1.44)	11.44 (0.28)	2.45 1.56-3.85)	11.31 (0.33)	2.68 (1.59-4.54)	11.19 (0.29)	2.93 (1.85-4.64)

002283	hsa-let-7d	hsa-let-7d-5p	11.05 (0.20)	1.00 (0.74-1.35)	11.10 (0.18)	0.97 (0.72-1.29)	11.19 (0.23)	0.90 (0.63-1.30)	11.29 (0.35)	0.84 (0.49-1.46)
002285	hsa-miR-186	hsa-miR-186-5p	12.29 (0.16)	1.00 (0.79-1.27)	11.69 (0.32)	1.51 (0.90-2.53)	11.31 (0.19)	1.97 (1.46-2.66)	11.53 (0.34)	1.69 (0.99-2.90)
002289	hsa-miR-139-5p	hsa-miR-139-5p	9.44 (0.18)	1.00 (0.77-1.30)	9.29 (0.16)	1.11 (0.86-1.42)	9.86 (0.14)	0.75 (0.60-0.94)	9.59 (0.24)	0.90 (0.62-1.30)
002295	hsa-miR-223	hsa-miR-223-3p	-0.80 (0.23)	1.00 (0.71-1.41)	-1.67 (0.26)	1.82 (1.20-2.77)	-2.15 (0.22)	2.54 (1.79-3.62)	-1.80 (0.24)	2.00 (1.38-2.89)
002296	hsa-miR-885-5p	hsa-miR-885-5p	9.88 (0.40)	1.00 (0.56-1.80)	8.95 (0.47)	1.90 (0.90-3.99)	9.80 (0.32)	1.06 (0.64-1.76)	9.73 (0.50)	1.11 (0.51-2.41)
002299	hsa-miR-191	hsa-miR-191-5p	5.33 (0.18)	1.00 (0.77-1.31)	4.88 (0.18)	1.36 (1.02-1.83)	4.67 (0.21)	1.58 (1.12-2.22)	5.06 (0.27)	1.21 (0.79-1.85)
002304	hsa-miR-199a-3p	hsa-miR-199a-3p / hsa-miR-199b-3p	9.79 (0.20)	1.00 (0.74-1.35)	9.56 (0.25)	1.18 (0.79-1.75)	9.87 (0.18)	0.95 (0.71-1.27)	9.70 (0.21)	1.07 (0.77-1.49)
002308	hsa-miR-17	hsa-miR-17-5p	4.59 (0.16)	1.00 (0.79-1.27)	4.32 (0.17)	1.21 (0.93-1.59)	4.52 (0.22)	1.06 (0.74-1.50)	4.17 (0.22)	1.34 (0.95-1.89)
002324	hsa-miR-744	hsa-miR-744-5p	12.48 (0.23)	1.00 (0.71-1.41)	12.36 (0.33)	1.09 (0.63-1.86)	11.99 (0.21)	1.41 (1.00-1.98)	11.99 (0.23)	1.40 (0.97-2.02)
002332	hsa-miR-409-3p	hsa-miR-409-3p	12.15 (0.19)	1.00 (0.76-1.32)	11.44 (0.18)	1.64 (1.22-2.19)	12.29 (0.30)	0.91 (0.56-1.46)	12.83 (0.30)	0.63 (0.39-1.00)
002338	hsa-miR-483-5p	hsa-miR-483-5p	11.63 (0.34)	1.00 (0.61-1.64)	10.53 (0.41)	2.15 (1.12-4.11)	11.43 (0.20)	1.15 (0.84-1.58)	10.79 (0.22)	1.80 (1.27-2.55)
002340	hsa-miR-423-5p	hsa-miR-423-5p	11.46 (0.22)	1.00 (0.72-1.39)	10.74 (0.29)	1.65 (1.03-2.66)	10.81 (0.15)	1.57 (1.24-1.99)	10.69 (0.34)	1.71 (1.00-2.92)
002367	hsa-miR-193b	hsa-miR-193b-3p	10.47 (0.29)	1.00 (0.65-1.54)	9.62 (0.30)	1.80 (1.11-2.93)	10.19 (0.20)	1.21 (0.88-1.66)	9.81 (0.21)	1.58 (1.13-2.19)
002398	hsa-miR-579	hsa-miR-579-3p	13.97 (0.11)	1.00 (0.85-1.17)	13.87 (0.15)	1.08 (0.85-1.37)	14.37 (0.08)	0.76 (0.66-0.87)	14.07 (0.11)	0.93 (0.78-1.12)
002844	HSA-MIR-320B	hsa-miR-320b	15.70 (0.12)	1.00 (0.84-1.20)	15.36 (0.19)	1.27 (0.94-1.72)	15.69 (0.21)	1.01 (0.72-1.41)	15.20 (0.29)	1.42 (0.91-2.22)
002884	HSA-MIR-1274B	NA[b]	7.12 (0.21)	1.00 (0.73-1.37)	6.31 (0.22)	1.76 (1.23-2.50)	6.21 (0.23)	1.87 (1.29-2.71)	6.38 (0.21)	1.67 (1.21-2.31)

Abbreviations: AU: HLA-B27-associated Anterior Uveitis, BU; Birdshot Uveitis, CI: confidence interval, ΔCrt: delta Cycle threshold value (ΔCrt = $Crt_{mean\ target}$ – $Crt_{mean\ spike\text{-}in}$), HC: Healthy Control, FC: Fold Change ($FC=2^{-\Delta Crt}$, where $\Delta\Delta Crt=\Delta Crt_{patient}-\Delta Crt_{HC}$), IU: Idiopathic Intermediate Uveitis, miRNA: microRNA, NA: not applicable, SE: Standard Error

[a] Abundant expression (amplification score >1.24, mean Crt<27) in >90% of all (n=44) samples.

[b] The putative mature miR-1274 sequence is a fragment of a Lys tRNA.[15]

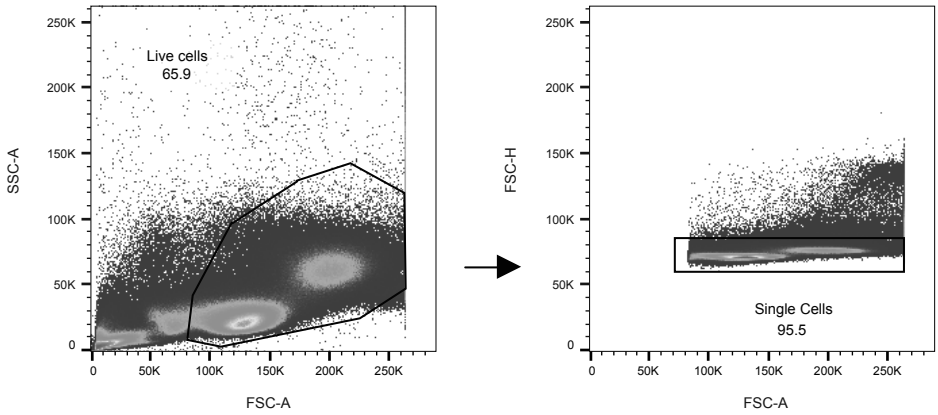
Supplementary Table 4. Discovery, validation and replication of 7 serum miRNAs in non-infectious uveitis patients

			Discovery (n=44)		Technical validation		Replication (n=36)	Cohorts combined[A] (n=80)	
microRNA			**FC (95% CI)**	**p-val.***	**rho**	**p-val. ****	**p-val.***	**p-val. *** 1**	**p-val. *** 2**
miR-140-5p	AU	HC	1.51 (0.91-2.49)	0.13			**0.01**		0.05
	IU	HC	**2.44** (1.51-3.95)	**<0.01**	**0.87**	**<0.0001**	**<0.01**	**<0.001**	**0.001**
	BU	HC	1.92 (1.31-2.83)	**<0.01**			0.17		**0.04**
	UV	HC	-	-			-		**<0.001**
U6 rRNA	AU	HC	**3.73** (1.63-8.51)	**<0.01**			**<0.05**		**0.02**
	IU	HC	**3.12** (1.64-5.92)	**<0.01**	**0.73**	**<0.0001**	**0.01**	**<0.001**	**<0.001**
	BU	HC	**3.56** (1.70-7.47)	**<0.01**			0.86		0.05
	UV	HC	-	-			-		**<0.001**
miR-223-5p	AU	HC	1.74 (0.94-3.23)	0.10			**0.04**		0.08
	IU	HC	**3.29** (2.05-5.30)	**<0.001**	**0.85**	**<0.0001**	**0.01**	**<0.001**	**<0.001**
	BU	HC	**2.22** (1.27-3.89)	**0.02**			0.44		**0.03**
	UV	HC	-	-			-		**<0.001**
miR-193a-5p	AU	HC	**2.45** (1.56-3.85)	**<0.01**			0.06		**0.02**
	IU	HC	**2.68** (1.59-4.54)	**<0.01**	**0.91**	**<0.0001**	**0.03**	**0.001**	**<0.01**
	BU	HC	**2.93** (1.85-4.64)	**<0.01**			0.28		**0.02**
	UV	HC	-	-			-		**<0.001**
miR-223-3p	AU	HC	1.82 (1.20-2.77)	**0.02**			**0.04**		0.26
	IU	HC	**2.54** (1.79-3.62)	**<0.001**	**0.97**	**<0.0001**	**0.02**	**0.001**	**<0.001**
	BU	HC	**2.00** (1.38-2.89)	**<0.01**			0.53		0.45
	UV	HC	-	-			-		**<0.01**
miR-491-5p	AU	HC	**2.39** (1.39-4.10)	**<0.01**			**0.04**		0.25
	IU	HC	**2.04** (1.51-2.75)	**<0.01**	**0.74**	**<0.0001**	**0.03**	**0.01**	**0.01**
	BU	HC	1.73 (1.08-2.78)	**0.04**			0.32		0.41
	UV	HC	-	-			-		**<0.01**
miR-29a-3p	AU	HC	1.48 (0.86-2.53)	0.19			0.34		0.50
	IU	HC	**2.20** (1.35-3.61)	**<0.01**	**0.55**	**=0.0001**	**0.04**	**0.03**	**0.03**
	BU	HC	**2.01** (1.31-3.08)	**0.01**			0.15		0.33
	UV	HC	-	-			-		**<0.01**
miR-127-3p	AU	BU	**2.04** (1.28-3.26)	**0.02**			0.72		-
	AU	IU	**2.05** (1.28-3.27)	**<0.05**	**0.84**	**<0.0001**	0.87	0.35	-
	AU	HC	1.61 (1.01-2.57)	0.12			0.97		-
miR-409-3p	AU	BU	**2.62** (1.96-3.50)	**<0.01**			0.55		-
	AU	IU	1.81 (1.35-2.42)	**0.03**	**0.90**	**<0.0001**	0.80	0.44	-
	AU	HC	1.64 (1.22-2.19)	**0.01**			0.56		-
miR-375	AU	BU	**2.07** (1.33-3.22)	**0.03**			-	-	-
	AU	IU	1.40 (0.90-2.19)	0.34	0.11	0.53	-		-
	AU	HC	1.50 (0.96-2.33)	0.24			-		-

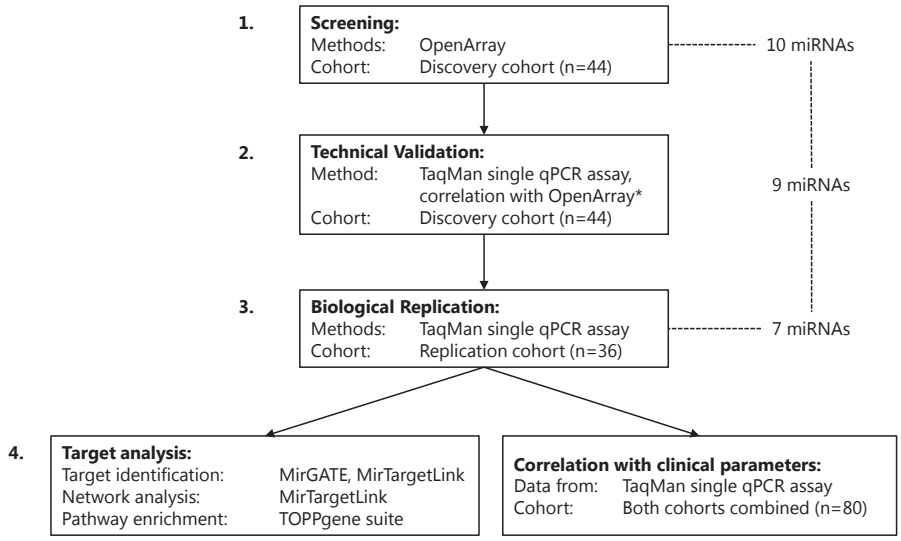
Abbreviations: AU: HLA-B27 associated acute anterior uveitis, BU: Birdshot uveitis, CI: confidence interval, FC: fold change, IU: idiopathic intermediate uveitis, HC: Healthy Control, miR: microRNA, UV: uveitis.
* Independent samples T test. ** Spearman's Rho. *** 1 Kruskal-Wallis. ***2 Individual p-values from Kruskal-Wallis with post hoc Dunn's test (AU, IU, BU, HC) or Mann Whitney U test (uveitis vs control)
[A] Data combined from TaqMan single qPCR assay. Analysis on ΔΔCt.

Supplementary Table 5. Proteins targeted by miR-29a and miR223-3p. List of target genes of mir-29a-3p and miR-223-3p that are present in proteomic data of Rieckmann et al[13] and used for figure 4C.

ABCB1	CCNT2	COL3A1	FBN1	IGF1R	PRDM1	SP3
ABL1	CD276	COL4A2	FGA	IL6	PTEN	SPARC
AKT2	CD93	CPEB2;CPEB3	FGB	IMPDH1	RAN	SRGAP2
ATM	CDC27	CPEB4	FGG	KLF4	RHOB	STAT5A
BCL2	CDC42	CYB5A	FOXO1	LIF	RNASEL	STMN1
BCL7A	CDC7	DICER1	FOXO3	LPL	S100B	TDG
CALR	CDK4	DNMT1	GLUL	MCL1	SCARB1	TET2
CAPRIN1	CDK6	DNMT3A	HBP1	PDGFRB	SERPINB9	TNFAIP3
CARM1	CEACAM6	ECT2	HSP90B1	POLR3G	SERPINH1	TOX
CCND2	CHUK	EPB41L3	IGF1;IGF-I	PPM1D	SP1	TRAF4
VDAC1	VEGFA	ZFP36	TRIM68			



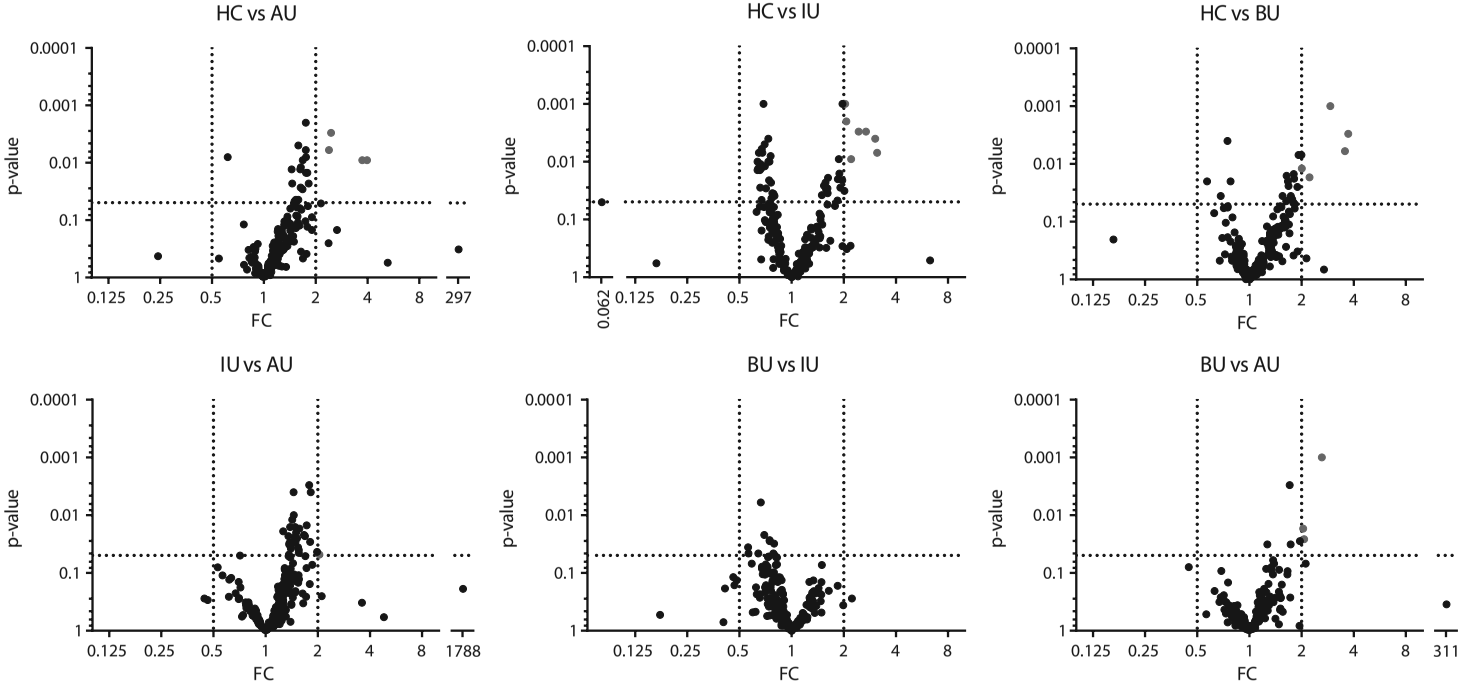
Supplementary Figure 1. Gating strategy flow cytometry for input into Citrus. Plots were generated in Flow Jo. First plot depicts forward (X axis) and sideward (Y-axis) scatter plot from which live cells are gated. The second (right) plot is a pulse geometry gate, using two forward scatter axes (FSC-A x FSC-H) to select single cells and eliminate double (clumped) cells.



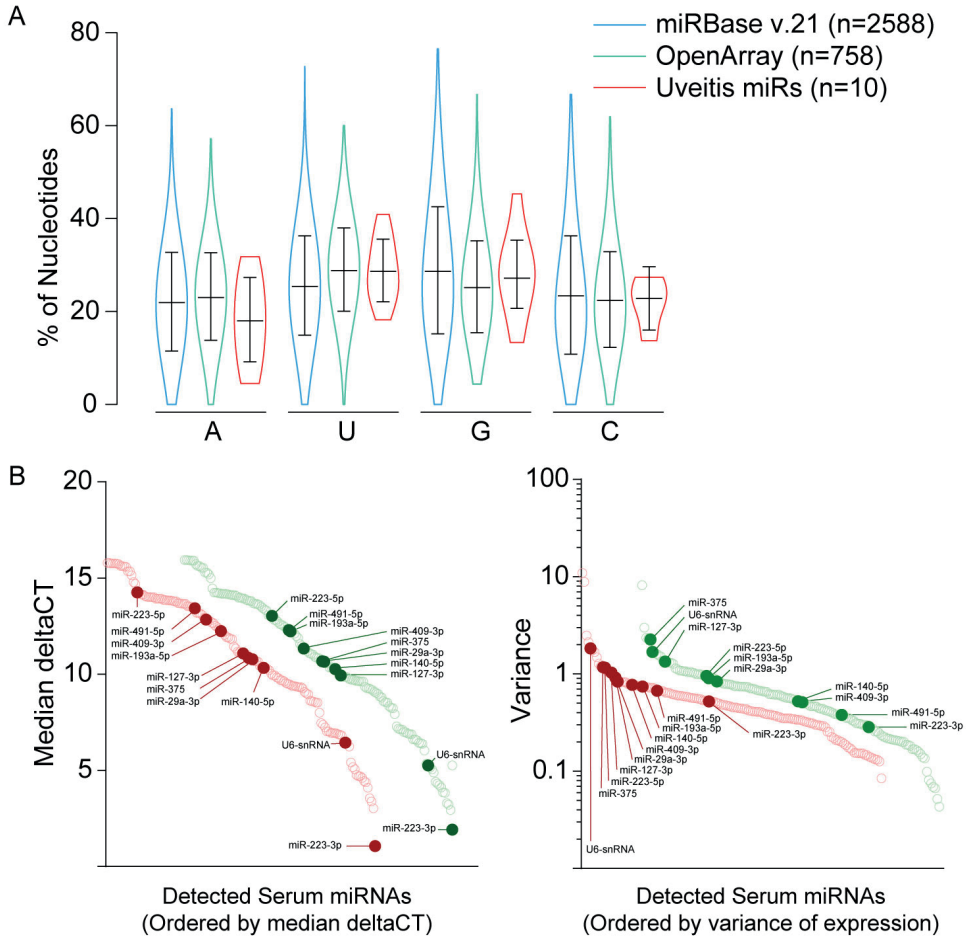
Supplementary Figure 2. Flow diagram of methods.

Abbreviations: miRNA: microRNA, qPCR: Quantitative Polymerase Chain Reaction

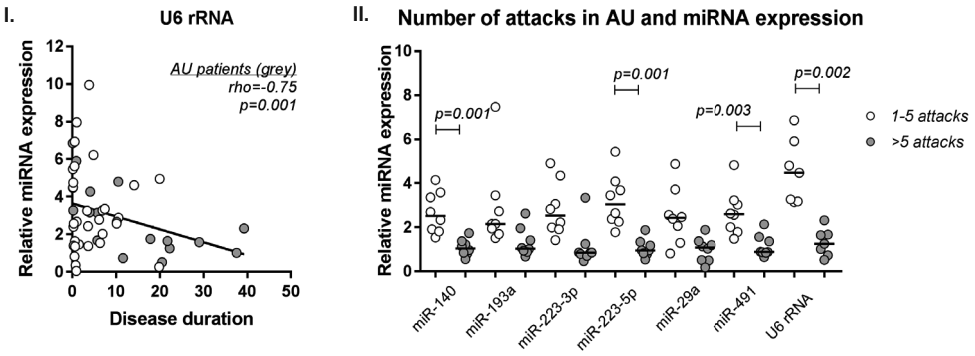
* TaqMan single qPCR was performed on leftover RNA from OpenArray: miR-223-5p, miR-140-5p, miR-193a-5p, U6 rRNA, miR-41-5p: technical validation performed on n=44 / miR-223-3p: Technical validation performed on n=42 / MIR-1274B, miR-127, miR-375, and miR-345: Technical validation performed on n=36



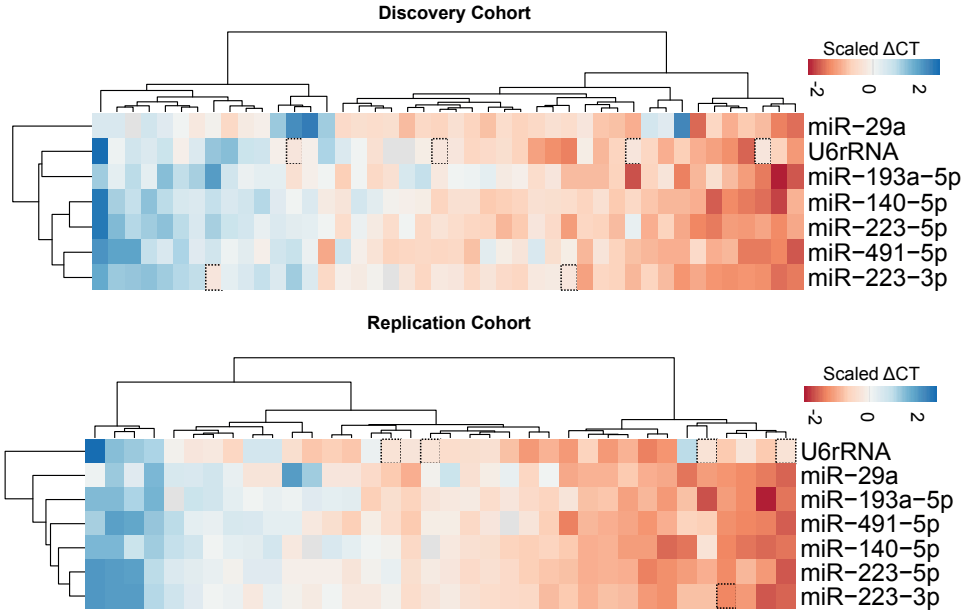
Supplementary figure 3. Volcano plots of circulating miRNA expression in uveitis versus controls and between different uveitis types. **Upper row:** Uveitis versus healthy controls. **Lower row:** Uveitis types compared with each other. Each dot represents a miRNA. Only miRNAs that meet amplification criteria are represented. In grey are the miRNAs that meet predefined criteria for replication (FC>2, p<0.05, represented by dented lines). **Abbreviations:** HC: Healthy Control, AU: HLA-B27-associated anterior uveitis, IU: Idiopathic Intermediate Uveitis, miR: microRNA.



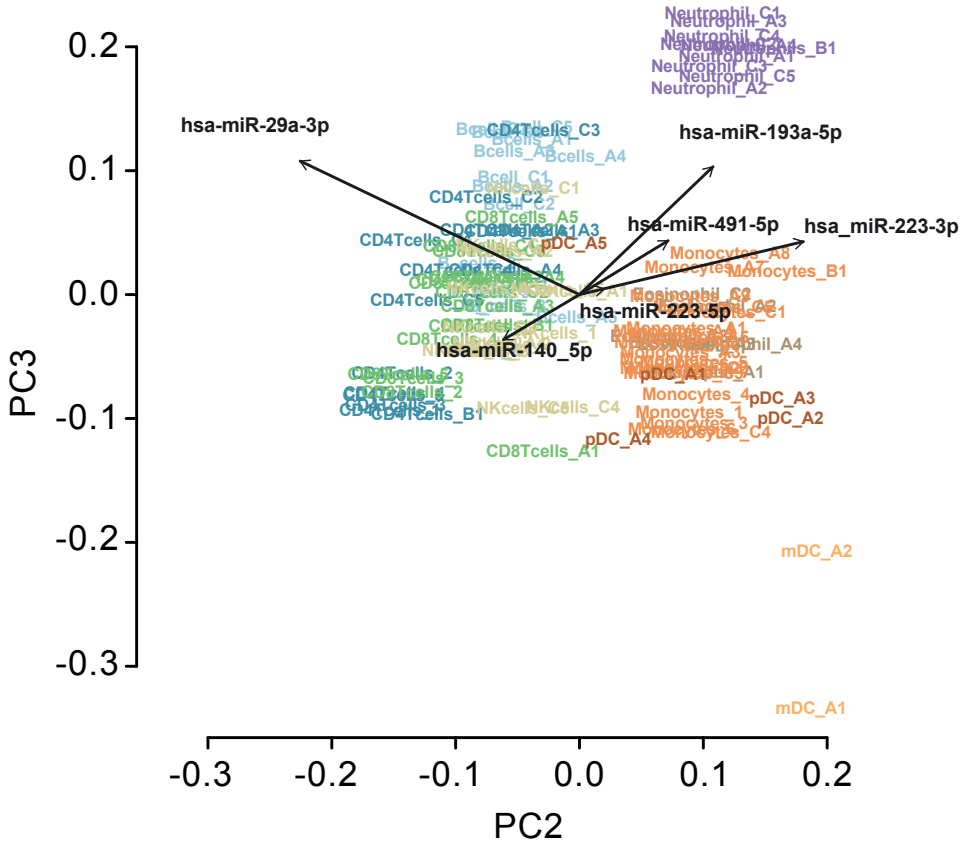
Supplementary Figure 4. Quality control of the miRNAs found to be differentially expressed in this study. **A.** The median ΔCt and Variance for each of the 102 detected miRNAs was ordered from low (light color) to high (intense color). The median ΔCt for each of the selected 10 differentially expressed small RNAs in serum of patients with non-infectious uveitis are indicated for controls and uveitis patients. Violin plots represent density estimates of data and extend to extreme values (mean and standard deviation in black). All comparisons were non-significant. The overall base composition of the 10 differentially expressed miRNAs was not distinct from the average base composition of non-coding RNAs present on the array or reported in miRBase (v.21). Likewise, the guanine-content of the selected 10 miRNAs was relatively low (mean guanine-percentage, 28.3% (range 13.6-45.5%). The sequence length of this panel (mean[SD]) was 22 [0.0], which is typical for miRNAs (mean[SD]; 21.6 [1.5] Mirbase v.21 (n=2,588); 21.9 [1.1] OpenArray™ Panel (n=758). **B** Although all 10 differentially expressed transcripts were detected well above background (mean Crt<27), their relative expression (left) or variance (right graph) indicated no obvious skewing compared to the entire set of detected serum miRNAs. All detected serum miRNAs (OpenArray) in uveitis patients (red circles) and controls (green circles) are ordered. Differentially expressed miRNAs are highlighted and labelled.



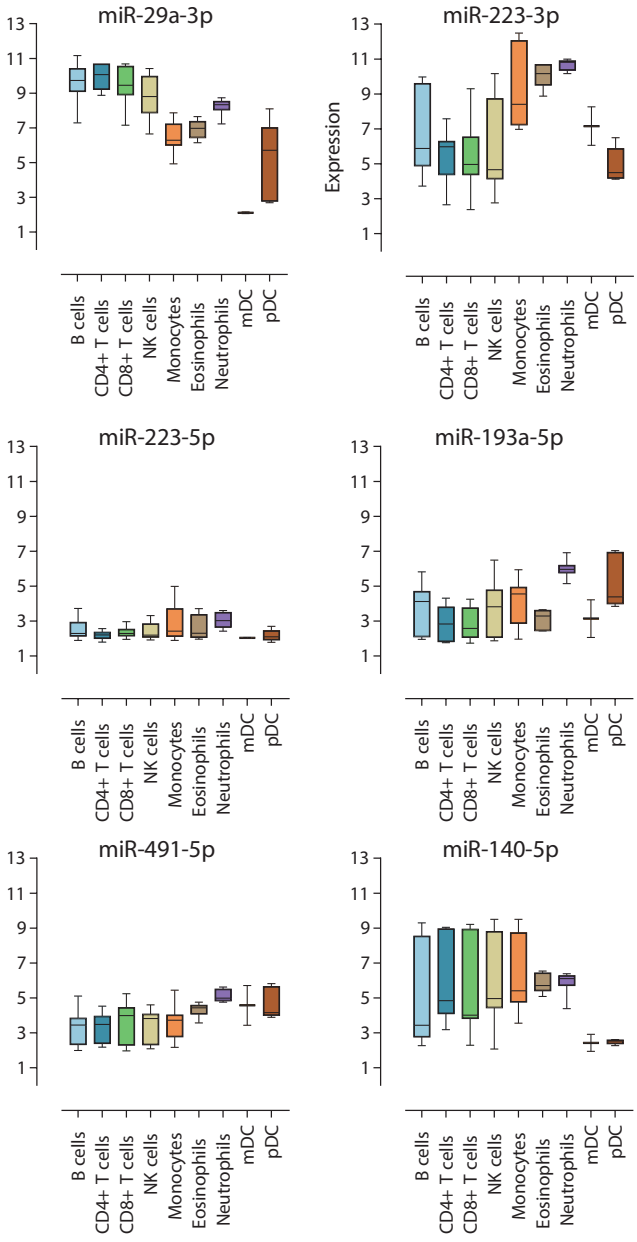
Supplementary Figure 5. Lower miRNA expression in late-stage anterior uveitis. **I.** Scatter plot showing correlation between U6 rRNA and disease duration in AU patients (grey). Correlation was tested using Spearman's Rho. **II.** miRNA expression levels in AU patients dichotomized to the number of experience uveitis attacks: 1-5 or >5. Differences between groups were tested using a Mann Whitney U test. Only p-values that are below the threshold for multiple testing ($p<0.0055$) are depicted. **Abbreviations:** AU: Anterior uveitis, BU: Birdshot uveitis, IU: Idiopathic Intermediate uveitis, miR: microRNA. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$



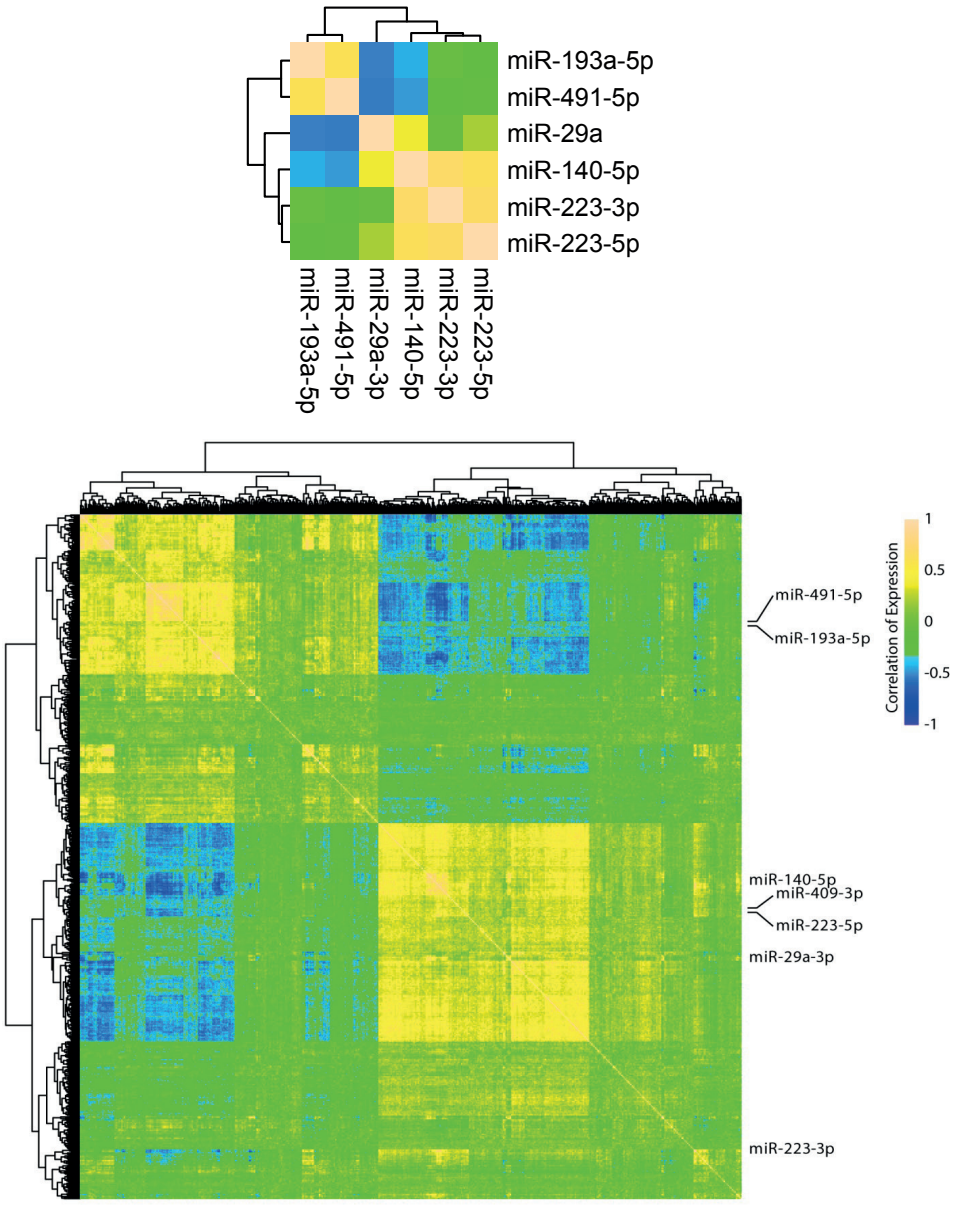
Supplementary Figure 6. miRNA expression levels for all noninfectious uveitis patients. Heatmaps of ΔCT values of seven sRNAs for each patient in the discovery and replication cohort. Rows are centered; unit variance scaling is applied to rows. Imputation is used for missing value estimation (indicated by dashed lining). Rows are clustered using Euclidean distance and complete linkage. Columns are clustered using Euclidean distance and Ward linkage. Heatmaps were made in Clustvis.[4]



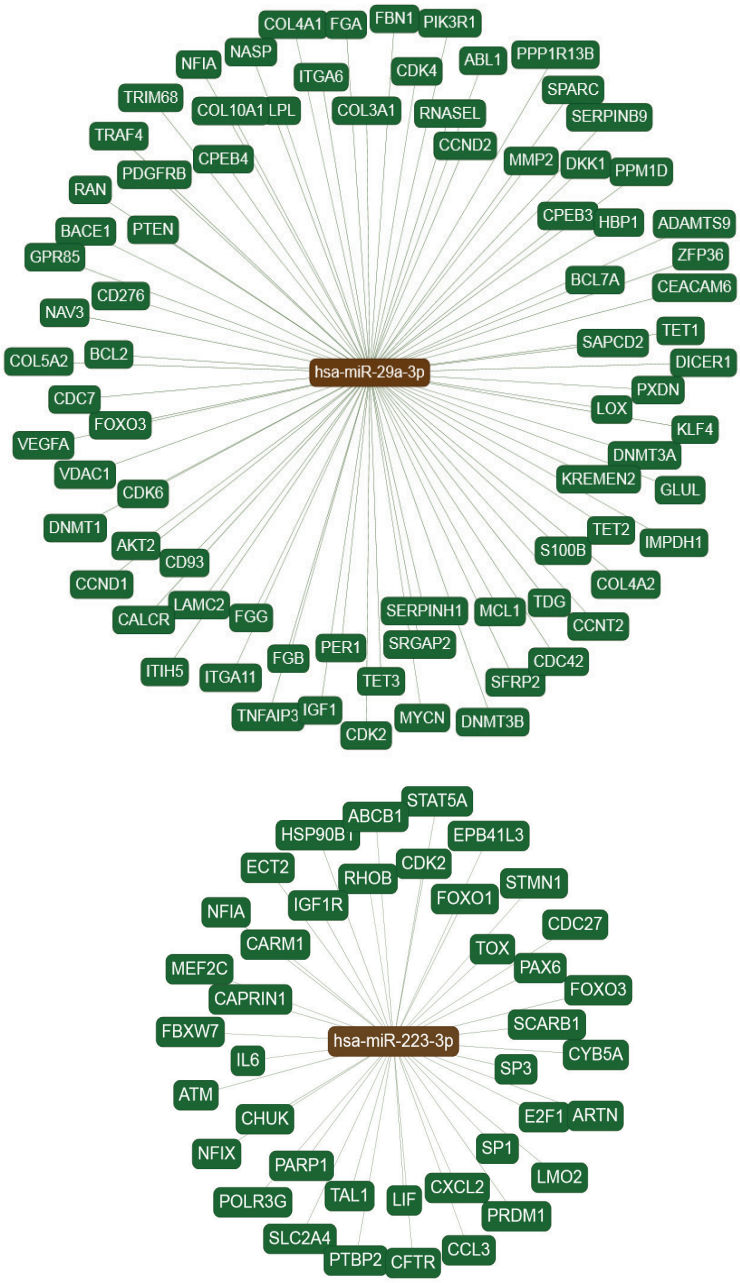
Supplementary Figure 7. PCA biplot of the expression data of six uveitis-associated miRNAs in nine leukocyte cell populations (n=113)**.** The PCA biplot is was generated using Metaboanalyst 3.0.[5] Colors match the populations of figure 4B and 4C. The direction of effect for each miRNA is highlighted.



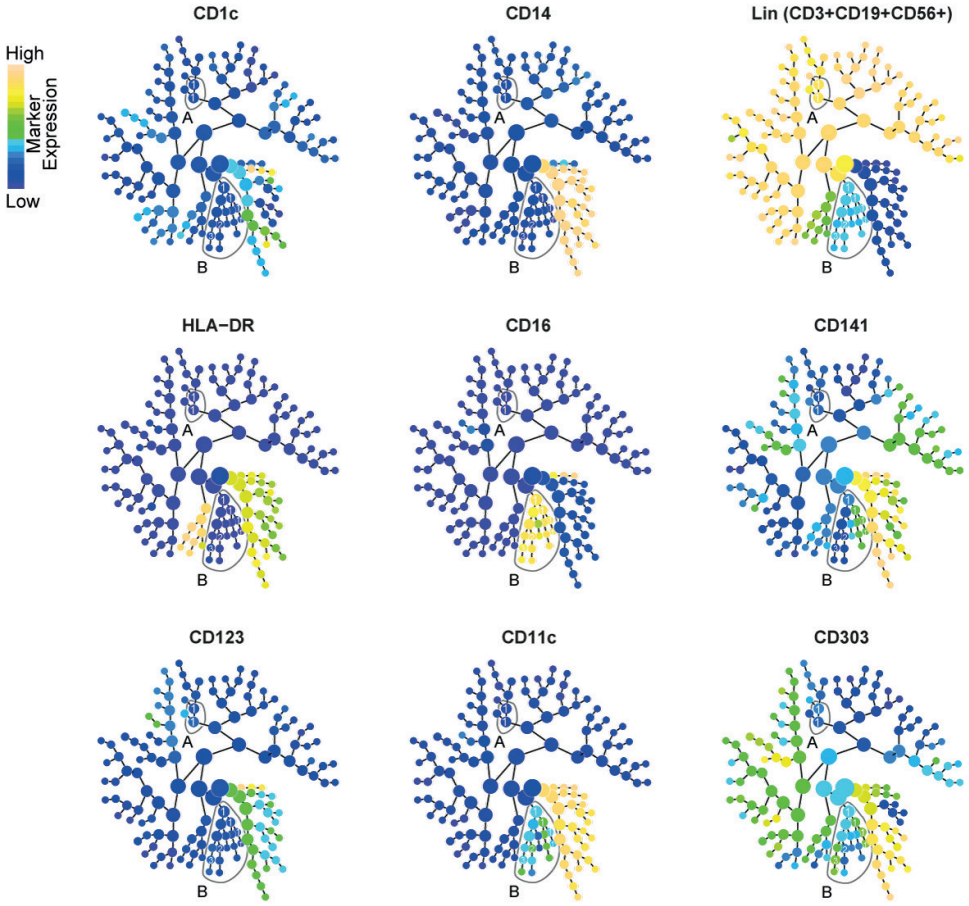
Supplementary figure 8. Normalized relative expression data for each of the uveitis-associated serum miRNAs in cell subsets. Six uveitis associated miRNAs were extracted from miRNA data from nine leukocyte cell subsets (see methods, n=113) and plotted to outline mean, IQR and range per cell subset. Boxplots were generated in Metaboanalyst 3.0 server.[5]



Supplementary Figure 9. Hierarchical clustering of miRNA expression profiles of leukocyte subsets. **Above:** Clustering according to Spearman's correlation for six differentially expressed miRNAs across all leukocyte populations (n=113). **Below:** Hierarchical clustering of miRNA-ome data across all leukocytes subsets. Relative location cluster for each of the Uveitis-associated serum microRNAs are indicated on the right. Plots were generated in Metaboanalyst 3.0 server.[5]



Supplementary Figure 10. Interaction target network for miR-29a-3p and miR-223-3p. The central brown node represents each miRNA surrounded by validated targets with strong experimental evidence (green). Figures were generated using miRTargetLink.[7]



Supplementary Figure 11. Flow cytometry data of peripheral mononuclear cells comparing individuals with high serum miRNA levels versus low. Citrus trees of the unsupervised hierarchical clustering using Citrus. The analysis used 9 cell surface markers: CD1c, CD14, lineage (CD3/CD19/CD56), HLA-DR, CD14, CD16, CD141, CD123, CD11c, CD303. Contours indicate clusters with significant differences in Significance Analysis of Microarrays (SAM), by FDR of 1%. Color scales indicate median intensity of marker expression.

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Chapter 8

CCR6+ T CELLS ARE AN EARLY PREDICTOR OF THE NEED FOR SYSTEMIC IMMUNOMODULATORY TREATMENT IN NON-INFECTIOUS UVEITIS

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ABSTRACT

BACKGROUND: Non-infectious uveitis (NIU) is a severe intra-ocular inflammation, which frequently requires prompt systemic immunosuppressive therapy (IMT) to halt the development of vision-threatening complications. IMT is considered when NIU cannot be treated with corticosteroids alone, which is unpredictable in advance. Previous studies have linked blood cell subsets to glucocorticoid sensitivity, which suggests that the composition of blood leukocytes may early identify patients that will require IMT.

PURPOSE: To map the blood leukocyte composition of NIU and identify cell subsets that stratify patients that required IMT during follow-up.

METHODS: We performed controlled flow cytometry experiments measuring 37 protein markers in the blood of 30 IMT free patients with active acute anterior uveitis, intermediate, and posterior uveitis, and compared these to 15 age- and sex-matched controls. Results from manual gating were validated by automatic gating using FlowSOM.

RESULTS: Patients with uveitis displayed a reduction of Natural Killer cells and an enrichment for memory T cells, in particular the CCR6+ lineages. These results were confirmed by automatic gating by unsupervised clustering using FlowSOM. We observed considerable heterogeneity in memory T cell subsets and abundance of CXCR3-CCR6+ (Th17) cells in the uveitis subtypes. Importantly, regardless of the uveitis subtype, patients that eventually required IMT in the course of the study follow-up exhibited increased CCR6+ T cell abundance before commencing therapy.

CONCLUSION: Using high-dimensional immunoprofiling in NIU patients, we show that CCR6+ T cell abundance can stratify patients who require IMT.

INTRODUCTION

Non-infectious uveitis (NIU) is an umbrella term for a family of intraocular inflammatory conditions that collectively affect more than one in 1000 individuals and account for ~10% of preventable blindness in Western countries.[1,2] This typically recurrent or chronic disorder may lead to poor visual outcome and low quality of life if undertreated.[3,4] Non-corticosteroid systemic immunosuppressive therapy (IMT) is often necessary to control inflammation and reduce the development of vision-threatening complications. According to the current guidelines, IMT should be introduced when NIU is not controlled with corticosteroids alone or corticosteroid-sparing agents are required because of intolerance to corticosteroids. Consequently, there may be a considerable treatment delay in the patients who require IMT to control their uveitis. Because these patients are not sufficiently treated, they have an increased risk of impairment of visual function and may experience significant side effects from systemic corticosteroid use.[5,6] Since, the need for IMT is not accurately predictable there is a clear medical need for early parameters that can identify patients who require IMT.

Several flow cytometry studies in blood of NIU patients have revealed increased numbers of inflammatory T cell populations that typically produce cytokines, such as Interleukin (IL)-17 (e.g., T helper (Th) 17 cells) and Interferon (IFN)-γ (e.g., Th1 cells). In addition, changes in the composition of principal subsets of human myeloid and plasmacytoid dendritic cells were observed that are linked to disease activity.[7,8] Both Th17 and Th1 cells are considered to drive uveitis in animal models, yet in humans the evidence of their contribution to pathology remains merely circumstantial.[9,10] IMT has been shown to selectively affect the phenotype or the function of specific leukocyte populations in blood (e.g. Th17 cells) and suggests that the frequency of these subsets in blood may be different in patients who require IMT.[11–13] To our knowledge, no studies have explored the potential link between the overall composition of blood cells and the need for IMT in clinically well characterized types of NIU.

Here, we conducted a high-dimensional profiling of myeloid and lymphoid cell populations of patients with three archetypical types of human non-infectious uveitis. Using clinical data acquired during follow-up of the patients, we show that the high-dimensional immune profiling can ***a priori*** identify patients who require IMT.

MATERIALS AND METHODS

Patients and patient material

This study was conducted in compliance with the Helsinki principles. Ethical approval was requested and obtained from the Medical Ethical Research Committee in Utrecht and all patients signed written informed consent before participation.

We collected heparinized venous blood from a total of 30 adult patients with HLA-B27-associated Acute Anterior Uveitis (AU, n=10), Idiopathic Intermediate Uveitis (IU, n=9) or Birdshot Uveitis (BU, n=11). Patients were seen at the outbound patient clinic of the uveitis center of excellence at the department of Ophthalmology of the University Medical Center Utrecht between July 2014 and July 2015. All patients had active uveitis (new onset or relapse) at the time of sampling. None of the patients had a related systemic auto-inflammatory or autoimmune disease, nor did they receive systemic immunomodulatory treatment in the last 3 months with the exception of a low dose of oral prednisolone (≤10mg) for 1 BU patient. Uveitis was classified and graded in accordance with the *Standardization of Uveitis Nomenclature* (SUN) classification.[14] Each patient underwent a full ophthalmological examination by an uveitis specialist and routine laboratory screening, including erythrocyte sedimentation rate, renal and liver function tests, serum angiotensin converting enzyme (ACE), and screening for infectious agents (e.g. syphilis, Borrelia, TB) in blood. A chest X-Ray was performed to exclude Sarcoidosis. All patients with BU were HLA-A29 positive in the presence of characteristic birdshot lesions and all patients with AU were HLA-B27 positive. Fifteen age and sex matched anonymous blood donors with no history of ocular inflammatory disease served as healthy controls (HC).

Medical records of uveitis patients were reviewed for demographic information. Follow up data were collected on the development of uveitis related complications (e.g., cystoid macular edema (CME), the development of ocular hypertension (defined as intraocular pressure >21 mm Hg without optic nerve damage or visual field abnormalities but requiring therapeutic intervention)) and the use of systemic immunomodulatory therapy (IMT) (n=23, with complete data). IMT was defined as the use of any systemic immunosuppressive agent (i.e., DMARD, biological etc.) other than oral or intravenous corticosteroid therapy. The necessity of IMT was mostly based on persistent uveitis despite local corticosteroid therapy. In three cases, IMT was necessary to replace periocular steroids because it resulted in high intraocular pressure. The details of the study cohort are shown in **Table 1**.

Table 1. Characteristics of the cohort investigated in this study

	AU	IU	BU	HC	p-value
N	10	9	11	15	NA
Male	30%	22%	36%	40%	0.86*
Age in years; mean ± SD	44.8 ± 18.4	39.3 ± 14.1	53.4 ± 12.7	41.9 ± 10.0	0.11**
Disease duration in years; median (range)	5.7 (0.1-39.3)	3.7 (0.2-20.0)	4.5 (0.2-15.1)	NA	0.19***
Follow-up after sampling in years; median (range)	2.1 (0.2-3.2)	2.8 (1.4-3.4)	2.7 (0.0-3.4)	NA	0.43***
Need for IMT during follow-up [A]	5 (50%)	2 (22%)	8 (73%)	NA	
Initial					
Methotrexate	5 (50%) [B]	0	8 (73%) [D]	NA	
Azathioprine	0	2 (22%) [C]	0	NA	
Switch or addition					
Mycophenolate mofetyl	0	0	2 (18%)	NA	
Mycophenolic acid	0	0	2 (18%)	NA	
Adalimumab	0	0	3 (27%)	NA	

Abbreviations: BU: Birdshot uveitis, AU: HLA-B27 associated anterior uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, NA: not applicable.

* Fishers exact test, ** Wilcoxon rank-sum test,*** Kruskal-Wallis test.

[A] the use of any non-corticosteroid systemic immunosuppressive agent.

[B] reasons for starting IMT: therapy resistance (n=3), persistent activity and steroid response (i.e. secondary rise of intraocular pressure) to parabulbar injections (n=2)

[C] reasons for starting IMT: persistent CME and steroid response to parabulbar injections (n=1), persistent vasculitis (n=1)

[D] reasons for starting IMT: Papillitis or vasculitis with or without CME (n=6), CME resistant to parabulbar steroids (n=1), persistent low-grade activity with visual field defects (n=1)

Flow cytometric phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated by standard ficoll gradient centrifugation immediately after blood withdrawal and stored in liquid nitrogen. To account for potential between-run variability, samples were randomized in 10 batches of 4-5 samples per experiment. Per batch, thawed PBMC cells for each sample were divided for staining with five multi-color antibody panels; a T-helper-panel (250,000 cells), a dendritic cell-panel (2.5 million cells), a T regulatory cell panel, a B-cell panel (both 400,000 cells) and a T cell intracellular cytokine panel (750,000-1,500,000 cells). For the T cell cytokine panel, PBMCs were first incubated for 4h with RPMI-1640 (10% Fetal calf serum) and ***Phorbol 12-myristate 13-acetate*** (PMA), ***Ionomycin*** calcium salt and BD GolgiPlug (BD Biosciences, San Jose, CA, USA). For the other panels, cells were incubated at room temperature (15 min) with 5% mouse serum to minimize non-specific binding of antibodies. Cells were then washed and suspended in phosphate buffered saline (PBS) supplemented with bovine serum albumin (1%) and 0.1% Sodium Azide and incubated (20 min., 4°C) in the dark in V-bottomed plates with Brilliant Stain Buffer (BD, #563794)

and the fluorescently-conjugated antibodies (**Supplementary Table 1**). For intracellular staining (Treg-panel and intracellular cytokine panel), cells were subsequently washed, fixed and permeabilized with a fixation and permeabilization solution according to the manufacturer's instructions (eBioscience, San Diego, CA, USA), and stained (30 min., 4°C) with the appropriate antibodies (**Supplementary Table 1**) in the dark. Flow cytometric analyses were performed on the BD LSR Fortessa™ Cell analyzer (BD Bioscience, San Jose, CA, USA). Data were analyzed using FlowJo software (TreeStar inc. San Carlos, CA, USA). The inter-assay variation was determined by measuring the same normal control (called 'internal control") in each of the 10 different days (separate batches see **Figure 1A**). Across all ten experiments, this sample revealed low inter-assay variation (relative standard deviation <15% for all leukocyte populations with >5% within any of the gates) between aliquots of the same sample.

We performed principal component analysis and hierarchical clustering on normalized frequency data of >100 leukocyte populations for NIU patients and controls. Based on the internal control, principal component analysis revealed high consistency between the 10 independent experiments performed (**Figure 1B**). A subpopulation was derived from its parent and grandparent population as indicated in **Supplementary Table 2**. The following markers were excluded for further analysis based on suboptimal performance (insufficient discriminative capacity by fluorescent intensity across the experiments compared to fluorescent intensity data obtained for the antibodies during optimization of the panels; CCR4 in the T helper panel, and IgD, IgG, IgA in the B cell panel. "Fluorescence-minus-one" (FMO) control experiments were performed for the intracellular cytokine staining before the start of analysis to determine the cut-off between positive and negative cell populations.

Statistical approaches

Statistical analyses on manually gated data were performed in SPSS version 21.0 (SPSS Inc., Chicago IL, USA) or the MetaboAnalyst v4.0.[15] To categorize patients and controls into groups with similar leukocyte composition in blood, data from all ten independent experiments were integrated, and subjected to principal component analysis and hierarchical clustering. For cluster analyses the data were log10-transformed, auto-scaled and compared using a correlation-based (Pearson's) dissimilarity measure, which considers two objects to be similar if their features are highly correlated regardless of their magnitudes and the average linkage method (combine clusters with the smallest average linkage distance). For hierarchical clustering, missing values for each leukocyte population were imputed with the median proportion (%) of all samples.

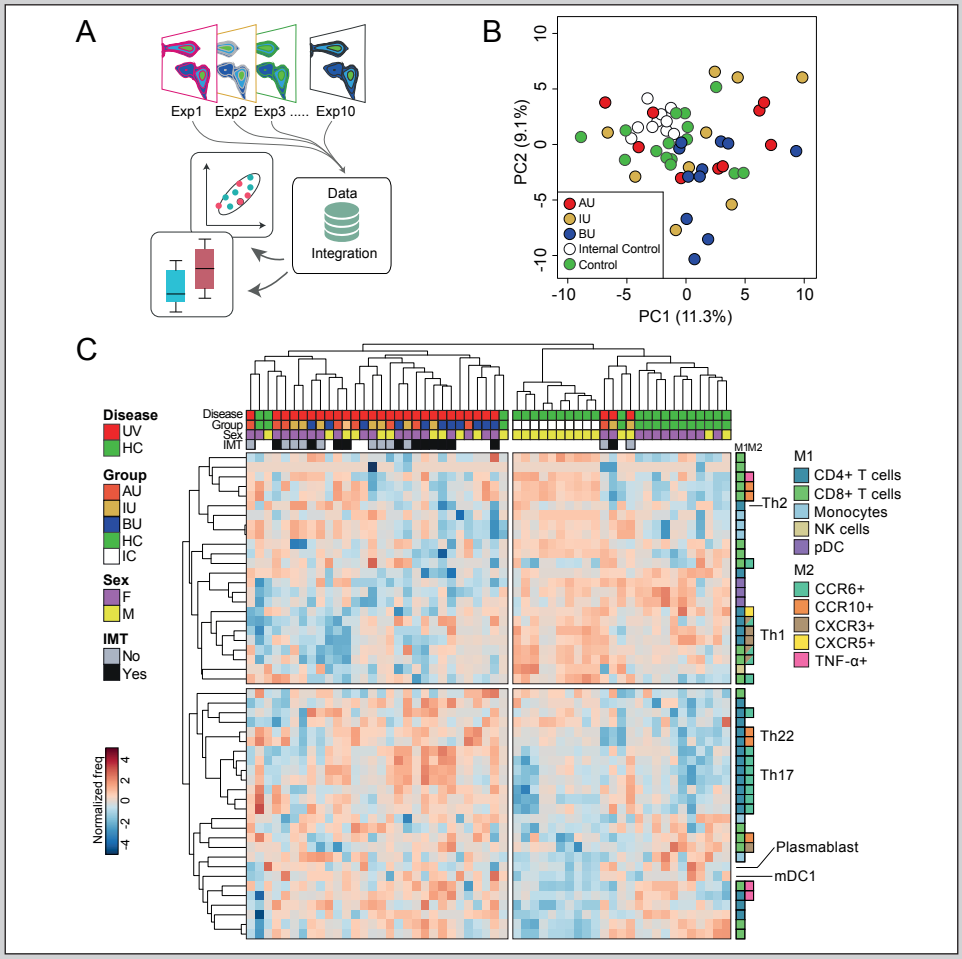


Figure 1. The overall blood leukocyte composition is changed in non-infectious uveitis. **A.** We performed high-dimensional immune profiling of blood of non-infectious uveitis and control samples by multi-color flow cytometry in ten independent experiments that were integrated for meta-analyses. **B.** Principal component analysis (projection onto first two principal components) of blood leukocyte populations of the 45 samples of this study, including one 'internal control' (i.e., the same patient-sample was taken along for each experiment to assess inter-assay variability [IC]). **C.** Heatmap of unsupervised hierarchical clustering of the top 50 immune cell subsets differentiating between uveitis (UV) patients and healthy controls (HC). Heatmap colors represent the changes in proportion from relatively lower proportion (in blue) to higher proportion (in red). Dendrograms indicating the clustering relationships between disease groups and leukocyte populations are shown to the left and above the heatmap. The sample meta-data are indicated above and the leukocyte subsets contributing to these clusters are indicated on the right. M1: first color marker to define leukocyte population. M2: additional color marker to define chemokine receptor and TNF-alpha production in T cells. **Abbreviations:** AU: HLA-B27 associated anterior uveitis, BU: Birdshot Uveitis, F: female, HC: healthy control, IC: internal control, IMT: requirement for systemic (non-corticosteroid) immunosuppressive therapy during follow-up, IU: idiopathic intermediate uveitis, M: male, PC: principal component, Th: T helper cell, TNF: tumor necrosis factor, UV: all uveitis patients combined.

The Wilcoxon rank-sum test or Kruskal–Wallis test with the Dunn's post-hoc test were used to assess group differences. In addition, we performed unsupervised clustering of single cell gated data from the T helper panel by Bioconductor package ***FlowSOM***. FlowSOM uses self-organizing map (SOM) algorithm to cluster cells into distinct clusters. Before executing the SOM algorithm, the flow cytometry data were subjected to logicle transformation and expression of each marker is scaled according to Z-score normalization. We first pooled all the cells together from each sample and performed 2,000 iterations to train the SOM (grid size=100). We then clustered all cells into 100 distinct clusters based on their similarities in high-dimensional space (i.e. using all 11 protein markers).[16] Cluster annotation was performed using consensus hierarchical clustering, as implemented in the ConsensusClusterPlus R package[17]. The number of meta-clusters was determined by FlowSOM algorithm using the elbow criterion allowing for a maximum of 90 clusters. To prevent overfitting of putative rare cell populations, we did not consider clusters with a mean proportion of <0.1% for further analyses.

Meta-analysis of frequency data across all 10 experiments for the 30 uveitis patients and the 15 unaffected controls were reported for subsets with an altered median frequency in the combined dataset below a type I error of 5% ($p<0.05$) and considered significant when manually gated cell populations and clusters identified (FDR <1%) by FlowSOM exhibited similar phenotypes (**Supplementary Table 2**).

RESULTS

Unsupervised hierarchical cluster analysis showed that the overall leukocyte composition was able to separate uveitis patients from healthy controls (**Figure 1C**) as a result of the accumulation of many - but modest - changes in the cell subsets (for detailed information see **Supplementary Table 2**).

Corroborating with prior studies, we noted a decrease in the proportion of plasmacytoid dendritic cells (pDCs, **Supplementary Figure 1**) and natural killer cells (NK, $CD56^+CD3^-$) cells (**Supplementary Figure 2**), and an increased proportion of $CD19^+$ B cells (**Supplementary Table 2**). The proportion of monocyte or myeloid dendritic cell subsets was comparable between NIU and healthy controls (**Supplementary Table 2**). Thus, we find that the composition of a subset of immune cells is altered in the blood NIU patients.

Heterogeneous changes in the T cell repertoire in patients with non-infectious uveitis

Within the $CD3^+$ T cell populations, the ratio of $CD4^+$ to $CD8^+$ T cells was higher in uveitis

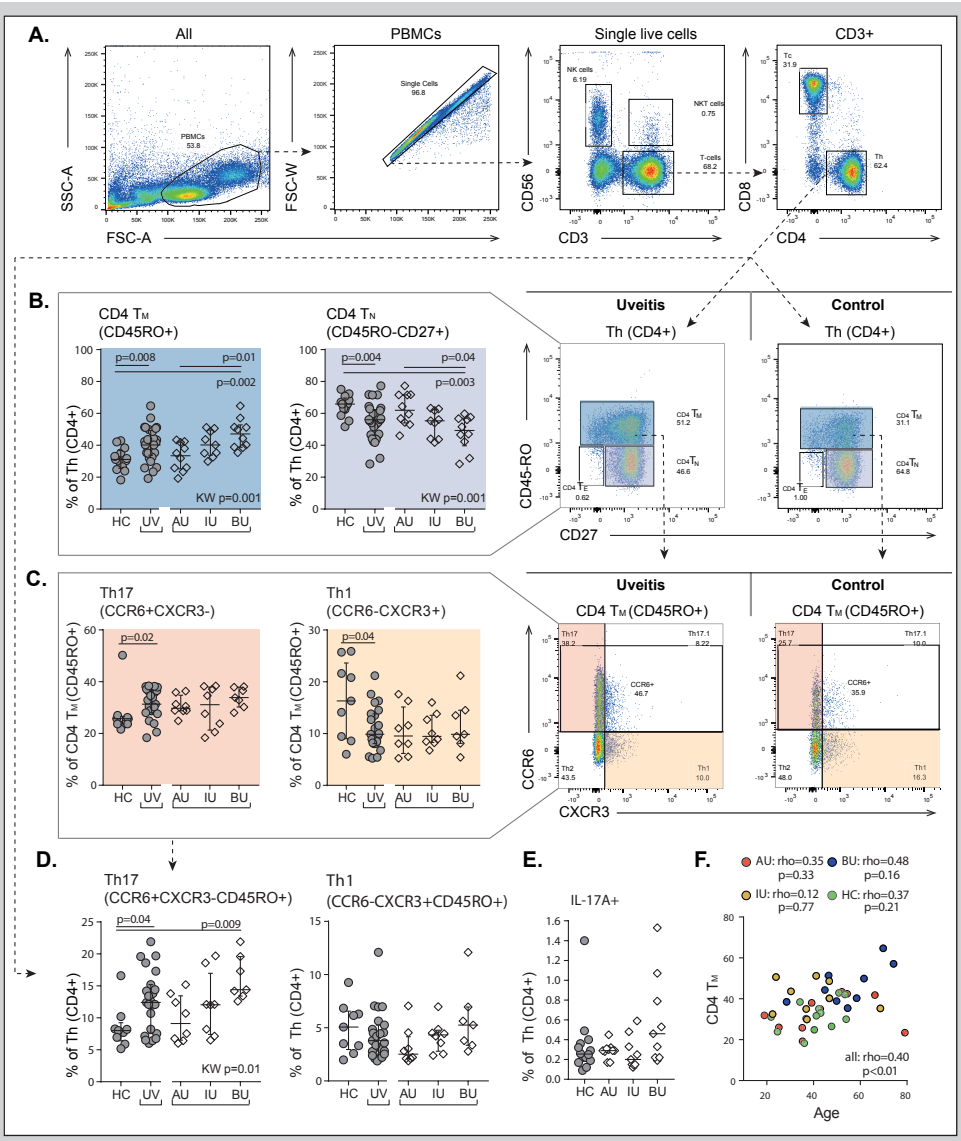


Figure 2. Heterogeneity in memory T cell subsets and abundance of CXCR3-CCR6+ (Th17) cells between uveitis subtypes. **A.** Gating strategy of a representative sample used to identify T helper (Th. $CD3^+CD4^+$) and cytotoxic T cells (Tc. $CD3^+CD8^+$) **B.** Heterogeneity in proportion of naïve and memory T helper cells in uveitis patients and controls. **C.** Within the $CD4^+T_M$ there is an increase of Th17 (CCR6+CXCR3-) and a decrease in Th1 (CCR6-CXCR3+) cells in uveitis patients compared to healthy controls. **D.** BU displayed an increase in Th17 cells within the total $CD4^+$ T cell population compared to the other patient groups **E.** Percentage of IL-17A+ cells within the total $CD4^+$ T cell population **F.** Correlation between the percentage of CD4 T_M cells and age. Dots represent each sample and are colored according to disease group. Bars indicate the median and interquartile range. *(Legend continues on next page)*

Legend Figure 2 continued. P-values between HC and UV are from Wilcoxon rank-sum test , p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing. **Abbreviations:** AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, HC: healthy control, IL: interleukin, IU: idiopathic intermediate uveitis, T_E: effector T cell (CD45RO-CD27-), Th: T helper cell (CD3+CD4+), T_M: memory T cell (CD45RO+), T_N: naïve T cell (CD45RO-CD27+), PBMC: peripheral blood mononuclear cell, TNF: tumor necrosis factor, UV: all uveitis patients combined.

patients (ratio [range]= 2.5 [1.4-9.4] vs 1.5 [1.1-3.3], p=0.03), due to a reduction in $CD8^+$ cells in the $CD3^+$ population (**Supplementary Figure 3A**). Using the expression of the surface markers CD45RO and CD27 on $CD4^+$ and $CD8^+$ T cells, we categorized naïve (T_N, $CD27^+CD45RO^-$), effector (T_E, $CD27^-CD45RO^-$), and memory (T_M, $CD27^{+/-}CD45RO^+$) phenotypes (**Figure 2A-B** and **Supplementary Figure 4)**. The decrease in the proportion of $CD8^+$ T cells is primarily due to diminished $CD8^+$ T_E cells in NIU patients (**Supplementary Figure 3B**). Apart from the differences between NIU and healthy controls, we found that different NIU types exhibited differences within subsets of CD8+ T cells. $CCR10^+$ $CD8^+$ T_M cells were significantly increased in BU patients compared to IU patients (median % [range] IU = 0.9 [0.7-1.3], BU = 2.3 [1.6-3.9], p=0.01 after post-hoc Dunn's correction, **Supplementary Figure 3C**).

In $CD4^+$ T cells, we noted a marked change in the proportion of naïve and memory cells in BU patients, but not for AU patients (**Figure 2A-B**). The frequency of $CD4^+$ T_M cells is known to be associated with age and we found a similar correlation between $CD4^+$ T_M cells and age (rho=0.4, p=0.009, **Figure 2F**).[18] To decouple the effect of age and disease, we used a linear regression model and found that the increased proportion of $CD4^+$ T_M cells was independently associated with uveitis (p=0.02). Within the $CD4^+$ T_M subset, uveitis patients showed a significant increase in the proportion of $CCR6^+CXCR3^-$ cells (Th17) and a decreased proportion of $CCR6^-CXCR3^+$ (Th1) cells (**Figure 2C,** p=0.02 and p=0.04 respectively). BU displayed an increase in $CCR6^+CXCR3^-$ cells (Th17) within the total $CD4^+$ T cell population compared to the other patient groups (**Figure 2D**). A similar trend was noted when considering IL-17-producing $CD4^+$ T cells (**Figure 2E**). We found no specific enrichment for $CD161^+$ Th17 lineage cells in uveitis patients (**Supplementary Table 3**).[19,20]

Finally, we observed a significant decrease in $CD4^+$ T_M $CD27^+CXCR5^+$ cells (i.e., follicular T helper cells) in uveitis patients (**Supplementary Table 3**). There were no changes in the percentage of regulatory ($CD3^+CD4^+CD25^{hi}CD127^{low}FoxP3^+$) T cells (**Supplementary Figure 5**). In summary, we find that the composition of multiple $CD4^+$ and $CD8^+$ T cell subsets were altered in different types of NIU patients.

Computational approach fine map the T and NK cell phenotypes linked to non-infectious uveitis

We further explored the T helper cell panel by automatic gating using ***FlowSOM***[16] that performs multivariate clustering of cells based on the self-organized map (SOM) algorithm and categorizes cells into relevant meta-clusters based on their surface markers. Using FlowSOM, we clustered all individual cells into 100 distinct clusters based on the surface expression marker proteins. Using unsupervised approaches, we further clustered these 100 clusters into 22 meta-clusters that are outlined in the minimal spanning tree shown in **Figure 3** (for details see **Supplementary Figure 6** and methods). This high-dimensional automated gating identified multiple meta-clusters that were linked to uveitis and corroborate the subsets determined by manual gating. We observed a

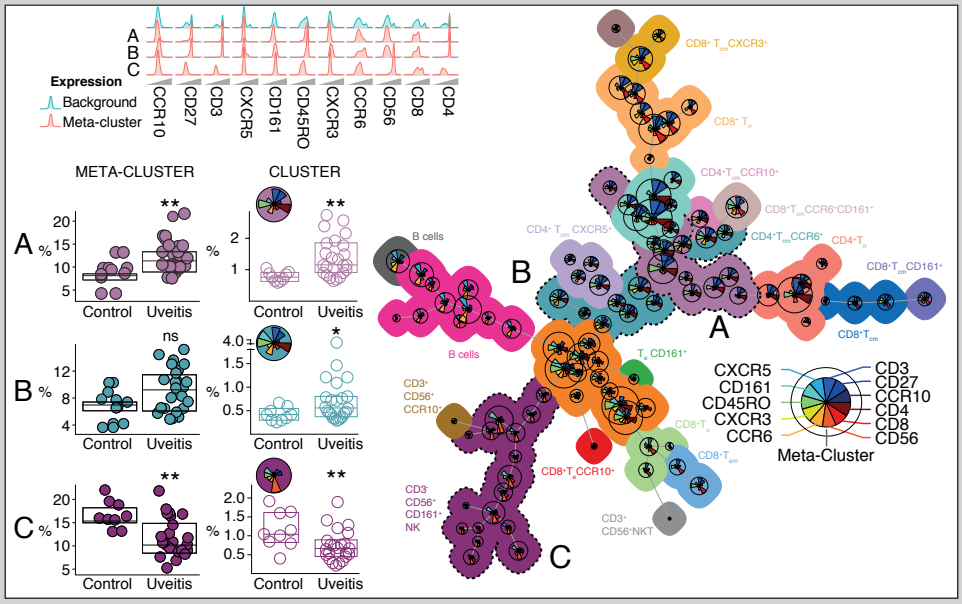


Figure 3. Automatic gating by unsupervised clustering using FlowSOM identifies cell (meta)clusters associated with non-infectious uveitis. Using FlowSOM, we clustered all individual cells within the single-cell gates of all samples into 100 distinct clusters based on the surface protein expression. Using unsupervised clustering, the 100 clusters were clustered into 22 meta-clusters of different cell types and organized in the minimal spanning tree on the right. The meta-clusters are represented by unique colors (all meta-cluster comparisons between uveitis and controls are provided in **Supplementary Figure 6)**. For each cluster (i.e., cell population), pie charts indicate the relative expression for each of the different surface markers and the pie size corresponds to the number of cells in each population. Three clusters and their associated meta-clusters (A-C) are indicated. P-values between HC and UV are from Wilcoxon rank-sum test (*P<0.05, ** P<0.01, ns = non-significant). For these three meta-clusters, the relative densities of normalized expression of the surface protein markers for from the T cell panel are indicated above and for each associated cluster the pie charts provide the relative expression of each surface marker.

significant increase in the proportion of ***meta-cluster A,*** pertaining to memory $CD4^+$ T cells expressing CD45RO and CD27[21] in uveitis patients (**Figure 3**). Analysis by FlowSOM further confirmed a significant decrease in a cluster reminiscent of NK cells ($CD3^-CD56^+$, ***meta-cluster C***, **Figure 3**). We also noted a $CD4^+$ memory meta-cluster (***meta-cluster B***) that contained subsets of Th17 enriched ($CXCR3^-CCR6^+$) clusters that were significantly increased in NIU (**Figure 3**). In short, the leukocyte populations associated to NIU by FlowSOM are similar to the populations identified by manual gating.

CCR6+ T cell subset composition associated with immunosuppressive treatment in uveitis patients before the initiation of therapy

Building on the manual gating, we first explored if the composition of blood leukocytes was different between patients that subsequently required IMT during follow-up. We first subjected the manual gated data from the T helper cell panel to hierarchical clustering to investigate the T cell composition in 23 NIU patients with complete data. Unsupervised clustering distinguished two overarching groups; the first cluster contained near exclusively patients (8/9, 89%) that during follow-up required IMT and the second cluster contained mostly patients that did not require IMT during follow-up (14/17, 82%). The cluster associated with IMT was characterized by increased proportions of $CD4^+$CCR6+ T(h17) populations and decreased proportions of $CD8^+$CCR6+ T cell populations (**Figure 4A**). These findings indicate that patients that will require IMT are characterized by distinct composition of T cells in blood. To further support these observations, we used FlowSOM for head-to-head comparison of IMT+ and IMT- patients. Analysis by FlowSOM supported that a cluster of Th17 cells was significantly more abundant in patients who required IMT (**Figure 4B, Supplementary Figure 6**). Also using manual gating we could establish that Th17 cells were more abundant in patients that require IMT during follow-up (**Figure 4C**). In summary, patients with non-infectious uveitis that in the near future will be treated with IMT show increased Th17 cells in blood.

DISCUSSION

In this study, we demonstrated that patients with anatomically distinct types of non-infectious uveitis (NIU) exhibit shared and unique perturbations in circulating immune cell subsets, most notably, subtype-specific changes in the T cell repertoire. The overall changes in proportions of leukocyte subsets were modest, which is typically reported in flow cytometry studies of non-infectious uveitis.[8,12,22] Importantly, patients that required systemic immunomodulatory treatment (IMT) after sampling could be distinguished ***a priori*** by $CD4^+$ populations expressing CCR6.

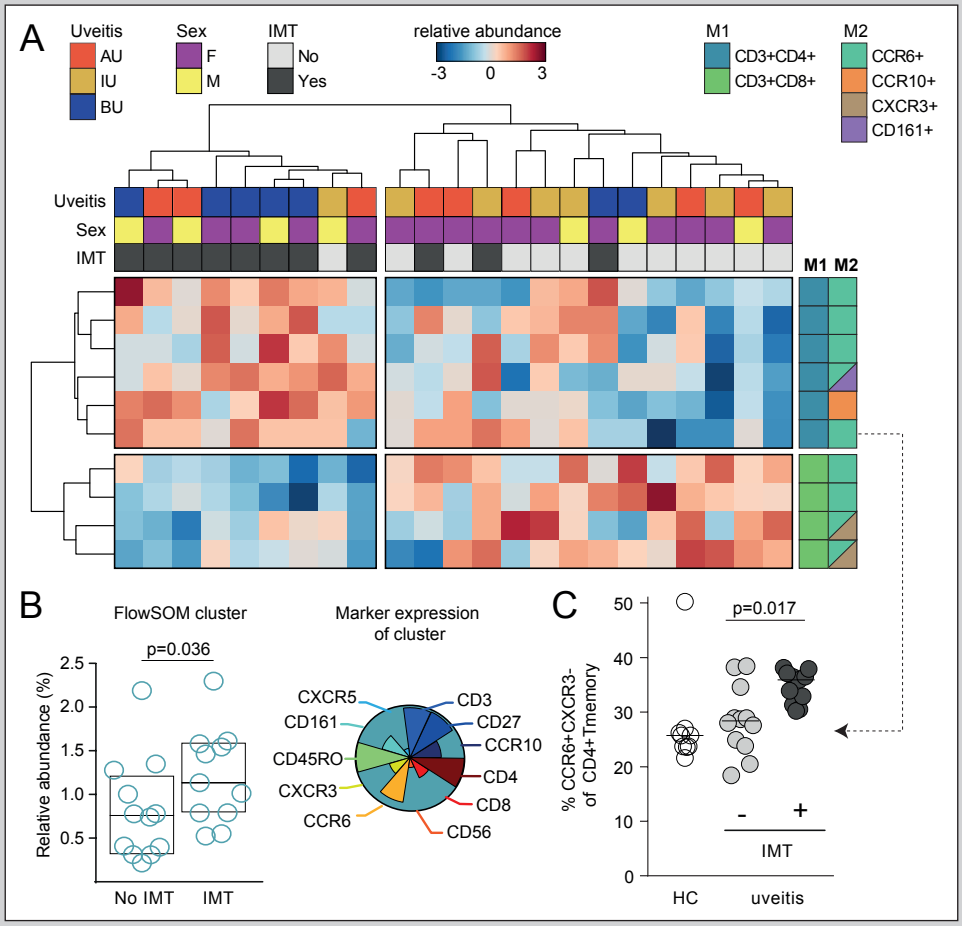


Figure 4. CCR6+ T cells are an early predictor of the need for systemic immunomodulatory treatment.
A. Unsupervised hierarchical clustering of the top 10 T cell subsets in 23 patients with complete data distinguishes two clusters of patients that are characterized by altered proportion of $CD4^+$ and $CD8^+$ T cell populations and differential requirement of IMT during follow-up. Heatmap colors represent the relative difference in proportion from relatively low (in blue) to high (in red) for each manually gated population. Data are normalized using quantum normalization and log transformation. Data are scaled using Auto scaling. The sample meta-data and the leukocyte subsets contributing to these clusters are indicated. M1: first color marker to define CD4 or CD8 expression in T cell populations. M2: additional color marker to define chemokine receptor or CD161 expression for each T cell population. **B.** FlowSOM analysis identified a cluster in meta-cluster B with significantly higher abundance in patients that required IMT during follow-up before commencing therapy. This IMT associated FlowSOM cluster has a phenotype consistent with Th17 cells (CD3+CD4+CXCR3-CCR6+). The scatterplot depicts the proportion of all cells (live single cells) used for FlowSOM analysis. Bars indicate median with interquartile range. **C.** Scatterplot of manually gated Th17 (CXCR3-CCR6+ of CD4+ T_M cells) cells shown for controls and the treatment groups. Bars indicate the median. **Abbreviations:** AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, F: female, HC: healthy control, IMT: requirement for systemic (non-corticosteroid) immunosuppressive therapy during follow-up, IU: idiopathic intermediate uveitis, M: male.

Our understanding of the pathogenic mechanisms underlying NIU remains incomplete, but has unequivocally shown to be immune-mediated.[9,23] In humans, multiple lines of evidence have linked type 17 immune responses (e.g. IL-17-producing T cells) to NIU.[10,22,24–28] In line with this, we observed an increase of ($CCR6^+CXCR3^-$) Th17 cells within CD4 T_M cells that was noticeable for all uveitis types. However, we observed a relatively large uveitis-subtype specific difference in memory CD4 (T_M) cell proportion, which suggests that the involvement of T helper populations might be different between uveitis subtypes.[29] The enrichment for memory CD4 T cells in BU contributed to a hitherto unappreciated larger Th17 fraction in $CD4^+$ T cells in BU over AU. This is an important observation, because to date, studies reporting on increased Th17 cells in human NIU have not accounted for uveitis subtype specific differences or lack of markers that differentiate between memory populations (e.g., define Th17 populations solely on $CD4^+IL\text{-}17^+$ cells).

There remains a lack of understanding which patients will require IMT for durable disease control.[30] Here, we provide evidence that the proportion of $CCR6^+$ T cells, which are enriched for Th17 cells, may harbor predictive capacity for the need of IMT. A recent study demonstrated that flow cytometry phenotyping of Th17 cells can potentially stratify patients to better match the choice of biological (e.g., ***Ustekinumab***) after MTX resistance in patients with psoriatic arthritis, an inflammatory conditions associated with uveitis.[31] Here, we demonstrate that Th17 cells in uveitis harbor a much broader clinical potential for stratifying patients for personalized treatment. Similarly, prospective evaluation of the proportion of Th17 cell populations at disease onset (or recurrence) might enable stratification of patients in need of more prompt IMT intervention. In addition, immunophenotyping of Th17 populations may be particularly useful for identifying patients that may benefit from therapeutic drugs targeting the IL-23/IL-17 axis (e.g. ***Ustekinumab***) or the Th17-related JAK-STAT-pathways.[32–34] It is tempting to speculate on the outcome of previous anti-IL-17 trials in NIU if patients groups were matched according to their T cell repertoires.[32]

High resolution phenotyping of T cell populations using a mass cytometry (e.g., CyTOF) to study a wide array of activation and signaling molecules might be of additive value to better define the T cell fingerprints of NIU, and improve prediction and choice of IMT for individual patients.[35] Also, future studies should not be limited to T cell populations: we recently linked serum microRNA levels of NIU patients to decreased frequency of blood $CD16^+$NK cells.[36] Strikingly, we confirmed a decrease in the proportion of $CD56^+$ NK cells in patients with active uveitis. NK cells (which can be roughly divided in $CD16^+CD56^{dim}$ and $CD16^-CD56^{hi}$ cells[37]) can directly infiltrate the eye during uveitis, perhaps by binding

of their ***Killer immunoglobulin-like*** (KIR) receptors, a class of immune receptors that have been genetically linked to Uveitis.[38,39] Of note, Ustekinumab also targets IL-12, a cytokine elevated in uveitis serum and by which $CD56^+$ NK cells are strongly activated.[40]

We confirmed a decrease in the proportion of circulating plasmacytoid (p)DCs in human non-infectious uveitis, but were unable to ascertain a significant increase in CD1c-positive myeloid DCs (mDC1).[8,41,42] This is likely to be explained due to a different uveitis population. Other novel observations of interest include an increase in ($CD19^+$) B cells and decrease in ($CXCR5^+CD27^+$) T follicular helper cells, a T helper subset linked to B cell function.[43] Note, inhibition of B cells by rituximab has been shown to be effective for some types of NIU.[44,45] Most flow cytometry studies in NIU have focused on the frequency or function of regulatory T cells (Tregs) in blood.[46,47] In our study, we did not observe changes in the proportion of these cells which adds up to the conflicting results in the literature on this topic.[12,22,27,28,48–50]

The results of this study should be considered in light of the following limitations. We deliberately included only uveitis patients with active disease and free from systemic treatment at the time of sampling. Although these stringent selection criteria contributed to several unique observations, the overall small changes in leukocyte percentages in blood combined with the relatively small sample size hampered statistical power. However, by using unsupervised algorithms were able to validate our main observations in T cell and NK cell proportions. The absence of CCR4 in our data may have hampered accurate Th2 frequency estimation, but Th17 and Th1 subsets are well identified using CCR6 and CXCR3.[51] Finally, flow cytometry is limited in number of simultaneous markers (~12-16-colors per panel) and prevents deep phenotyping of cell status (e.g., activation, intra-cellular signaling) of cells. Although FlowSOM supported our gating strategy based on the here investigated markers, subsequent detailed phenotyping focusing on specific subsets (e.g., T and NK populations) or other subsets (e.g., innate-like lymphoid cells, neutrophils) may benefit from using mass cytometry (CyTOF) to obtain more in-depth information on the cell signatures that can predict disease outcome of NIU patients.

In conclusion, NIU is characterized by changes in circulating immune cell subsets, particularly T cell populations that were associated with future IMT requirement. A deeper functional understanding of these observations will aid in optimizing timely therapeutic interventions of this potentially blinding disease.

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Supplementary Table 1. Antibody panel used for Flow Cytometric staining of PBMCs

Panel	Fluorochrome	Marker	Dilution	Clone	Company
Dendritic cell panel	FITC	CD123	1:20	7G3	BD Pharmingen
	PerCP-CY5.5	-	-	-	-
	APC	CD1c	1:10	AD5-8E7	Miltenyi
	AF700	CD3	1:40	UCHT1	BioLegend
	AF700	CD19	1:50	HIB19	eBioscience
	AF700	CD56	1:50	B159	BD Pharmingen
	eFluor780	CD14	1:40	61D3	eBioscience
	BV421	HLA-DR	1:100	L243	BioLegend
	BV510	CD16	1:100	3G8	BD Horizon
	BV605	-	-	-	-
	BV711	CD141	1:50	1A4	BD Horizon
	PE	CD169	1:20	7-239	eBioscience
	PE-Cy7	CD303	1:40	201A	BioLegend
	PE-CF594	CD11c	1:200	B-ly6	BD Horizon
Thelper panel	FITC	CXCR3	1:40	G025H7	BioLegend
	PerCP-CY5.5	CD4	1:25	SK3	BD
	APC	CCR10	1:10	314305	R&D Systems
	AF700	CD3	1:40	UCHT1	BioLegend
	eFluor780	CD27	1:20	L128	eBioscience
	BV421	CXCR5	1:40	RF8B2	BD
	BV510	CD161	1:20	DX12	BD Horizon
	BV605	CCR4	1:40	1G1	BD Horizon
	BV711	CD45RO	1:100	UCHL1	BioLegend
	PE	CCR6	1:40	11A9	BD Pharmingen
	PE-Cy7	CD8	1:200	SK1	BD
	PE-CF594	CD56	1:20	B159	BD
T regulatory panel	FITC	-		-	-
	PerCP-CY5.5	CD4	1:25	SK3	BD
	APC	FoxP3	1:25	PCH101	eBioscience
	AF700	CD3	1:40	UCHT1	BioLegend
	eFluor780	-	-	-	-
	BV421	CD127	1:40	HIL-7R-M21	BD Horizon
	BV510	-	-	-	-
	BV605	-	-	-	-
	BV711	CD45RO	1:100	UCHL1	BioLegend
	PE	CD25	1:50	2A3	BD
	PE-Cy7	CD8	1:200	SK1	BD
	PE-CF594	-	-	-	-
B cel panel	FITC	IgG	1:500	NA	Southern Biotech
	PerCP-CY5.5	CD38	1:100	HIT2	BD Pharmingen
	APC	CD27	1:50	L128	BD
	AF700	CD3	1:40	UCHT1	BioLegend
	eFluor780	CD19	1:20	HIB19	eBioscience
	BV421	IgM	1:50	G20-127	BD
	BV510	IgD	1:500	IA6-2	Biolegend
	BV605	-	-	-	-
	BV711	CD21	1:20	B-ly4	BD
	PE	IgA	1:5000	NA	Southern Biotech
	PE-Cy7	CD10	1:100	HI10A	BD
	PE-CF594	CD24	1:200	ML5	BD Horizon
Intracellular cytokine panel	FITC	IL-17A	1:40	eBio64DEC17	eBioscience
	PerCP-CY5.5	IFN-γ	1:40	4S.B3	eBioscience
	APC	IL-22	1:40	IL22JOP	eBioscience
	AF700	CD3	1:40	UCHT1	BioLegend
	eFluor780	CD4	1:40	RPA-T4	eBioscience
	BV421	TNF-α	1:100	MAb11	BD Horizon
	BV510	CD27	1:50	L128	BD Horizon
	BV605	-		-	-
	BV711	CD45RO	1:100	UCHL1	BioLegend
	PE	IL-21	1:10	3A3-N2.1	BD Pharmingen
	PE-Cy7	CD8	1:200	SK1	BD
	PE-CF594	IL-9	1:20	MH9A3	BD Horizon

Supplementary Table 2. Frequencies of manually gated cell subsets

Cell subset	% of	N	HC median (IQR)	UV median (IQR)	p-val	AU median (IQR)	IU median (IQR)	BU median (IQR)	p-val	posthoc group	p-adj*
PBMCs (live single)	all cells	42	51.8 (46.7-60.7)	61.1 (54.1-68.0)	**0.023**	60.9 (52.5-68.3)	61.2 (54.4-69.9)	61.6 (53.3-69.9)	0.16		
NK ($CD3^-CD56^+$)	PBMCs	32	14.4 (11.8-16.3)	9.6 (7.4-12.8)	**0.005**	9.7 (7.5-12.0)	10.5 (8.2-14.8)	7.4 (6.2-12.8)	**0.02**	**HC-BU**	**0.04**
NKT ($CD3^+CD56^+$)	PBMCs	32	1.00 (0.6-2.6)	1.0 (0.6-2.6)	0.95	1.0 (0.6-1.7)	1.1 (0.5-2.2)	0.9 (0.5-5.0)	0.97		
Tcells ($CD3^+CD56^-$)	PBMCs	42	55.9 (52.4-62.5)	55.3 (49.3-65.1)	0.69	59.1 (50.0-66.3)	52.8 (47.0-58.5)	59.4 (49.5-65.6)	0.46		
$CD4^+$	Tcells	42	57.6 (51.1-70.5)	64.8 (56.5-73.6)	0.09	64.8 (55.3-73.3)	64.8 (53.2-71.7)	64.9 (59.4-77.8)	0.28		
T_N ($CD45RO^-CD27^+$)	Th (CD4+)	42	65.9 (60.6-69.6)	56.0 (46.3-62.4)	**0.004**	62.0 (54.1-71.7)	55.4 (43.9-62.4)	49.3 (39.7-56.9)	**0.001**	**HC-BU**	**0.003**
										AU-BU	**0.04**
T_E ($CD45RO^-CD27^-$)	Th (CD4+)	42	1.1 (0.7-1.5)	0.9 (0.7-1.7)	0.96	0.9 (0.6-1.5)	1.5 (0.8-2.7)	0.8 (0.6-2.0)	0.30		
T_M ($CD45RO^+$)	Th (CD4+)	42	31.1 (25.7-36.8)	40.2 (33.8-49.3)	**0.008**	33.5 (25.2-42.0)	40.2 (33.8-49.6)	47.1 (38.8-52.8)	**0.001**	**HC-BU**	**0.002**
										AU-BU	**0.01**
Th1 ($CCR6^-CXCR3^+$)	CD4 T_M	32	16.3 (9.0-23.7)	9.9 (8.1-13.8)	**0.044**	9.5 (6.2-15.1)	9.5 (8.3-13.5)	9.9 (8.1-14.5)	0.22		
Th1 ($CCR6^-CXCR3^+$ $CD45RO^+$)	Th (CD4+)	32	5.1 (3.0-6.6)	3.8 (2.5-5.3)	0.44	2.5 (2.1-4.2)	4.3 (2.8-4.8)	5.3 (3.4-7.0)	0.16		
Th2 ($CCR6^-CXCR3^-$)	CD4 T_M	32	41.7 (37.6-54.0)	46.8 (41.8-55.5)	0.40	52.0 (47.1-55.4)	43.1 (41.9-64.8)	39.6 (31.4-50.9)	0.17		
Th2 ($CCR6^-CXCR3^-$ $CD45RO^+$)	Th (CD4+)	32	12.7 (10.5-17.5)	18.6 (13.6-21.4)	**0.049**	16.6 (11.8-20.8)	19.9 (15.8-24.3)	16.5 (14.6-32.0)	0.15		
Th17 ($CCR6^+CXCR3^-$)	CD4 T_M	32	25.7 (23.7-26.6)	31.3 (28.0-36.5)	**0.018**	29.7 (28.8-34.8)	31.1 (21.3-37.9)	33.9 (30.2-37.9)	0.07		
Th17 ($CCR6^+CXCR3^-$ $CD45RO^+$)	Th (CD4+)	32	8.0 (6.4-9.2)	12.4 (7.5-15.2)	**0.038**	9.1 (6.5-13.5)	12.1 (7.4-17.0)	14.4 (13.4-19.6)	**0.010**	**HC-BU**	**0.009**
Th17.1 ($CCR6^+CXCR3^+$)	CD4 T_M	32	10.0 (7.0-14.6)	8.2 (5.4-13.7)	0.52	7.2 (5.1-9.3)	9.3 (5.1-13.1)	16.5 (5.9-17.6)	0.31		
Th17.1 ($CCR6^+CXCR3^+$ $CD45RO^+$)	Th (CD4+)	32	2.8 (2.1-4.5)	3.5 (2.0-6.0)	0.82	2.1 (1.3-3.3)	3.8 (1.7-5.6)	6.4 (2.1-9.8)	0.06		
Th22 ($CCR6^+CCR10^+$)	CD4 T_M	32	1.7 (1.1-2.5)	1.9 (1.1-3.1)	0.93	2.3 (1.6-3.2)	1.3 (0.7-2.3)	1.8 (1.1-5.3)	0.57		
Th22 ($CCR6^+CCR10^+$ $CD45RO^+$)	Th (CD4+)	32	0.5 (0.3-0.9)	0.8 (0.4-1.2)	0.54	0.9 (0.4-1.2)	0.5 (0.3-1.1)	1.2 (0.4-2.1)	0.36		
Tfh ($CXCR5^+CD27^+$)	CD4 T_M	42	25.0 (17.4-29.7)	17.0 (14.1-22.2)	**0.002**	16.1 (13.8-22.9)	14.8 (13.2-18.5)	19.2 (16.2-23.7)	**0.011**	**HC-IU**	**0.01**
Tfh ($CXCR5^+CD27^+$ $CD45RO^+$)	Th (CD4+)	42	7.8 (5.0-10.0)	7.0 (5.4-8.5)	0.51	6.4 (3.6-7.1)	6.5 (5.1-7.4)	9.0 (8.0-10.3)	**0.033**	-	
$CCR6^+$	CD4 T_M	32	35.9 (34.9-41.1)	40.1 (36.8-47.5)	0.20	38.0 (36.9-40.1)	42.1 (26.7-49.3)	47.5 (40.1-53.3)	0.18		

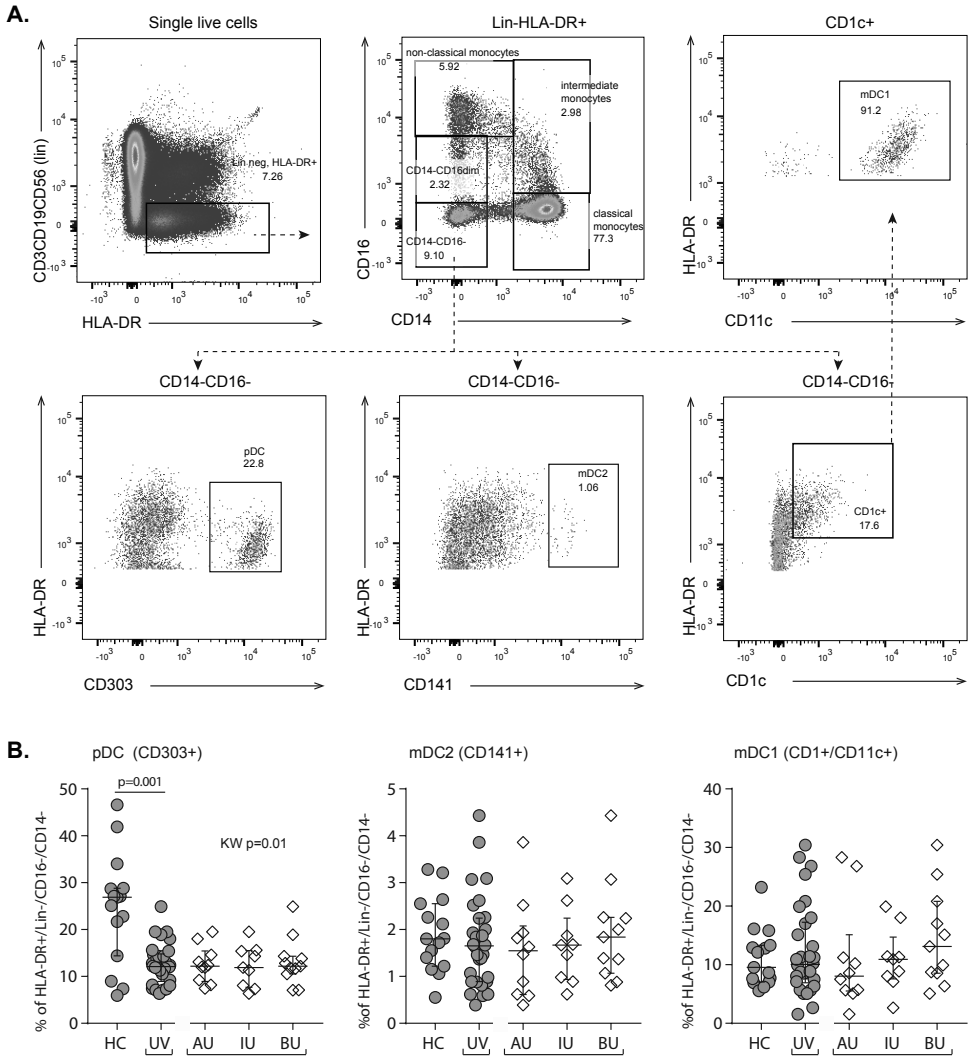
CCR6$^+$CD45RO$^+$	Th (CD4+)	32	10.4 (9.3-14.5)	15.7 (10.0-20.7)	0.10	11.5 (8.3-15.8)	16.0 (9.2-21.4)	20.7 (15.7-27.1)	**0.009**	**HC-BU**	**0.02**
										AU-BU	**0.02**
CD161$^+$	CD4 T_M	42	22.3 (16.7-25.5)	19.9 (15.7-23.9)	0.41	17.1 (13.7-21.7)	21.7 (15.9-23.6)	18.8 (15.3-27.3)	0.48		
CD161$^+$CD45RO$^+$	Th (CD4+)	42	5.7 (5.2-8.0)	7.8 (6.0-10.4)	0.17	6.0 (3.8-7.9)	9.8 (6.0-10.9)	9.2 (7.6-11.3)	**0.022**	**AU-BU**	**0.04**
CD161$^+$CCR6$^+$	CD4 T_M	32	10.3 (6.4-14.2)	8.2 (5.5-12.9)	0.46	8.2 (6.5-9.7)	9.7 (4.0-14.4)	9.9 (5.5-15.4)	0.73		
CD161$^+$CCR6$^+$ CD45RO$^+$	Th (CD4+)	32	3.0 (2.2-3.9)	2.9 (2.0-6.0)	0.71	2.6 (1.9-3.0)	3.9 (1.5-7.0)	3.8 (2.9-7.4)	0.23		
CD161$^-$CCR6$^+$	CD4 T_M	32	28.1 (20.4-30.5)	30.6 (26.4-34.6)	0.22	28.4 (26.8-31.3)	28.5 (18.3-35.7)	34.6 (31.6-41.6)	0.08		
CD161$^-$CCR6$^+$ CD45RO$^+$	Th (CD4+)	32	6.7 (6.1-11.2)	12.1 (7.1-15.3)	0.08	9.0 (6.3-11.8)	10.9 (6.3-14.5)	16.3 (12.9-21.4)	**0.012**	**HC-BU**	**0.01**
										AU-BU	**<0.05**
CD161$^+$ Th17 (CCR6+CXCR3-)	Th17 cells	32	31.4 (20.9-37.4)	29.4 (22.2-33.6)	0.54	28.5 (22.8-32.2)	32.8 (20.4-36.3)	25.7 (21.1-36.1)	0.87		
CD161$^+$ Th17 (CCR6+CXCR3-)	CD4 T_M	32	8.1 (6.1-10.2)	8.6 (7.0-12.2)	0.54	8.0 (7.3-9.4)	9.3 (5.3-12.6)	8.8 (7.0-13.4)	0.88		
CD161$^+$ Th17 (CCR6+CXCR3-)	Th (CD4+)	32	2.4 (1.9-2.9)	3.4 (2.1-4.7)	0.06	2.4 (1.8-3.4)	3.5 (2.1-5.6)	4.5 (4.1-5.0)	**0.033**	**HC-BU**	**0.04**
CD161$^-$ Th17 (CCR6+CXCR3-)	Th17 cells	32	68.2 (61.9-79.1)	73.1 (66.2-77.0)	0.41	73.5 (67.8-76.7)	67.9 (64.5-79.6)	74.1 (63.8-80.3)	0.85		
CD161$^-$ Th17 (CCR6+CXCR3-)	CD4 T_M	32	16.4 (15.1-20.0)	22.4 (19.8-26.4)	**0.014**	22.0 (21.1-23.8)	20.3 (15.6-26.2)	24.2 (22.4-27.3)	**0.035**	**HC-BU**	**0.03**
CD161$^-$ Th17 (CCR6+CXCR3-)	Th (CD4+)	32	5.6 (4.0-7.2)	9.0 (5.6-11.8)	**0.029**	6.5 (4.6-9.4)	7.6 (5.7-12.0)	10.2 (9.0-14.8)	**0.014**	**HC-BU**	**0.01**
CD161$^+$CCR6$^-$	CD4 T_M	32	4.3 (2.3-6.0)	2.6 (1.8-4.0)	0.10	2.1 (1.5-3.6)	2.9 (2.0-6.8)	2.9 (1.0-4.0)	0.24		
CD161$^+$CCR6$^-$ CD45RO$^+$	Th (CD4+)	32	1.1 (0.7-1.7)	1.0 (0.6-1.8)	0.65	0.6 (0.4-1.0)	1.3 (0.7-2.7)	1.4 (0.6-2.0)	0.27		
Treg (FoxP3$^+$CD25^{hi})	CD4 T_M	36	9.1 (6.6-12.4)	9.4 (6.8-11.1)	0.86	9.6 (8.6-12.1)	8.5 (6.1-11.1)	9.4 (5.6-12.1)	0.67		
Treg (FoxP3$^+$CD25^{hi})	Th (CD4+)	36	5.5 (3.4-5.9)	5.3 (4.5-6.2)	0.48	4.8 (4.4-6.1)	5.2 (4.3-6.5)	5.8 (4.3-7.9)	0.86		
CD8+	Tcells (CD3$^+$)	42	35.1 (23.6-40.7)	26.9 (19.3-35.2)	**0.027**	25.3 (16.6-35.9)	26.9 (22.3-35.9)	27.2 (18.2-32.8)	0.15		
T_N (CD45RO$^-$CD27$^+$)	Tc (CD8+)	42	49.0 (31.5-63.4)	58.8 (35.5-73.9)	0.30	61.5 (36.1-81.8)	44.1 (35.5-73.9)	59.0 (28.8-64.4)	0.61		
T_E (CD45RO$^-$CD27$^-$)	Tc (CD8+)	42	20.3 (14.7-29.0)	9.2 (3.5-21.9)	**0.029**	8.5 (3.2-24.3)	6.8 (3.1-20.8)	9.9 (4.1-43.4)	0.12		
T_M (CD45RO$^+$)	Tc (CD8+)	42	20.8 (16.0-36.5)	26.4 (19.5-32.8)	0.77	23.3 (11.7-31.0)	31.9 (19.9-38.8)	26.0 (22.6-27.6)	0.52		
CXCR3$^-$CCR6$^+$	CD8 T_M	32	13.1 (6.9-21.4)	9.8 (6.4-15.3)	0.69	13.4 (8.9-16.8)	8.8 (6.4-30.1)	6.4 (5.4-15.3)	0.78		
CXCR3$^+$CCR6$^+$	CD8 T_M	32	4.7 (2.5-6.2)	3.9 (1.4-5.8)	0.30	3.9 (1.2-5.8)	4.1 (1.7-5.8)	2.3 (1.9-5.8)	0.77		
CXCR3$^+$CCR6$^-$	CD8 T_M	32	20.4 (15.0-26.5)	16.4 (10.5-24.6)	0.23	16.8 (7.1-23.7)	15.1 (8.7-24.4)	14.3 (13.7-27.6)	0.66		
CXCR3$^-$CCR6$^-$	CD8 T_M	32	58.3 (48.5-71.8)	68.3 (45.6-80.7)	0.41	68.2 (55.9-79.9)	67.2 (42.2-83.1)	68.3 (45.6-79.0)	0.86		

$CD161^+CCR6^+$	CD8 T_M	32	10.8 (4.8-20.5)	7.0 (3.1-13.8)	0.41	9.1 (5.2-13.6)	5.7 (1.0-27.1)	3.1 (1.6-13.8)	0.63		
$CD161^-CCR6^+$	CD8 T_M	32	6.0 (4.9-7.9)	6.8 (5.4-8.1)	0.65	6.2 (4.9-8.3)	6.8 (6.1-8.1)	6.7 (4.5-8.0)	0.92		
$CCR10^+$	CD8 T_M	42	1.2 (0.8-2.3)	1.6 (1.0-2.8)	0.45	1.9 (1.1-5.2)	0.9 (0.7-1.3)	2.3 (1.6-3.9)	**0.011**	**IU-BU**	**0.01**
$CXCR3^-CCR6^+$	CD8 T_E	32	4.1 (2.2-6.7)	4.3 (2.4-7.3)	0.79	3.1 (2.5-7.2)	4.9 (4.3-12.5)	2.2 (1.8-14.9)	0.33		
$CXCR3^+CCR6^+$	CD8 T_E	32	1.5 (0.6-2.9)	1.0 (0.3-1.6)	0.16	0.3 (0.2-2.2)	1.3 (0.8-1.6)	0.8 (0.3-1.3)	0.39		
$CXCR3^+CCR6^-$	CD8 T_E	32	4.1 (1.9-9.9)	3.0 (1.3-4.3)	0.15	1.7 (0.7-6.3)	2.8 (1.6-6.5)	3.3 (2.9-4.3)	0.40		
$CXCR3^-CCR6^-$	CD8 T_E	32	91.1 (83.3-93.1)	92.5 (83.3-95.7)	0.43	94.7 (84.7-97.0)	90.5 (78.8-92.9)	93.5 (80.6-96.1)	0.34		
$CD161^+CCR6^+$	CD8 T_E	32	1.4 (1.0-2.1)	0.9 (0.3-2.7)	0.57	1.2 (0.6-1.9)	2.0 (0.1-7.3)	0.4 (0.2-6.8)	0.74		
$CD161^-CCR6^+$	CD8 T_E	32	3.6 (2.8-4.6)	3.4 (2.1-5.4)	0.95	2.9 (1.6-5.2)	5.2 (3.2-5.5)	3.3 (1.2-3.4)	0.36		
$CCR10^+$	CD8 T_E	42	0.4 (0.3-1.0)	0.5 (0.1-0.8)	0.84	0.4 (0.2-1.0)	0.3 (0.1-0.6)	0.6 (0.1-1.2)	0.57		
$IL9^+$	Th (CD4+)	42	0.03 (0.02-0.05)	0.04 (0.02-0.06)	0.64	0.04 (0.02-0.06)	0.02 (0.01-0.06)	0.04 (0.02-0.07)	0.75		
$IL17^+$	Th (CD4+)	42	0.28 (0.19-0.37)	0.29 (0.19-0.46)		0.29 (0.25-0.31)	0.20 (0.14-0.40)	0.46 (0.22-0.93)	0.16		
$IL21^+$	Th (CD4+)	42	1.9 (1.1-2.9)	1.6 (1.4-2.3)	0.69	1.7 (1.1-3.2)	1.6 (1.2-2.0)	1.6 (1.5-2.4)	0.86		
$IL22^+$	Th (CD4+)	42	0.18 (0.09-0.24)	0.19 (0.09-0.36)		0.19 (0.10-0.28)	0.14 (0.08-0.38)	0.38 (0.11-1.00)	0.26		
$TNFa^- IFN^+$	Th (CD4+)	42	1.3 (0.5-2.1)	1.4 (1.1-2.0)	0.34	1.2 (0.9-1.8)	1.4 (1.0-2.4)	1.5 (1.2-2.5)	0.61		
$TNFa^+ IFN^+$	Th (CD4+)	42	5.3 (2.9-9.9)	7.3 (4.5-11.0)	0.23	6.2 (3.6-9.5)	5.5 (4.1-13.3)	9.0 (7.1-14.5)	0.20		
$TNFa^+ IFN^-$	Th (CD4+)	42	29.4 (21.4-34.8)	27.5 (20.4-35.7)	0.92	25.3 (17.1-34.9)	20.5 (15.3-32.1)	33.4 (25.4-39.0)	0.25		
$TNFa^- IFN^-$	Th (CD4+)	42	65.5 (51.4-74.8)	61.0 (49.2-73.4)	0.69	61.8 (56.6-78.3)	70.2 (52.1-75.0)	54.1 (46.9-61.9)	0.18		
$IL9^+$	Tc (CD8+)	42	0.52 (0.44-0.74)	0.62 (0.42-0.92)	0.49	0.58 (0.40-0.74)	0.59 (0.27-0.84)	0.92 (0.57-1.06)	0.22		
$IL17^+$	Tc (CD8+)	42	0.08 (0.05-0.15)	0.06 (0.04-0.09)		0.06 (0.05-0.10)	0.06 (0.03-0.10)	0.06 (0.05-0.09)	0.31		
$IL21^+$	Tc (CD8+)	42	0.01 (0.00-0.02)	0.01 (0.01-0.02)	0.33	0.01 (0.01-0.02)	0.01 (0.01-0.02)	0.02 (0.01-0.04)	0.13		
$IL22^+$	Tc (CD8+)	42	0.02 (0.01-0.04)	0.02 (0.01-0.08)		0.02 (0.01-0.04)	0.02 (0.01-0.04)	0.05 (0.01-0.14)	0.55		
$TNFa^- IFN^+$	Tc (CD8+)	42	11.8 (6.3-16.8)	10.8 (4.7-15.4)	0.63	5.1 (4.2-14.5)	10.7 (5.1-17.0)	12.8 (6.2-16.3)	0.68		
$TNFa^+ IFN^+$	Tc (CD8+)	42	24.8 (12.8-34.7)	22.7 (11.6-34.4)	0.77	20.6 (7.7-35.3)	20.6 (9.4-29.3)	24.5 (18.2-40.2)	0.55		
$TNFa^+ IFN^-$	Tc (CD8+)	42	6.7 (4.4-9.4)	7.3 (4.7-12.4)	0.54	6.5 (4.3-9.2)	9.0 (3.2-14.1)	6.9 (5.0-13.8)	0.64		
$TNFa^- IFN^-$	Tc (CD8+)	42	56.0 (41.0-72.0)	51.1 (43.8-71.2)	0.75	53.9 (38.8-83.8)	53.2 (45.8-73.9)	50.4 (41.3-57.8)	0.57		
$HLA\text{-}DR^+Lin^-$	PBMCs	45	14.9 (11.0-20.5)	17.9 (12.6-24.3)	0.32	16.1 (12.1-22.5)	23.5 (18.0-27.8)	13.7 (12.4-23.2)	0.19		
Classical monocytes ($CD14^+CD16^-$)	$HLA\text{-}DR^+Lin^-$	45	75.1 (68.9-77.3)	76.4 (74.1-80.4)	0.20	76.8 (74.8-79.8)	76.6 (75.3-83.5)	73.4 (68.0-78.6)	0.14		
Class. monocytes ($CD14^+CD16^-HLA\text{-}DR^+Lin^-$)	PBMCs	45	11.5 (8.3-15.0)	12.5 (9.3-19.3)	0.32	12.5 (9.7-17.5)	18.4 (13.9-21.9)	9.4 (8.5-19.8)	0.12		

Intermediate monocytes ($CD14^+CD16^+$)	$HLA\text{-}DR^+Lin^-$	45	3.8 (3.5-5.7)	4.1 (2.8-5.6)	0.72	3.9 (2.7-4.9)	2.9 (2.7-4.7)	5.2 (3.9-7.0)	0.33		
Intermediate Monocytes ($CD16^+CD14^+HLA\text{-}DR^+Lin^-$)	PBMCs	45	0.6 (0.5-1.0)	0.7 (0.5-1.0)	0.57	0.6 (0.4-0.8)	0.8 (0.6-1.1)	0.6 (0.5-1.1)	0.57		
Non-class. monocytes ($CD16^+CD14^-$)	$HLA\text{-}DR^+Lin^-$	45	5.9 (4.8-11.8)	5.7 (2.2-8.3)	0.06	5.9 (2.3-7.6)	3.7 (1.1-7.1)	7.9 (2.9-10.5)	0.11		
Non-class. monocytes ($CD16^+CD14^-HLA\text{-}DR^+Lin^-$)	PBMCs	45	1.2 (0.6-2.1)	0.9 (0.3-1.6)	0.24	1.0 (0.2-1.8)	0.5 (0.3-1.7)	1.1 (0.4-1.4)	0.69		
pDC ($CD303^+$)	$CD14^-CD16^-HLA\text{-}DR^+Lin^-$	45	26.9 (14.4-28.8)	12.1 (9.0-14.8)	**0.001**	12.2 (9.0-15.5)	11.9 (7.7-15.5)	12.2 (10.5-14.3)	**0.013**	-	
pDC ($CD303^+CD14^-CD16^-$)	$HLA\text{-}DR^+Lin^-$	45	2.4 (1.7-3.2)	1.2 (0.8-1.6)	**0.001**	1.1 (0.8-1.5)	1.1 (0.8-1.4)	1.2 (0.7-1.9)	**0.010**	**HC-AU**	**<0.05**
										HC-IU	**0.03**
pDC ($CD303^+CD14^-CD16^-HLA\text{-}DR^+Lin^-$)	PBMCs	45	0.3 (0.2-0.5)	0.2 (0.1-0.3)	**0.035**	0.2 (0.1-0.3)	0.3 (0.1-0.4)	0.2 (0.1-0.3)	0.11		
mDC2 ($CD141^+$)	$CD14^-CD16^-HLA\text{-}DR^+Lin^-$	45	1.8 (1.2-2.6)	1.7 (0.9-2.2)	0.41	1.6 (0.6-2.1)	1.7 (0.9-2.2)	1.8 (1.1-2.3)	0.70		
mDC2 ($CD141^+$)	$HLA\text{-}DR^+Lin^-$	45	0.2 (0.1-0.2)	0.1 (0.1-0.2)	0.19	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.27		
mDC2 ($CD141^+$)	PBMCs	45	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.84	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.45		
mDC1 ($CD1^+CD11c^+$)	$CD14^-CD16^-HLA\text{-}DR^+Lin^-$	45	9.6 (7.1-13.3)	10.1 (6.9-17.3)	0.89	8.1 (5.5-15.1	10.9 (7.6-14.7)	13.1 (8.6-20.8)	0.48		
mDC1 ($CD1^+CD11c^+$)	$HLA\text{-}DR^+Lin^-$	45	0.9 (0.7-1.5)	1.0 (0.6-1.4)	0.81	0.8 (0.5-1.3)	0.9 (0.5-1.4)	1.1 (0.9-1.9)	0.34		
mDC1 ($CD1^+CD11c^+$)	PBMCs	45	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.41	0.1 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.2)	0.38		
B cells ($CD19^+$)	Lympho's	31	8.9 (6.7-11.3)	12.1 (9.6-14.4)	**0.03**	11.1 (7.1-12.3)	13.1 (9.4-16.7)	12.2 (9.9-14.4)	0.10		
Transitional B cells ($CD24^+CD38^+CD10^+$)	Bcells	31	6.2 (4.6-9.8)	4.9 (2.6-7.2)	0.09	5.2 (2.0-7.3)	4.6 (3.2-5.5)	4.6 (1.6-7.6)	0.40		
Plasmablast ($CD10^-CD38^{hi}CD27^{hi}$)	Bcells	31	1.4 (0.5-2.1)	0.6 (0.5-1.1)	0.09	0.6 (0.4-1.1)	0.6 (0.5-1.0)	0.7 (0.5-1.1)	0.38		
Bregs ($CD28^{hi}CD24^{hi}$)	Bcells	31	6.5 (4.3-10.0)	4.6 (2.4-7.5)	0.12	3.6 (2.3-8.8)	4.7 (3.4-5.3)	4.6 (1.5-8.0)	0.45		

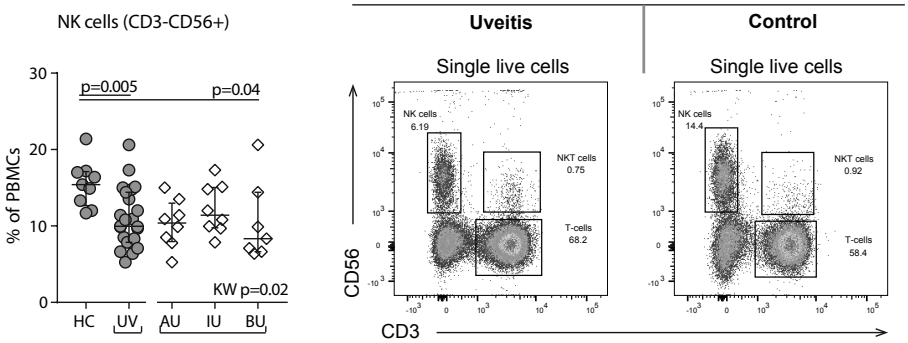
P-values between HC and UV are from Wilcoxon rank-sum test , p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing (*).

Abbreviations: Breg: regulatory B cell, AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, HC: healthy control, IFN: interferon, IL: interleukin, IQR: interquartile range, IU: idiopathic intermediate uveitis, mDC: myeloid dendritic cell, Tc: cytotoxic T cell, T_E: effector T cell, Th: T helper cell, T_M: memory T cell, T_N: naïve T cell, Treg: T regulatory cell, PBMC: peripheral blood mononuclear cell, pDC: plasmacytoid dendritic cell, TNF: Tumor Necrotis Factor



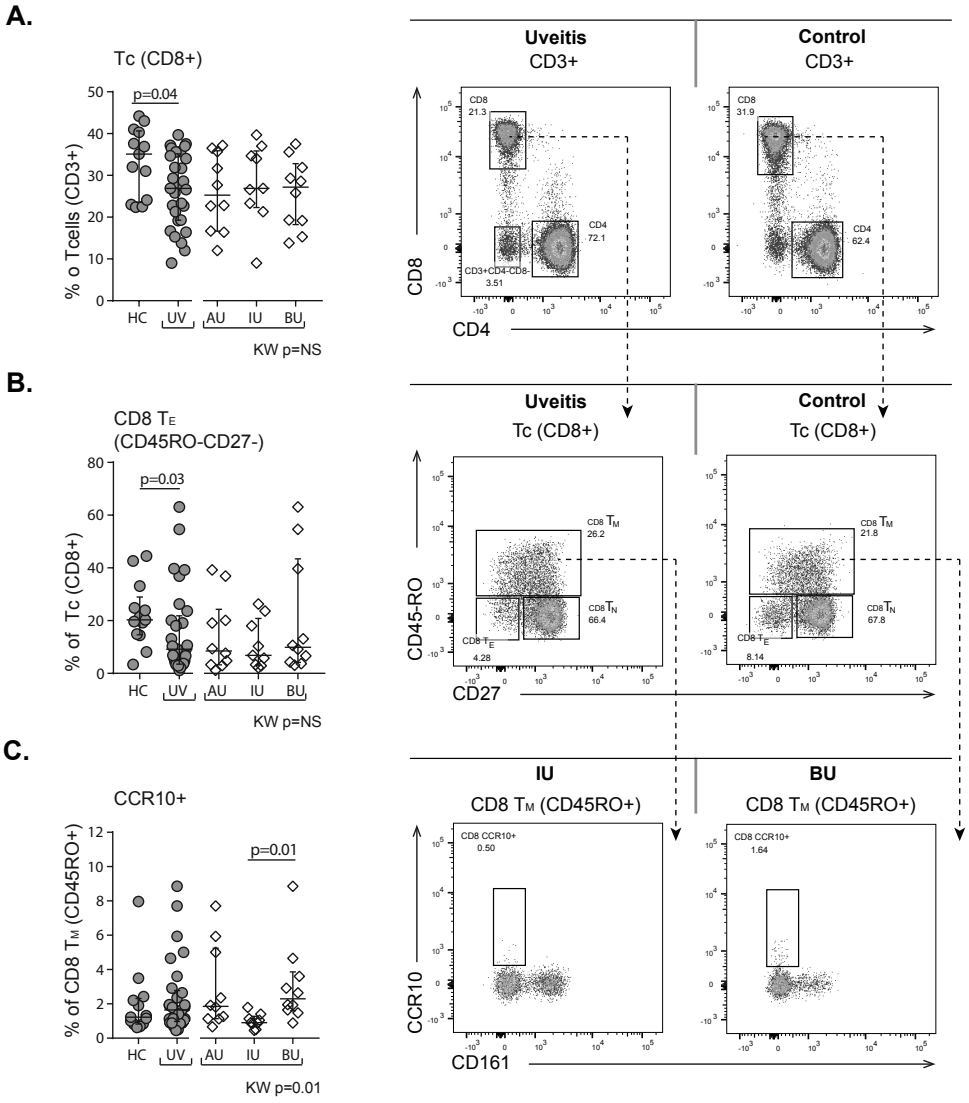
Supplementary Figure 1. Flow cytometry of peripheral blood mononuclear cells shows a decrease of plasmacytoid dendritic cells in non-infectious uveitis. **A.** Gating strategy used to identify dendritic cell subsets (representative sample). **B.** The frequency of circulating plasmacytoid and myeloid dendritic cell subsets as percentage of the HLA-DR$^+$Lineage(CD3,CD19,CD56)$^-$CD16$^-$CD14$^-$ cells.

Bars in plots indicate median, error bars indicate interquartile range. P-values between HC and UV are from Wilcoxon rank-sum test , p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing. **Abbreviations:** AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, KW: Kruskal-Wallis test, mDC; myeloid dendritic cell, pDC; plasmacytoid dendritic cell, UV: Combined Uveitis samples.



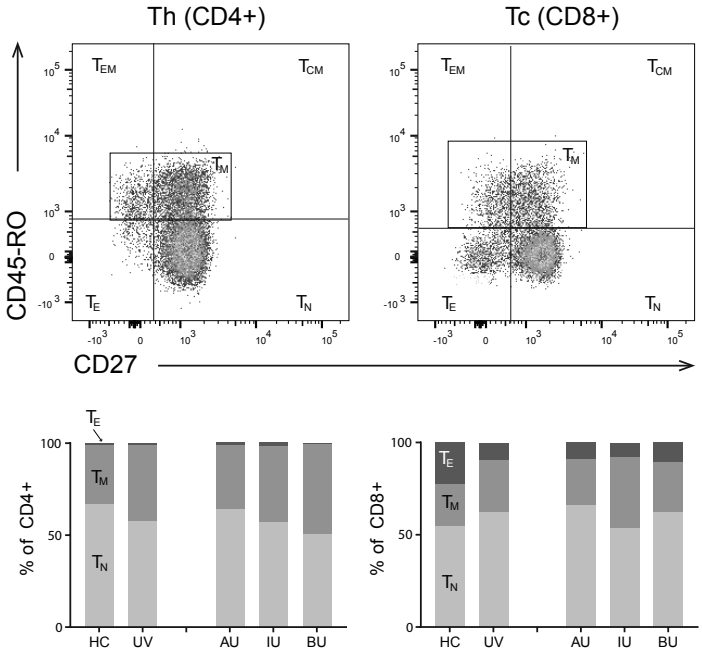
Supplementary Figure 2. Flow cytometry of peripheral blood mononuclear cells shows a decrease of Natural Killer (NK) cells in uveitis. **Left:** levels of circulating NK (CD56+CD3-) cells as % of PBMCs. **Right:** gating strategy used to identify NK cells.

Bars in plots indicate median, error bars indicate interquartile range. P-values between HC and UV are from Wilcoxon rank-sum test, p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing. **Abbreviations:** AU: HLA-B27 associated anterior uveitis. BU: Birdshot uveitis. HC: healthy control. IU: idiopathic intermediate uveitis, PBMC's: peripheral blood mononuclear cells, UV: Combined Uveitis samples.

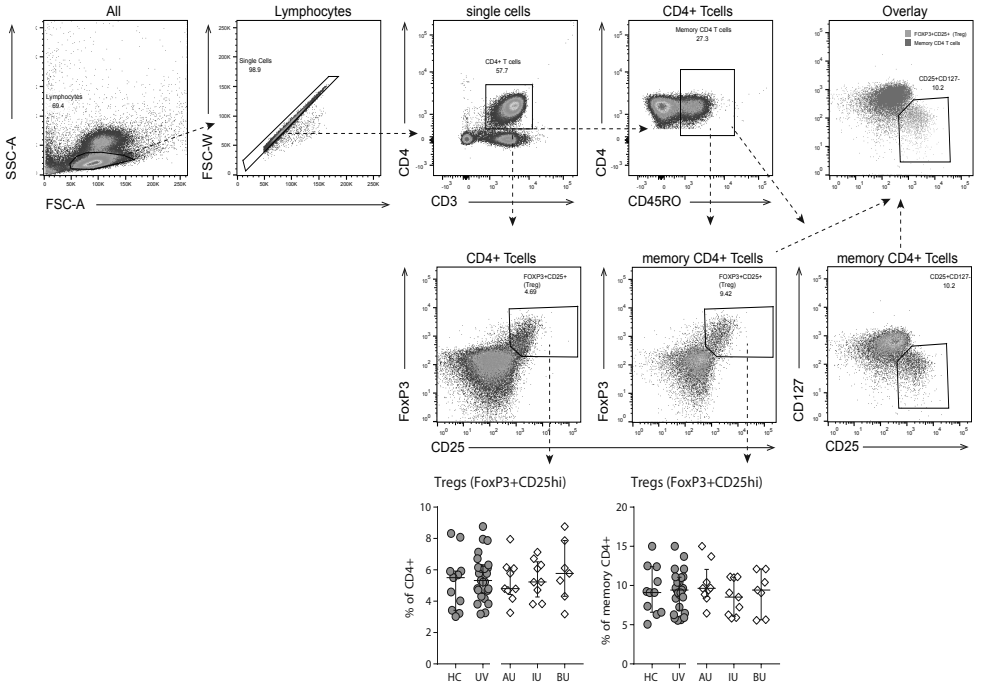


Supplementary Figure 3. Flow cytometry of peripheral blood mononuclear cells shows changes in the proportion of cytotoxic T cell subsets. **A.** Decrease of CD8+ (cytotoxic) T cells in uveitis compared to healthy controls. Representative patient and control samples are indicated. **B.** The decrease in CD8+ T cells is caused by a decrease in the CD45RO-CD27- T_E population. **C.** CCR10+ memory CD8+Tcells are significantly higher in BU compared to IU.

Bars in plots indicate median, error bars indicate interquartile range. P-values between HC and UV are from Wilcoxon rank-sum test, p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing. **Abbreviations:** AU: HLA-B27 associated anterior uveitis. BU: Birdshot uveitis. HC: healthy control. IU: idiopathic intermediate uveitis. Tc; cytotoxic Tcell. Th; Thelper cell. T_E; effector Tcells (CD45RO-CD27-). T_N; naïve (CD45RO-CD27+). T_M; memory (CD45RO+), UV: Combined Uveitis samples.

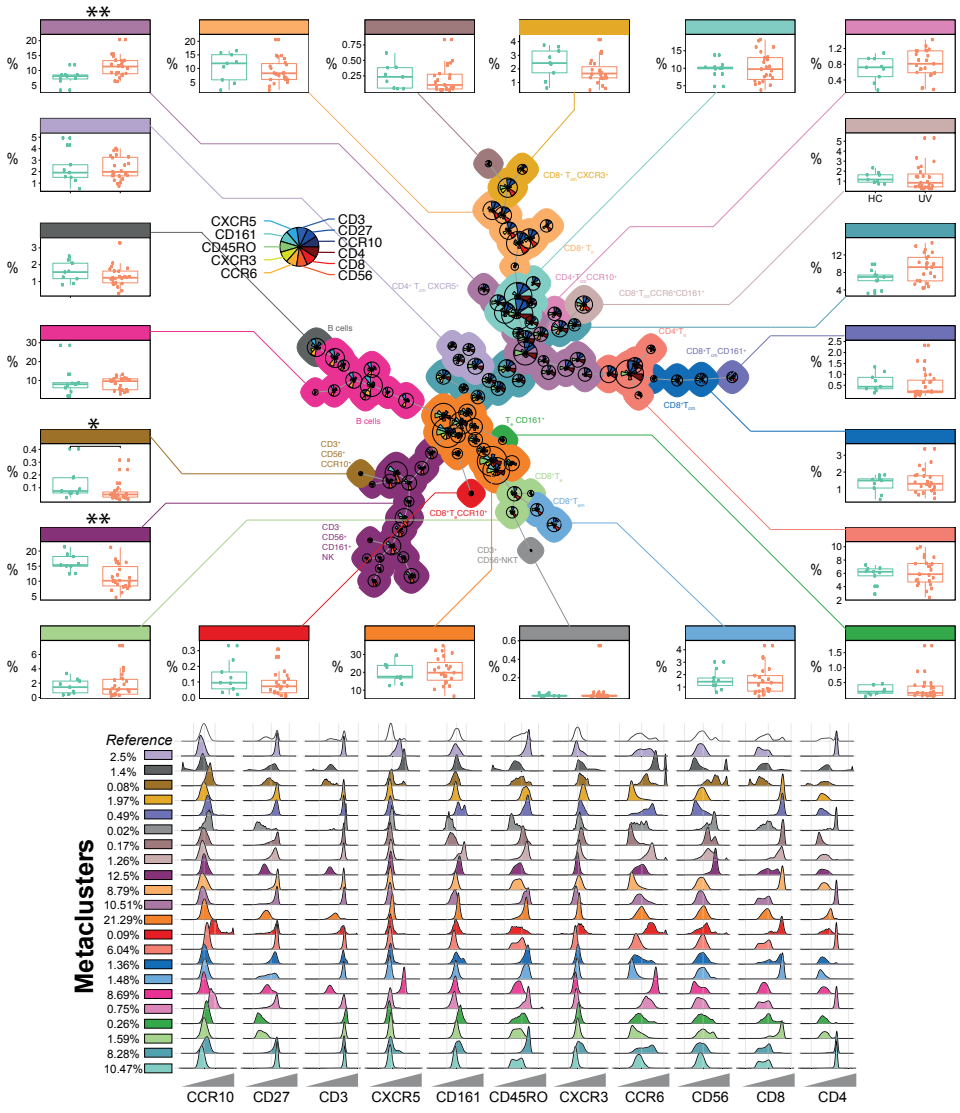


Supplementary Figure 4. The proportions of naive and memory cells in Th ($CD4^+$) and Tc ($CD8^+$) cells. **Top:** gating strategy used to identify naïve (T_N, $CD45RO^-CD27^+$), memory (T_M, $CD45RO^+$) and effector T cells (T_E, $CD45RO^-CD27^-$) within the CD3+CD4+ and CD3+CD8+ gates. **Bottom:** distribution of CD4 and CD8 T_E, T_M, T_N for all groups. **Abbreviations:** AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, UV: Combined Uveitis samples, Tc; cytotoxic Tcell, Th; Thelper cell, T_E; effector Tcells (CD45RO-CD27-), T_N; naïve (CD45RO-CD27+), T_M; memory (CD45RO+)

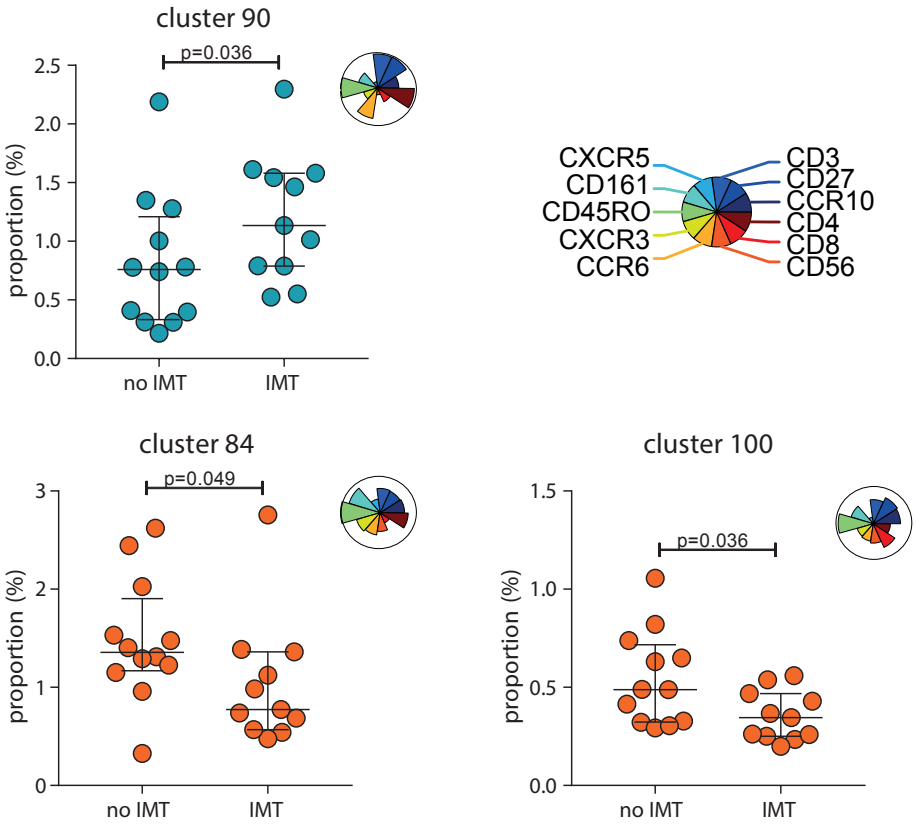


Supplementary Figure 5. Flow cytometry analysis of T regulatory cells. **Top and second row:** gating strategy used to identify T regulatory (Treg, $CD25^{high}FoxP3^{+}$) cells. **Bottom row:** levels of circulating Tregs as % of (memory) CD4+Tcells. All Tregs are also CD127- (see overlay).

Bars in plots indicate median, error bars indicate interquartile range. P-values between HC and UV are from Wilcoxon rank-sum test, p-values between uveitis subgroups are from Kruskal-Wallis (KW) test with post-hoc Dunn's correction for multiple testing. **Abbreviations:** AU: HLA-B27 associated anterior uveitis. BU: Birdshot uveitis. HC: healthy control. IU: idiopathic intermediate uveitis, UV: Combined uveitis samples.



Supplementary Figure 6. Details from unsupervised automatic gating using FlowSOM used in main Figure 3. Using FlowSOM, we clustered all individual cells within the single-cell gates of all samples into 100 distinct clusters based on the surface protein expression. Using unsupervised clustering, the 100 clusters were clustered into 22 meta-clusters of different cell types and organized in the minimal spanning tree on the right. The meta-clusters are represented by unique colors. For each cluster (i.e., cell population), pie charts indicate the relative expression for each of the different surface markers and the pie size corresponds to the number of cells in each population. Scatter plots of each cluster comparing healthy controls (blue) and uveitis patients (orange) are given for each respective meta-cluster. Clusters that differed significantly between the groups (based on Wilcoxon rank-sum test) are indicated by an asterisk (*).Relative densities of marker expression (logicle-transformed) for 11 surface markers and proportion (% in single cell gate) are given for all meta-clusters below.



Supplementary Figure 7. Clusters identified by FlowSOM differentiating between patients that need prolonged systemic IMT after inclusion. Dot plots are given for 3 clusters significantly differentiating between IMT+ and IMT- patients. For each cluster (i.e., cell population), pie charts indicate the relative expression for each of the different surface markers. Cluster 84 and 100 showed relatively low CD3 expression and were not considered to represent T cell populations. Colors of the dots represent the metaclusters these clusters belong to (see also **Figure 3**). Bars in plots indicate median, error bars indicate interquartile range. P-values between HC and UV are from Wilcoxon rank-sum test.


9

Chapter 9

A CD1C+ DENDRITIC CELL TRANSCRIPTOMIC PROGRAM IS LINKED TO HUMAN NON-INFECTIOUS UVEITIS

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Manuscript in preparation

ABSTRACT

BACKGROUND: Peripheral blood myeloid dendritic type 1 cells (CD1c+ mDCs) have been recently linked to Non-infectious uveitis (NIU), but their role in the pathogenesis remains to be elucidated.

METHODS: CD11c+ CD1c+ mDCs were purified from blood from 29 adult patients and 16 age- and sex-matched controls. We performed RNA sequencing to study the differences between transcriptomes of CD1c+ mDCs isolated from uveitis patients and healthy control. We further identified uveitis associated gene modules (sets of genes exhibiting similar transcriptomic profiles) by generating data-driven weighted gene co-expression networks. To identify robust gene modules and uveitis specific gene signatures, we replicated our findings in an independent cohort of 22 patients and 13 controls.

RESULTS: Unsupervised clustering revealed that the CD1c+ mDCs isolated from the blood of uveitis patients have a distinct functional phenotype compared to those isolated from the healthy individuals. We identified and replicated a uveitis linked 'gene signature' of 147 co-expressed genes. These genes included transcriptional regulators such as *RUNX3*, *NFKB1*, *ATF4*, *GNAS*, *JDP2* and *IRF8,* innate immune receptors such as *TLR7, IFI16, CD180,* and chemokine receptors *CCR5, CX3CR1* linked to TLR cascades, NF-κB1- and interleukin signalling. Furthermore, it was possible to distinguish between different subtypes of uveitis using a gene signature (comprising >200 genes).

CONCLUSION: These data show that non-infectious uveitis is hallmarked by substantial changes in the transcriptome of CD1c+ myeloid dendritic cells and that eye inflammatory disease leaves a footprint in the blood circulating immune cells.

INTRODUCTION

Non-infectious uveitis (NIU) refers to a collection of severe inflammatory eye diseases that are among the leading causes of vision loss in the Western world.[1,2] The cause of NIU is poorly understood. Although NIU is considered to be a predominantly T cell-mediated condition, emerging studies suggest also a pivotal role for CD11c+ (myeloid) dendritic cells (mDCs) in human NIU.[3–5] CD1c$^+$ mDCs (also called mDC1, or - confusingly - cDC2 cells) account for the majority of the circulating dendritic cells. The CD1c+ mDCs promote Th17 responses, a T helper subset we and others have linked to human noninfectious uveitis (see also **chapter 8**).[67,89,10] Interestingly, the proportion of CD1c$^+$ mDCs has previously been shown to be increased in blood of patients and is associated with uveitis activity.[11,12] In addition, myeloid dendritic cells increase in abundance in eye fluid of patients with anterior uveitis.[5] However, the role of CD1c$^+$ mDCs in the pathogenesis of NIU remains to be elucidated.

To this end, we performed transcriptional profiling (RNA sequencing) of purified peripheral blood CD1c$^+$ mDCs from two independent cohorts of NIU patients. We constructed data-driven gene co-expression networks to identify clusters of genes altered in different groups of uveitis patients. Using an independent cohort, we further show robust replication of several of these co-expressing genes validating their associations with uveitis disease groups.

MATERIAL AND METHODS

Patients and patient material

This study was conducted in compliance with the Helsinki principles. Ethical approval was requested and obtained from the Medical Ethical Research Committee in Utrecht. All patients signed written informed consent before participation. We collected blood from a discovery cohort of 29 and a replication cohort (± 2 years later) of 22 adult patients (**Table 1**) with HLA-B27-associated Acute Anterior Uveitis (AU), Idiopathic Intermediate Uveitis (IU), or Birdshot Uveitis (BU). Patients were recruited at the outbound patient clinic of the department of Ophthalmology of the University Medical Center Utrecht between July 2014 and January 2017. Twenty-nine age, sex, and ancestry matched anonymous blood donors with no history of ocular inflammatory disease were recruited at the same institute and served as unaffected controls (HC: 16 for the discovery cohort and 13 for the replication cohort). Uveitis was classified and graded in accordance with the Standardization of Uveitis Nomenclature (SUN) classification.[13] Each patient underwent a full ophthalmological examination by an ophthalmologist experienced in uveitis, routine laboratory screening, and an X-Ray of the lungs.

Table 1. Characteristics of the discovery and replication cohort

Discovery cohort	AU	IU	BU	HC	p-value
N	9	9	10	16	n.a.
Male / Female	3/6	2/7	4/6	6/10	0.90*
Age in years; mean ± SD	47.7 ± 17.0	39.3 ± 14.0	52.9 ± 13.2	41.4 ± 9.8	0.09**
Disease duration in years; median (range)	5.8 (0.1-39.3)	3.7 (0.2-20.0)	1.3 (0.2-15.1)	n.a.	0.14***
Replication cohort	**AU**	**IU**	**BU**	**HC**	**p-value**
N	10	5	8	13	
Male / Female	2/8	3/2	5/3	5/8	0.25*
Age in years; mean ± SD	45.9 ± 16.1	29.9 ± 10.7	42.1 ± 10.5	42.2 ± 13.3	0.20**
Disease duration in years; median (range)	8.1 (0.2-22.3)	3.4 (0.4-14.1)	0.9 (0.2-19.9)	n.a.	0.36***

Abbreviations: BU: Birdshot uveitis, AU: HLA-B27 associated anterior uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, n.a.: not applicable, * Fisher's exact test, ** ANOVA,*** Kruskal-Wallis.

Laboratory screening included erythrocyte sedimentation rate, renal and liver function tests, angiotensin converting enzyme (ACE), and screening for various infectious agents in the serum including syphilis, Lyme disease, and an Interferon-Gamma Release Assay (IGRA) tuberculosis test. Two digit HLA typing of the ***HLA-A*** and ***HLA-B*** locus was done for all patients with anterior or posterior involvement to confirm HLA-B27 or HLA-A29 status. All patients had active uveitis (new onset or relapse) at the time of sampling. There was no clinical evidence for uveitis-associated systemic inflammatory disease (e.g., rheumatic condition). None of the patients received systemic immunomodulatory treatment in the last 3 months, other than low dose (≤10mg) oral prednisolone in one BU patient of the discovery cohort and one AU patient of the replication cohort. All patients with AU and BU were HLA-B27 or HLA-A29 positive, respectively.

Flow cytometry

The proportion of $CD11c^{+}CD1c^{+}$ mDCs in the discovery cohort was evaluated by flow cytometry as described previously (***this thesis, chapter 8***). Briefly, nitrogen stored PBMCs were quickly thawed and pre-incubated with 5% mouse serum for 5 minutes at room temperature and immediately stained with a fluorochrome-conjugated antibody body panel as described in **Supplementary Table 1**. Flow cytometric analyses were performed on the BD LSR Fortessa™ Cell analyzer (BD Bioscience, San Jose, CA, USA). Data were analyzed using FlowJo software (TreeStar inc. San Carlos, CA, USA). The gating strategy for $CD1c^{+}$ CD1c+ mDCs is illustrated in **Supplementary Figure 1**.

CD1c+ myeloid dendritic cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by standard ficoll gradient

centrifugation from 80mL heparinized blood immediately after blood withdrawal (GE Healthcare, Uppsala, Sweden). For the discovery cohort, fresh PBMCs were immediately subjected to magnetic-activated cell sorting (MACS) for the removal (by positive selection) of $CD304^+$ ($BDCA4^+$) cells, followed by $CD19^+$ cells, and subsequently isolation of $CD1c^+$ cells by using the $CD1c^+$ (BDCA1) isolation kit (all from Miltenye Biotec, Germany) according to the manufacturer's instructions. Patient and control samples were isolated in a total of 32 batches. The isolated CD1c-positive fraction contained on average 147,114 cells (range 46,000-773,000) and had a mean (SD) purity of 92.9(4.6)% as determined by flow cytometry (**Supplementary Figure 2**). For the replication cohort, ten batches (individual days) of 4-5 randomly selected patient and control samples of nitrogen stored PBMCs (mean storage time of 11 [range 0-31] months) were quickly thawed and washed once in ice cold FACS Buffer (1% bovine serum albumin (BSA) and 0.1% sodium azide in phosphate buffered saline (PBS)) and incubated with a mix of surface antibodies (**Supplementary Table 2**). Subsequently, $CD3^-CD14^-CD19^-CD1c^+$ cells were sorted by flow cytometry (FACS) following the gating strategy as illustrated in **Supplementary Figure 3**. The average number of collected cells by sorting was 56,881 (range 6,669-243,385). MACS or FACS purified CD1c+ cells were immediately taken up in lysis buffer (RLT plus, Qiagen, Venlo, Netherlands) containing 1% β-mercaptoethanol, snap frozen on dry ice, and stored at -80°C until RNA extraction was performed.

RNA isolation and RNA sequencing

Total RNA from $CD1c^+$ cell lysates from patients and controls were randomly divided for RNA extraction on different days using the AllPrep Universal Kit (Qiagen) on the QIAcube (Qiagen) according to the manufacturer's instructions. cDNA synthesis, library preparation, and Illumina sequencing was performed on 44 samples of the discovery cohort at BGI (Hong Kong). RNA-seq libraries were generated with the TruSeq RNAseq RNA Library Prep Kit (Illumina Inc., Ipswich, MA, USA), and were sequenced using Illumina NextSeq 500 generating approximately 20 million 100bp paired ended reads for each sample. For the replication cohort (a total of 36 samples), RNA-seq libraries were generated by GenomeScan (Leiden, the Netherlands) with the TruSeq RNAseq RNA Library Prep Kit (Illumina Inc., Ipswich, MA, USA), and were sequenced using Illumina HiSeq 4000 generating ~20 million 150bp paired ended reads for each sample.

Differential gene expression and gene co-expression network analysis

Quality check of the raw sequences was performed using the ***FastQC*** tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to the human genome (GRCh38 build 79) using ***STAR aligner***[14] and Python package ***HTSeq*** was used to

count the number of reads overlapping each annotated gene.[15] The obtained raw count data was subsequently used by ***DESeq2*** Bioconductor/R package to identify differentially expressed genes (DEGs) between the four disease groups (AU, IU, BU, HC). Using DESeq2, we modelled the biological variability and overdispersion in the gene expression data as a negative binomial distribution.[16] We then used Wald's test to identify DEGs in each pair-wise comparison and used likelihood ratio test (LRT) to identify DEGs considering multiple disease groups. We further used regularized log transformation to obtain normalized gene counts (regularized log transformed data or RLD), which were used for subsequent analysis.

To study the interdependence between genes and their interactions, we constructed two gene co-expression networks (one for discovery cohort and another for replication cohort) using ***Weighted Gene Co-expression Network Analysis*** (WGCNA) Bioconductor/R package.[17] The gene co-expression networks were constructed using all the genes that were differentially expressed ($p<0.05$) either in the pairwise analysis (Wald's test) or in the multi-group comparison (LRT). WGCNA allows us to define '***modules***' or clusters of highly correlated genes (as described in more detail in[17–19]). The modules are defined by first performing an unsigned pair-wise spearman's correlation between genes and subsequently transforming the co-expression similarities into an adjacency matrix (that provides the relative connection strengths between genes) and scaling the adjacency matrix to achieve a scale-free topology (scaling power=6 selected to have a network that fits the scale-free topology criterion with $R^2 > 0.9$).

For each module the ***eigengene*** or the first principal component was calculated and plotted using barplot function in R. For each gene, the module membership (MM) reflects the correlation of the gene's expression data with the module eigengene. We calculated the intersect between the modules constructed from the two cohorts and used Fisher's exact test to identify modules that exhibited significant overlap in their genes. The two independent cohorts had several possible differences between them such as storage conditions, purification methods and sequencing facility. When we observed significant overlap between gene modules of the discovery and replication cohort, we considered the overlapping genes and gene modules robust and relevant for non-infectious uveitis. We performed pathway enrichment analysis on DEGs from modules that were replicated in ToppGene Suite (BMI CCHMC, Cincinnati, OH, USA)[20]. In order to visualize interactions between DEGs as well as the pathways that the individual DEGs are involved in, a functional enrichment network analysis was performed using the String (Search Tool for the Retrieval of Interacting Genes/Proteins) database on the replicated DEGs from Signature A.[21]

Transcription factor network

To study whether the co-expressed genes in a given module are regulated by common transcription factors (TFs), we generated a module specific TF-target network using TFhunter (Python based pipeline, Pandit and associates, ***manuscript in preparation***). Briefly, we first curated TF-target interactions (both experimentally determined and predicted) from several literature resources (such as TRRUST[22], ENCODE[23], REGNET[24], among others). For each module, we then extract the list of TFs that are predicted to regulate one or more genes from the module. We filter the transcription factors which are not co-expressed with their targets (i.e. not found in the module of interest). We plot the TF-target network where each gene (node) is scaled based upon the number of their TF-target interactions.

Proteomic profiling of serum

Serum was collected from all patients and controls of the discovery and replication cohorts as described in **chapter 7** and stored at -80ºC. A total of 100 ul serum of each sample was subjected to SOMAscan 1.3 k assay (performed by SomaLogic Inc. ,Boulder, CO, USA) to determine the relative concentrations of 1,305 protein analytes (47 % secreted proteins, 28 % extracellular domains, 25 % intracellular proteins). Following SomaLogic's guidelines, we performed Hybridization Control Normalization to adjust for between-well variance.[25] We choose the recommended conservative cutoff of a 200 relative fluorescent unit (RFU) output from the array as a threshold for detection. Serum protein data was considered for further analyses if the median RFU was >200 in ≥1 of the 4 disease groups (n=921 protein targets). Two BU samples were considered outliers after inspection of the serum proteome by principle component analysis and removed from the dataset. We used a likelihood ratio test (LRT) to identify differentially expressed serum proteins (DEPs, at FDR 5%) considering multiple disease groups. We used the location function of the Universal Protein Resource (UniProt[26]) to sort DEPs that are known to be secreted, or considered DEPs if they were known to be secreted based on an NCBI search. Next, we further filtered for DEPs that have been reported to bind to receptors that were well-expressed in the mDC transcriptomic data.

RESULTS

We aimed to characterize circulating CD1c+ myeloid dendritic cells (mDCs) from patients with non-infectious uveitis (NIU). Therefore, we first questioned whether the frequency of mDCs was changed in blood patients with NIU. The relative abundance of blood $CD1c^+$ mDCs cells measured by flow cytometry was not significantly different between the patients and the controls in the discovery cohort (**Figure 1A**). For the discovery

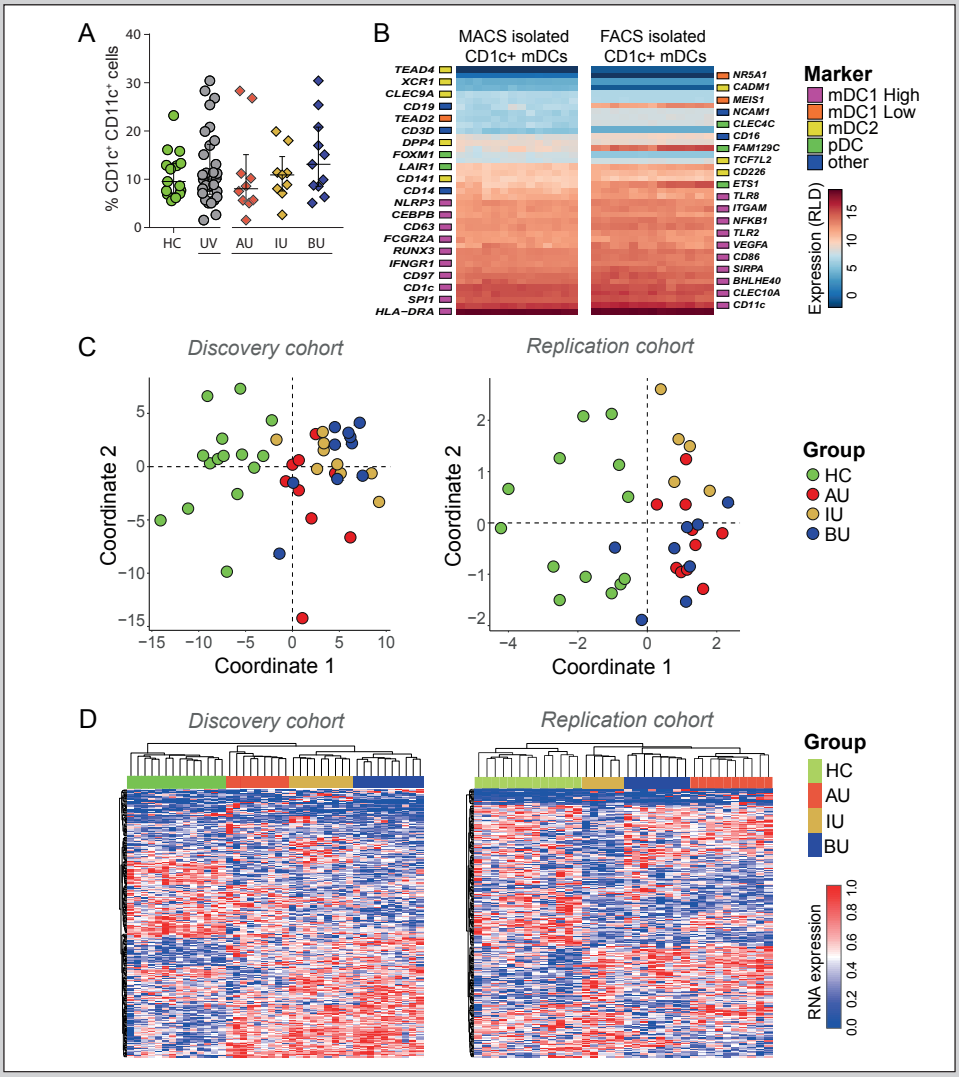


Figure 1. The blood CD1c+ myeloid dendritic cell (mDC1) transcriptome is changed in patients with non-infectious uveitis. **A.** Flow cytometry of the proportion of CD1c+CD11c+ myeloid dendritic cells in the disease groups. **B.** The relative gene expression for lineage markers and dendritic cell signature genes (indicated by colors) in the 16 controls of the discovery cohort and 13 controls of the replication cohort. **C.** Multidimensional scaling plots (MDS) of the first two coordinates based on the top 50 differentially expressed genes (DEG) for each of the six group comparisons (resulting in 244 and 234 unique genes, respectively). **D.** Hierarchical clustering based on top 50 DEGs for each comparison (n=244 and n=234 after removal of duplicates) using Eucledian distance and Ward linkage method revealed disease specific gene signatures. The Heatmap colors represent the relative RNA expression from low (blue) to high (red). Dendrograms indicate the clustering relationships between the DEGs and the disease groups**. Abbreviations:** BU: Birdshot uveitis, AU: HLA-B27 associated anterior uveitis, HC: healthy control, IU: idiopathic intermediate uveitis. MACS: Magnetic-activated cell sorting. FACS: Fluorescence-activated cell sorting.

cohort, we obtained purified CD1c$^+$ mDCs leukocytes from fresh PBMCs using MACS (**Supplementary Figure 2**). For the replication cohort, we obtained purified CD1c$^+$ mDCs from nitrogen stored PBMCs using FACS.

To verify the claim that these cells are CD1+ expressing CD1c+ mDCs, we first determined the genomic makeup of the purified populations from both cohorts. In the transcriptomic data of the healthy controls (n=16 and n=13, respectively), we found high expression for signature genes of the CD1c+ mDC lineage including ***CD1c***, ***ITGAX*** (encoding CD11c), ***HLA-DRA, VEGFA, FCGR2A***, ***TLR2,*** and the recently reported CD1c+ mDC-specific receptor ***CLEC10A*** (CD301a) (**Figure 1B**).[27,28] We also observed moderate expression for ***CD14*** and ***CD141*** genes (albeit low protein levels are implicated by the FACS sorting procedure which excludes CD14 expressing cells in the process, see **Supplementary Figure 3**), and low expression for CD141$^+$ mDC2 genes ***CLEC9A***, ***CADM1***, ***XCR1*** (**Figure 1B**). We also noted that FACS isolated CD1c$^+$ mDCs from the replication cohort showed expression for ***CD19*** and ***FAM129C***. Indeed, evaluation of reference transcriptomic data revealed that these genes can be expressed in this dendritic cell subset (**Supplementary Figure 4**). Importantly, a transcriptional regulator cluster, which defines principal subsets of human dendritic cells[29], showed a signature consistent with CD1c$^+$ mDC cells (e.g., high ***RUNX3, NFKB1, SP11, CEPBP, BHLHE40,*** low ***MEIS1, TEAD2, NR5A1***). Based on these results, we considered the here investigated cell population highly reminiscent of CD1c$^+$ mDCs and comparable between the cohorts.

Human non-infectious uveitis is characterized by changes in the transcriptome of circulating CD1c$^+$ myeloid dencritic cells

We aligned the reads of the RNA sequencing data sets from both cohorts to 65,217 annotated genes (see **methods**). Global assesment of the DEGs by multidimensional scaling (MDS) evidently revealed a distinct functional genome for blood CD1+ mDCs (CD1c+ mDCs) of uveitis patients (**Figure 1C**). Differences in the transcriptomic composition between each of the uveitis entities became perceivable by head-to-head comparison (**Supplementary Figure 5**) and comprises a gene signature of at least 244 unique genes (resulting from head-to-head cmparisons of the top 100 DEGs between each of the groups and then subtracting the DEGs that present in more than one comparison) (**Figure 1D**). The mean expression of the 1,800 DEGs of the replication cohort was markedly higher compared to the DEGs of the discovery cohort (mean RLD ± SD = 10.54 ± 2.2 compared to 6.82 ± 4.0; **Supplementary Figure 6**), suggesting that moderate and low expressed mRNA was less sufficiently detected, most likely as a consequence of the lower number of cells obtained by FACS sorting (i.e., lower RNA yield). Regardless, a total of 613 DEGs

(Jaccard similarity index of 7.8%) were differentially expressed in both cohorts, representing 8.8% of all DEGs of the discovery and 30.7% of all DEGs detected in the replication cohort (**Figure 2**). Of note, 18 of the replicated DEGs were consistently differentiating between the uveitis subtypes, including ***HLA-B,*** the primary risk locus for AU (**Supplementary Table 3**). Collectively, these data show that NIU is hallmarked by reproducible changes in the transcriptome of CD1c^{+} dendritic cells, which is able to discern anatomically distinct types of eye inflammation in blood.

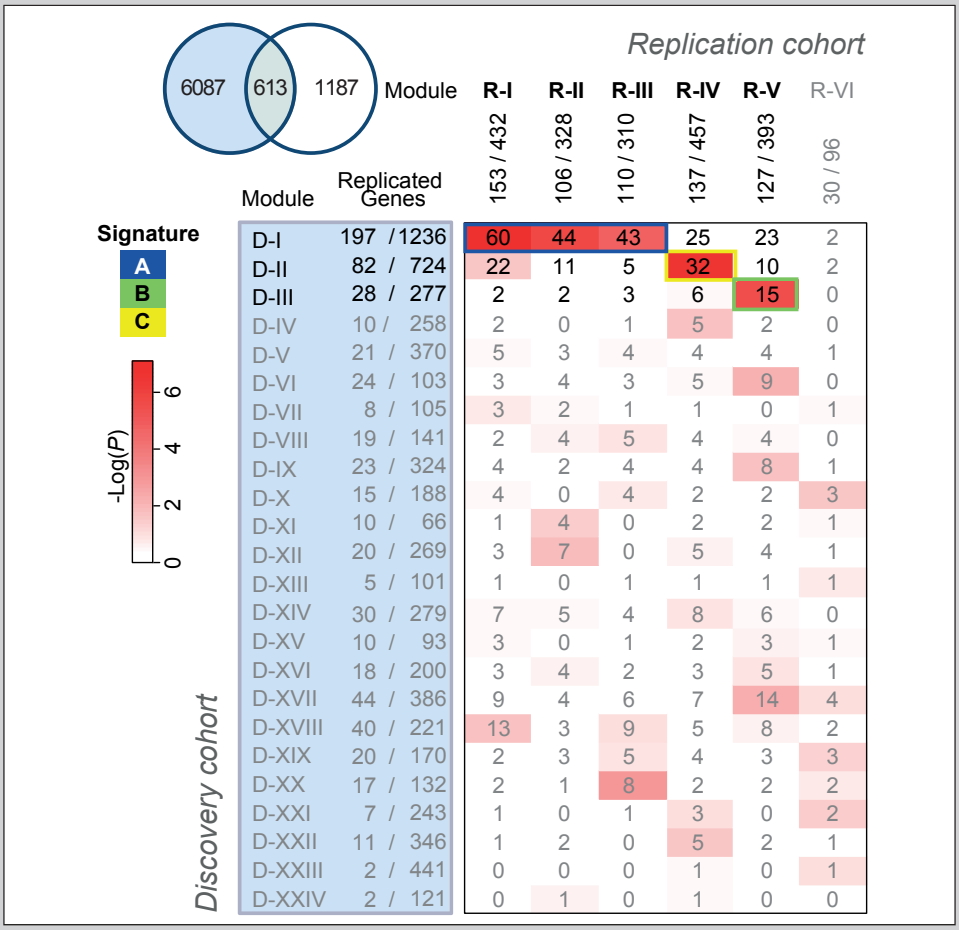


Figure 2. Overlap in mDC1 gene modules identified in the discovery and replication cohort. Gene co-expression networks based on differentially expressed genes of the discovery and replication cohort were constructed using WGCNA (ref). For the DEGs of the discovery cohort, WGCNA analysis discerned 24 discreet gene modules (D-I to D-XXIV). The DEGs of the replication cohort clustered into six gene modules. Significant overlap in genes is highlighted in colors. The number of overlapping genes and statistical significance are indicated. The gene modules will be further referred to as Signature A (n=147 genes, Module D-I and R-I-II-III), signature, signature B (n=15 genes, Module D-III and R-V) and C (n=32 genes, Module D-II and R-IV).

Identifcation and validation of a uveitis-associated dendritic cell gene network

Next, we constructed gene co-expression networks from the DEGs using WGCNA.[17] Gene co-expression network analysis allows us to catalog the transcriptomic data into biologically meaningful clusters (i.e., gene modules). Using the gene modules, we can further identify important pathways, master regulatory circuits (.e.g., transcription factors), hub genes, and evaluate their association with NIU. For the DEGs of the discovery cohort, WGCNA analysis discerned 24 discreet gene modules (**Figure 2**). Similarly, a total of six gene modules were clustered in the replication cohort (**Figure 2**). Importantly, we replicated several gene modules identified in the discovery cohort based on a significant number of overlapping module members and similar group direction of the eigengene values. In other words, we replicated clusters of genes, which exhibit consistent alterations in both cohorts (**Figure 3**). These replicated gene modules will be further referred to as ***Signature A*** (n=147 genes, Module D-I and R-I-II-III, see for details **Supplementary Table 4**), and ***C*** (n=15 genes, Module D-III and R-V). Although signature C a sufficient number of overlapping genes between modules from the discovery and replication cohort, the eigengene values (i.e., the 'direction' of association) did not show a consistent pattern across the cohorts and was therefore not considered for further analysis.

Closer examination of signature A by unsupervised hierarchical clustering substantiates the identification of a ***bona fide*** disease network as evidenced by the consistent clustering of samples (**Figure 4**). The pattern of the direction of eigengene values in the discovery and replication cohort indicate that signature A is associated with NIU, regardless of disease subtype. This is also evident when comparing the relative gene expression for this signature between each of the disease groups (**Supplementary Figure 7**). The top 10 most differentially expressed genes of signature A include increased levels of Toll-like receptor family members ***CD180*** and ***TLR7***, chemokine receptors ***CX3CR1*** and ***CCR5,*** the non-coding ***HCP5*** (HLA complex P5) transcript, Interferon Gamma Inducible Protein 16 (***IFI16***), and decreased expression for GNAS complex locus (***GNAS***), Spermatogenesis Associated 2 (***SPATA2***), SH3 omain binding protein 5 (***SH3BP5***), and Heterogeneous Nuclear Robonucleoprotein D like (***HNRNPDL***). The molecular interactions between the 147 DEGs from Signature A as well as the putative pathways that they are involved in are visualized in **Figure 5A**. Signature A was enriched with genes that were involved in (MyD88 dependent) TLR signaling, positive regulation of cytokine production and gene regulation processes.

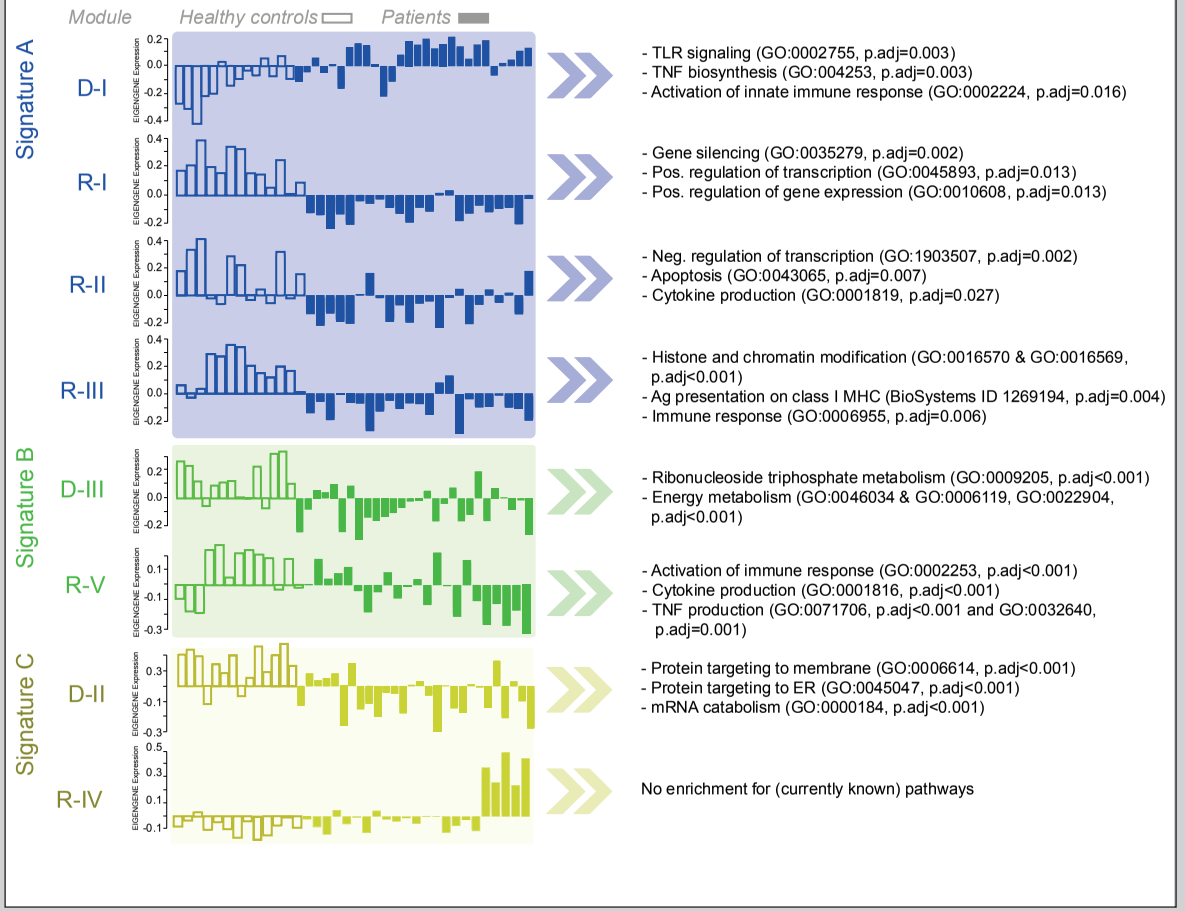


Figure 3. The eigengene values of the three overlapping module signatures (A-C) reveal consistent direction for signature A and B. **Left:** For each module (see **Figure 2**) the eigengene or the first principal component was calculated and plotted for each sample. The pattern of eigengenes value for signature A distinguised patients from controls in the discovery cohort (D-I) and replication cohort (R-I to R-III) and was therefore considered to be replicated. For signature X, the eigengene value did not show a consistent pattern across the cohorts and was therefore not considered for further analysis. **Right:** Pathway enrichment for all DEGs of each module. Pathway enrichment analysis was performed for Gene Ontology (GO) bioloical processes. Selected signficant pathways (FDR 5%).

To identify key transcription factors involved in signature A, we constructed a module specific TF-target network (**Figure 5B**; see **methods** for details). The gene modules (D-I and R-I to Ⅲ), on which Signature A was based, contained 117, 54, 40, and 43 transcription factors respectively (254 in total). We replicated a total of 23 transcription factors, six of which were highly connected in both cohorts (≥35 DEG connections within their respective module): Nuclear Factor Kappa B Subunit 1 (***NFKB1***) was increased while the levels of POZ/BTB and AT hook containing zinc finger 1 (***PATZ1***), Jun Dimerization Proteitn 2 (***JDP2***), Interferon Regulatory Factor 8 (***IRF8***), Runt Related Transcription Factor 3 (***RUNX3***) and Activating Transcription Factor 4 (***ATF4***) were decreased in CD1c⁺ mDC

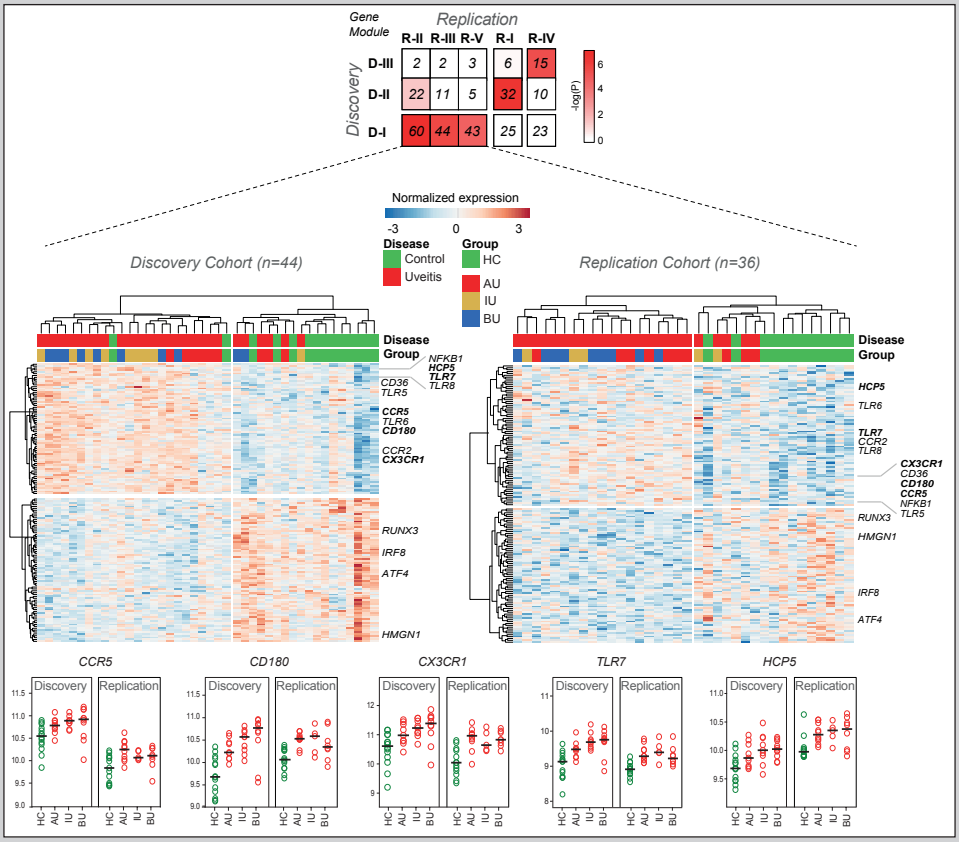


Figure 4. Unsupervised hierarchical clustering of the 147 replicated genes from signature**.** Unit variance scaling was applied to rows. Clustering was performed with the ClustVis server using Pearson distance and Ward linkage. A selection of the top 10 DEGs from Signature A are indicated by scatterplots below. **Abbreviations:** ATF4: Activating Transcription Factor 4, AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, CCR: CC cytokine subfamily receptor, CX3CR1: CX3C cytokine subfamily receptor, HC: healthy control, HCP5: HLA complex P5, HMGN1: High-mobility group nucleosome binding domain 1, IRF8: interferon regulatory factor 8, IU: idiopathic intermediate uveitis, NFKB1: Nuclear Factor Kappa B Subunit 1, RUNX3: Runt Related Transcription Factor 3,TLR: Toll-like receptor.

cells of uveitis patients (**Figure 5C**). Pathway enrichment for the 15 replicated genes representing signature B (within modules D-III & R-V) did not yield significant pathway annotations.

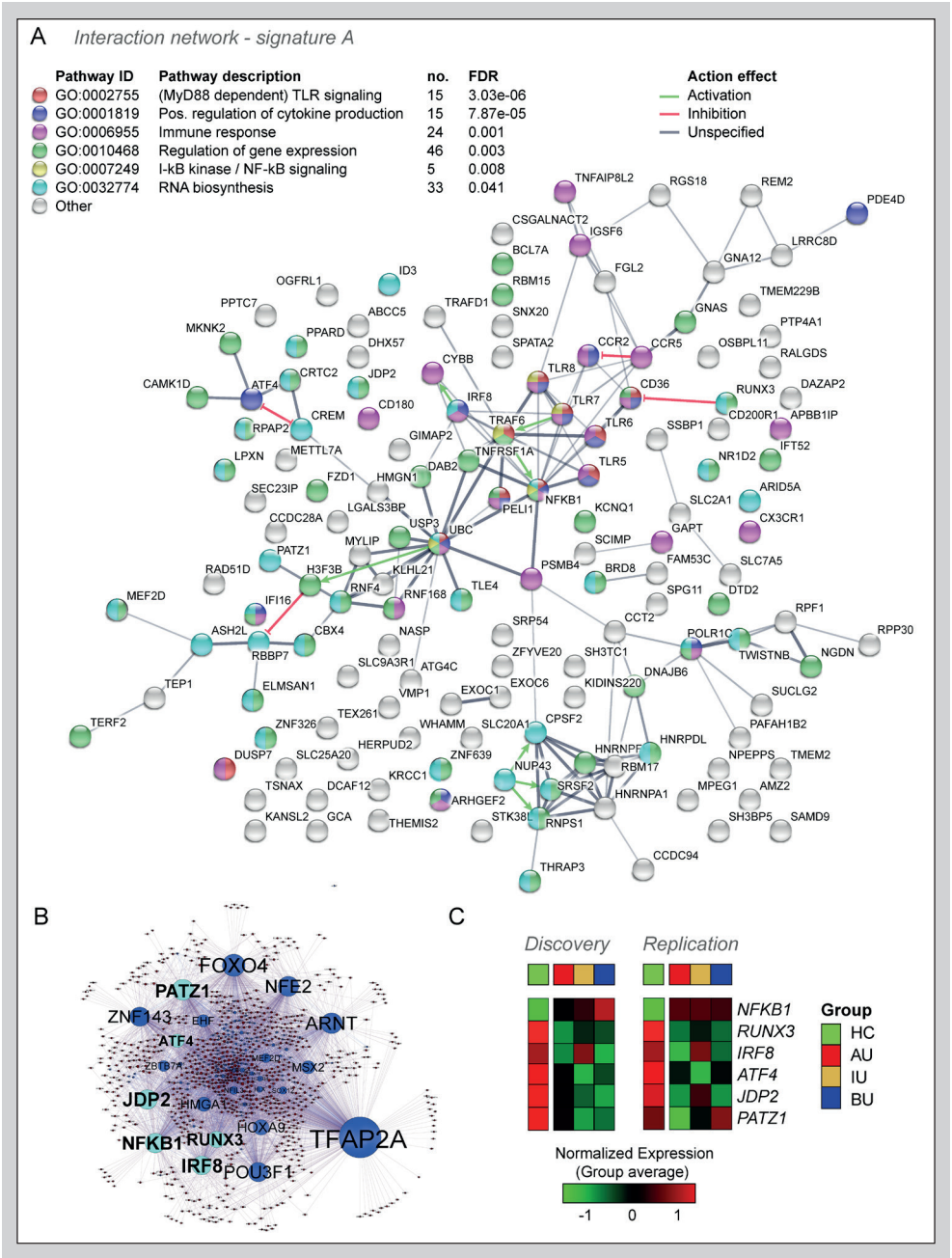


Figure 5. Network analysis of signature**. A.** Interaction network computed in STRING[21]. Nodes represent DEGs from signature A. Lines between DEGs signify interaction data supporting the network, displayed by confidence (thicker lines indicate higher confidence) and colored by interaction bype: red lines indicate inhibitory interaction, green lines indicate activating interaction, grey lines indicate interaction that is unspecified. Pathways in which DEGs are active are indicated in a color coded manner. **B.** Transcription factor-target network gene module D-I (discovery cohort). For the differentially expressed genes belonging to module D-I of the discovery cohort, the predicted and validated transcription factor targets interactions were determined by TFhunter. Each gene (node) is scaled based upon the number of their TF-target interactions. Six of the top 15 transcription factors were replicated in the second cohort (module R-I, R-II or R-III) and are indacted in light blue. **C.** hierarchical clustering of replicated top transcription factors (from panel B) shows increased expression levels of NFKB1 and decreased expression levels of ATF4, JDP2, PATZ1, RUNX3 and IRF8. Rows are centered and unit variance scaling is applied to rows. Rows are clustered using Euclidean distance and Ward linkage. No clustering is applied to collums. **Abbreviations:** ATF4: activating transcription factor 4, AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, HC: healthy control, IU: idiopathic intermediate uveitis, IRF8: interferon regulatory factor 8, JDP2: Jun dimerization protein 2, NFKB1: nuclear factor kappa B subunit 1, PATZ1: POZ/BTB and AT hook containing zinc finger 1, RUNX3: runt related transcription factor 3, TFAP2A: transcription factor AP-2 alpha.

Serum proteome linked to the dendritic cell signature

Finally, we were interested to determine if specific stimuli might be related to the uveitis-associated gene network in mDCs. Therefore, the serum proteome of each patient and control was characterized using the SOMAscan Assay (see **methods**). A total of 115 differentially expressed proteins (DEPs) were observed between the groups. The DEPs clustered in three overarching groups based on co-expression (**Figure 6A**). Consistent with the mDC RNA-sequencing data, global assessment of the DEPs by principle component analysis revealed a distinct serum proteome in NIU patients (**Figure 6B**). Among the DEPs were the scavenger receptor CD36 and the TLR4-ligand ***High-mobility group nucleosome binding domain 1*** (HMGN1), which were also DEGs in signature A of the CD1c+ mDC RNA sequencing data (**Figure 6C**). CD36 in serum was well expressed (log2[RFU]>11), while HMGN1 was detected just above the threshold of detection in patients (log2[RFU<8]). Finally, we identified four abundant DEPs that were increased in serum of patients. These four DEPs can bind to receptors that were well expressed in the transcriptomic data of the mDCs: The alarmins ***heat shock protein 90*** (binds to receptor encoded by ***SCARF1***) and ***S100A12*** protein (can bind to receptores encoded by ***CD36***, ***AGER*** and ***TLR4***), the TLR regulatory ligand ***Annexin A1*** (binds FPR1 receptor), and the ***adrenomedullin***, a neuropeptide with immunoregulatory functions (binds receptor encoded by ***CALCRL***) (**Figure 6D**). Collectively, these data show that NIU is characterized by a distinct serum proteome that contains DEPs that can target receptors implicated in the transcriptome of CD1c^{+} dendritic cells.

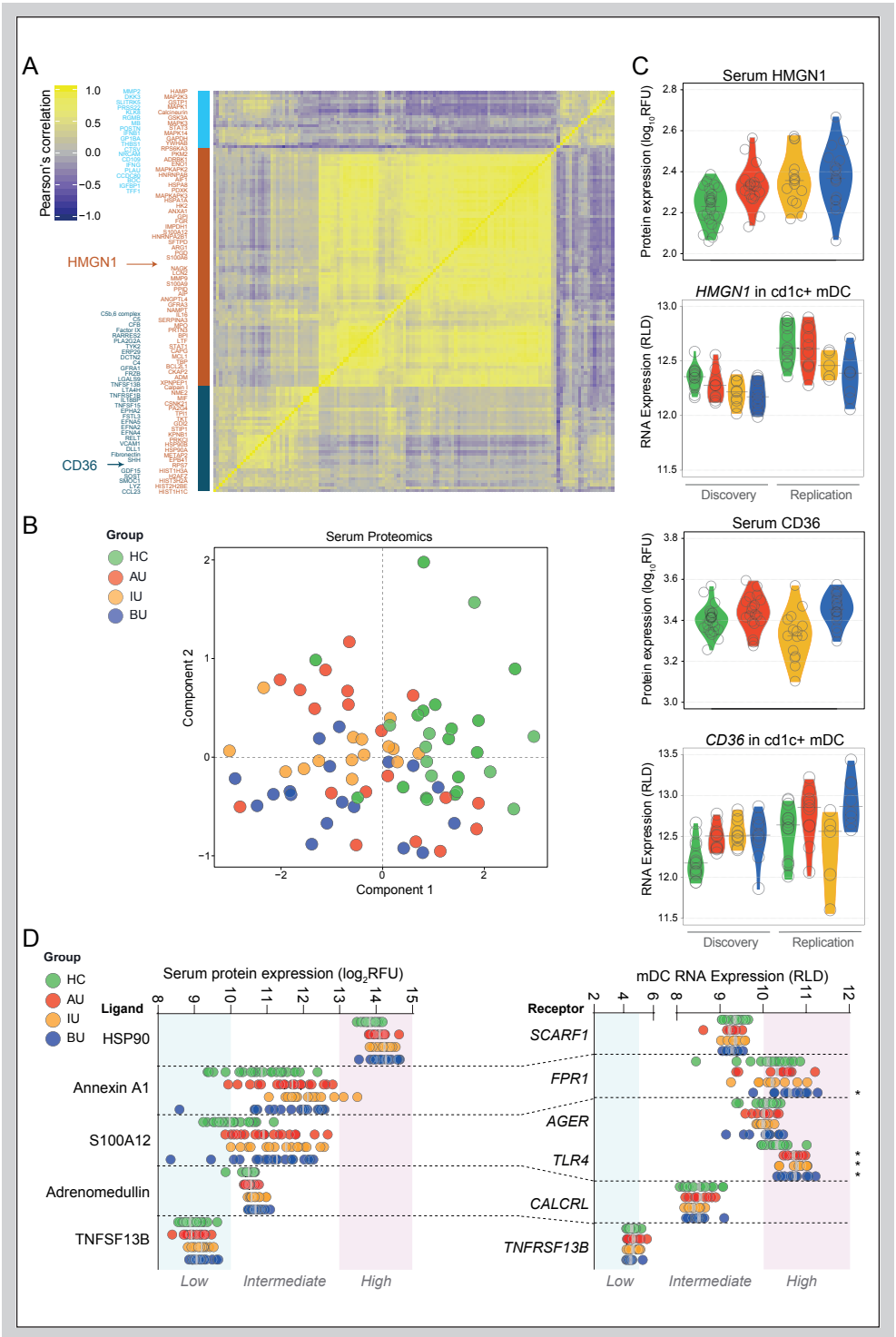

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Serum HMGN1
Protein expression (log10RFU)
HMGN1 in cd1c+ mDC
RNA Expression (RLD)
Discovery
Replication
Serum CD36
CD36 in cd1c+ mDC
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Figure 6. Changes in the serum proteome are associated with the dendritic cell transcriptome of patients with non-infectious uveitis. **A.** Correlation matrix of 115 differentially expressed proteins (DEPs) in serum of patients from the discovery and replication cohort. Three large clusters of co-expressing proteins are indicated by colors. **B.** Principal component analysis of the 115 DEPs in serum seperates patients from controls. **C.** The serum protein expression and gene expression levels for CD36 and HMGN1. **D.** The serum protein levels for four abundant DEPs (log2[RFU>10]) and gene expression levels from their respective well-expressed receptors in mDCs from the discovery cohort. TNFSF13B is shown as a reference DEPs with relatively low serum expression and low gene expression of its receptor TNFRSF13B in mDCs. **Abbreviations:** AGER: Advanced Glycosylation End-Product Specific Receptor, AU: HLA-B27 associated anterior uveitis, BU: Birdshot uveitis, CALCRL: Calcitonin Receptor Like Receptor, FPR1: Formyl Peptide Receptor 1, HC: healthy control, HMGN1: High-mobility group nucleosome binding domain 1, HSP90; heat shock protein 90, IU: idiopathic intermediate uveitis, S100A12: S100 Calcium Binding Protein A12, TLR: Toll-like receptor, TNFSF13B: TNF Superfamily Member 13b, TNFRSF13B: TNF Receptor Superfamily Member 13B, SCARF1: Scavenger Receptor Class F Member 1.

DISCUSSION

We revealed that the CD1c$^+$ mDC isolated from the blood of uveitis patients have a distinct functional transcriptome that shows a distinct 'gene signature' of 147 co-expressed transcriptional regulators, innate immune receptors, and chemokine receptors.

In uveitis, myeloid dendritic cells have been shown to be enriched in aqueous humor of anterior uveitis[5] and elevated proportions of circulating CD1c+ mDCs have been associated with uveitis activity[12]. In this study, we investigated the transcriptional phenotype of CD1c$^+$ myeloid dendritic cells in three archetypical types of human NIU and observed substantial changes in the functional genetic make-up of these immune sentinels. Our results show it is possible to discern eye-restricted disease using only transcriptional data from relevant immune cells in blood.

We identified and replicated a uveitis associated gene module consisting of 147 genes that function in Toll-like receptor (TLR) signaling, cytokine production, and regulation of gene expression and translation ("signature A"). More specifically, we observed higher expression levels of several Toll-like receptor family members, including ***CD180,*** and ***TLR-5, -6, -7, and -8*** and chemokine receptors, such as ***CX3CR1, CCR5,*** and ***CCR2***. Furthermore, there were six transcription factors that were considered central to signature A: we found increased expression levels of ***NFKB1*** and decreased expression of ***IRF8***, ***ATF4***, ***JDP2***, ***PATZ1*** and ***RUNX3***. Nuclear factor kappa B (NF-κB) is a transcription factor complex involved in the activation and maturation of DCs upon interaction of pathogen-associated molecular patterns (PAMPs) with Toll-like receptors.[30] and is considered central to inflammation.[31,32] The exact implications of the observed increased gene expression levels of ***NFKB1*** have to be tested further, especially since alternative splicing of results in multiple transcript variants encoding different functional isoforms.[33]

The transcription factor RUNX3 is an important transcriptional regulator of CD1c+ mDCs.[29] Loss of functional RUNX3 results in an hyperactivated and pro-inflammatory dendritic cell phenotype.[34] The function of the other transcription factors may be more context dependent. For example, IRF8 deletion in T cells exacerbates uveitis in mice but loss of IRF8 expression in retinal cells protects from the development of uveitis.[35] Therefore, functional studies are needed to assess the exact role of the ensemble of these transcription factors and elucidate the collective function of these factors and unravel if interfering with one of these signals might abrogate uveitis activity.

Little is known about the role of CD1c expressing myeloid dendritic cells (CD1c+ mDCs) in non-infectious uveitis (NIU). However, in chronic inflammatory conditions these cells are considered as potent antigen presenting cells which are known for their ability to promote effector functions in Th1 and Th17 cells (both considered key players in the pathogenesis of human NIU).[4,6] Genes linked to antigen presentation or required for T cell signaling (e.g., ***HLA***, ***CD80***, ***CD83, CD86***) were either downregulated or unchanged in patients with uveitis in this study. This may suggest that the CD1c+ mDCs are not fully activated or exhibit a mature phenotype. Neither did the CD1c+ mDC phenotype resemble the "semi-mature" phenotype, which is hallmarked by high expression of ***MHC*** and costimulatory molecules and low expression of inflammatory cytokines such as IL-12, IL-6 and TNF-α.[28,36] In contrast, the CD1c+ mDCs in NIU show elevated expression of pattern recognition receptors and chemokine receptors that are implicated in the recruitment DCs to inflamed tissues.[37] These genes are also upregulated in mDCs isolated from lymph nodes.[29] Therefore, the transcriptome data suggest the CD1c+ mDCs in NIU are 'primed' or in a state which render these cells hypersensitive to stimuli, most likely triggering migration to secondary lymphoid tissues.[38–41] The 'primed' CD1c+ mDC phenotype is further supported by the composition of the serum proteome. NIU showed higher concentrations of numerous ligands for receptors on mDCs, including multiple S100 proteins and heat-shock proteins, which bind TLR4 and CD36 (receptors part of signature A), and Annexin-A1 and Adrenomedullin, that bind FPR1 and CALCRL receptor, respectively. It is tempting to speculate that interfering with these ligands or receptors may prevent migration of cells and halt disease (progression). Upcoming functional studies will be essential to elucidate whether these expression levels indeed represent priming or phenotypes that influence migration.

The different types of uveitis are also characterized by unique transcriptional programs. Although the number of genes that were differentially expressed between the uveitis subtypes is a magnitude lower compared to controls, study of these genes may hold

the key for understanding the clinical distinct phenotypes of the subtypes investigated here (i.e., the anatomically distinct eye inflammation). One remarkable finding included the replicated lower expression of ***HLA-B*** in BU compared to AU. We note this because all the AU patients in this study were HLA-B(27) positive, while BU is associated with HLA-A29 (none were HLA-B27+). If the expression of ***HLA-B*** is directly related to antigen presentation in these patients will need further investigation.[42,43] Perhaps, the ***HLA-B*** expression in BU represents an underappreciated feature of this form of uveitis.

We used a combination of strategies to identify relevant disease related signals. Differentially expressed genes provided the input for WGCNA analysis to find clusters of co-expressed genes. These findings were then replicated in a second independent cohort. A relatively limited number of DEGs were replicated.[44] This is most likely caused by the overall lower number of DEGs in the replication cohort. Differences in sorting method, the freezing of cells, the sequencing batch effect will all have contributed to the lower replication potential. We hypothesize that the RNA yield for this rare blood population in the frozen (FACS sorted) samples of the replication cohort was perhaps less sufficient for detection of lower expressing transcripts. In support of this, we noted an underrepresentation of DEGs with lower expression levels (**Supplementary Figure 6**). This also explains why three modules of the replication cohort (R-I to R-III) overlap with one single module in the discovery cohort (D-I): The genes with high module membership (the highly connected drivers of the module) mostly exhibited relatively low expression in the discovery cohort (RLD<2) and may have been insufficiently captured in the replication cohort. (e.g., *TFAP2A*) (**Figure 4**). Regardless, the pattern of clustering of samples, and gene expression patterns of signature A were highly consistent in both cohorts and underscores that the identified gene cluster represent a genuine and robust disease signature in the CD1c^{+} myeloid dendritic cell population that form the basis for subseqeuent studies.

In summary, we found that the transcriptome of circulating CD1c+ mDCs is affected in NIU patients suggestive of a "hypersensitive" or "primed" state. The fact that we can distinguish eye- inflammation based on relevant circulating cells provides proof of concept of a systems wide dysregulation of the immune system in NIU. These results shed light on the pathogenic pathways implicated in NIU and serve as a starting point for further research into therapeutic targets.

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Supplementary Table 1. Antibody panel used for Flow Cytometric staining of Peripheral Blood Mononuclear Cells

Marker	Chanel	Dilution	Clone	Company
CD123	FITC	1:20	7G3	BD Pharmingen
-	PerCP-CY5.5	-	-	-
CD1c	APC	1:10	AD5-8E7	Miltenyi
CD3	AF700	1:40	UCHT1	BioLegend
CD19	AF700	1:50	HIB19	eBioscience
CD56	AF700	1:50	B159	BD Pharmingen
CD14	eFluor780	1:40	61D3	eBioscience
HLA-DR	BV421	1:100	L243	BioLegend
CD16	BV510	1:100	3G8	BD Horizon
-	BV605	-	-	-
CD141	BV711	1:50	1A4	BD Horizon
CD169	PE	1:20	7-239	eBioscience
CD303	PE-Cy7	1:40	201A	BioLegend
CD11c	PE-CF594	1:200	B-ly6	BD Horizon

Supplementary Table 2. FACS panel used for FACS sorting of mDC1 cells

Marker	Chanel	Dilution	Clone	Company
CD3	AF700	25	UCHT1	Biolegend
CD4	BV711	50	OKT4	Sony Biotechnology
CD8	V500	50	RPA-T8	BD
CD14	PerCP-Cy5.5	100	HCD14	Biolegend
CD19	BV605	50	SJ25C1	BD
BDCA-3 (CD141)	APC	20	AD5-14H12	Miltenyi
BDCA-4 (CD304)	PE	20	AD5-17F6	Miltenyi
CD123	FITC	20	6H6	eBioscience
CD1c	BV421	25	L161	Sony Biotechnology

Supplementary Table 3. Replicated differentially expressed genes differentiating between uveitis subtypes. The mean (RLD) expression and SD are shown for each gene. Colum one shows the gene names (Hgnc; HUGO Gene Nomenclature Committee).

Gene identification			Discovery cohort								Replication cohort							
Gene	Ensembl		HC		AU		IU		BU		HC		AU		IU		BU	
(hgnc)	gene ID		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
HLA-B	ENSG00000234745		16.16	0.17	16.06	0.13	15.94	0.17	15.87	0.13	16.44	0.19	16.52	0.17	16.54	0.21	16.33	0.15
TLE4	ENSG00000106829	BUvsAU	10.45	0.11	10.49	0.08	10.57	0.12	10.61	0.12	10.46	0.29	10.71	0.20	10.59	0.33	10.40	0.27
TSC22D3	ENSG00000157514	BUvsAU	13.74	0.30	13.76	0.49	13.52	0.43	13.41	0.29	14.42	0.49	14.28	0.34	14.24	0.30	13.94	0.21
C16orf54	ENSG00000185905	BUvsAU	10.11	0.21	9.99	0.19	10.04	0.23	10.19	0.17	10.16	0.32	10.58	0.24	10.60	0.14	10.32	0.19
SENP7	ENSG00000138468	BUvsAU	9.86	0.17	10.03	0.15	9.93	0.09	9.89	0.15	10.16	0.25	9.95	0.21	10.24	0.15	10.27	0.26
TMEM2	ENSG00000135048	IUvsAU	10.00	0.20	10.01	0.27	9.66	0.21	9.81	0.32	10.82	0.40	10.34	0.28	10.68	0.30	10.50	0.24
SIK1B	ENSG00000275993	IUvsAU	10.75	0.43	10.80	0.56	10.27	0.30	10.50	0.41	11.67	0.28	11.11	0.31	11.47	0.43	11.28	0.60
ROGDI	ENSG00000067836	IUvsAU	11.18	0.19	10.91	0.19	11.17	0.22	10.94	0.20	11.37	0.34	11.31	0.40	11.00	0.18	11.40	0.24
C1orf162	ENSG00000143110	IUvsAU	13.18	0.12	13.18	0.19	12.98	0.16	13.01	0.12	12.80	0.32	12.91	0.31	12.56	0.29	12.87	0.29
HERPUD2	ENSG00000122557	IUvsAU	10.07	0.12	10.01	0.10	10.13	0.08	10.08	0.09	10.13	0.24	10.36	0.24	10.03	0.23	10.32	0.27
HPS1	ENSG00000107521	IUvsAU	12.33	0.08	12.27	0.07	12.37	0.09	12.31	0.14	12.47	0.25	12.29	0.17	12.55	0.21	12.46	0.25
CLEC11A	ENSG00000105472	IUvsBU	7.78	0.43	7.48	0.32	7.97	0.82	7.63	0.24	8.16	0.14	8.05	0.09	8.01	0.09	8.19	0.15
ROGDI	ENSG00000067836	IUvsBU	11.18	0.19	10.91	0.19	11.17	0.22	10.94	0.20	11.37	0.34	11.31	0.40	11.00	0.18	11.40	0.24
MYADM	ENSG00000179820	IUvsBU	12.86	0.28	12.83	0.21	12.84	0.28	13.10	0.19	13.10	0.39	13.30	0.15	12.70	0.29	13.17	0.48
MTURN	ENSG00000180354	IUvsBU	9.11	0.11	9.07	0.17	9.21	0.23	9.07	0.10	9.54	0.24	9.63	0.18	9.42	0.19	9.67	0.25
PEX26	ENSG00000215193	IUvsBU	10.55	0.07	10.56	0.09	10.62	0.05	10.54	0.06	10.97	0.14	10.91	0.24	10.88	0.24	10.58	0.36
SEMA4G	ENSG00000095539	IUvsBU	6.97	0.25	6.95	0.21	6.90	0.20	7.10	0.29	4.18	0.03	4.17	0.00	4.17	0.00	4.23	0.09

Abbreviations: AU: HLA-B27-associated Anterior Uveitis, BU; Birdshot Uveitis, HC: Healthy Control, IU: Idiopathic Intermediate Uveitis, SD: standard deviation

Supplementary Table 4. Replicated differentially expressed genes from Signature A. The mean (RLD) expression and SD are shown for each gene. Colum one shows the gene names (Hgnc; HUGO Gene Nomenclature Committee). The ensemble ID and ensemble transcript IDs are shown in columns two and three respectively.

Gene identification		Discovery cohort								Replication cohort							
Gene	Ensembl	HC		AU		IU		BU		HC		AU		IU		BU	
(hgnc)	gene ID	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
ABCC5	ENSG00000114770	9.66	0.17	9.83	0.11	9.91	0.14	9.84	0.16	10.13	0.18	10.25	0.18	10.22	0.13	10.32	0.22
AMZ2	ENSG00000196704	10.02	0.14	10.07	0.14	9.93	0.11	10.02	0.10	10.25	0.27	10.14	0.14	10.45	0.34	10.03	0.39
APBB1IP	ENSG00000077420	11.48	0.12	11.58	0.11	11.64	0.11	11.67	0.12	11.18	0.31	11.46	0.24	11.55	0.27	11.40	0.20
ARHGEF2	ENSG00000116584	12.44	0.13	12.49	0.12	12.54	0.07	12.57	0.10	12.56	0.23	12.77	0.22	12.81	0.15	12.65	0.17
ARID5A	ENSG00000196843	10.91	0.24	10.82	0.20	10.68	0.16	10.89	0.28	10.93	0.31	10.67	0.14	10.72	0.17	10.59	0.32
ASH2L	ENSG00000129691	10.16	0.14	10.25	0.11	10.30	0.08	10.32	0.13	10.29	0.27	10.56	0.11	10.39	0.35	10.49	0.22
ATF4	ENSG00000128272	13.40	0.25	13.31	0.18	13.23	0.19	13.27	0.24	13.78	0.19	13.58	0.15	13.46	0.13	13.59	0.25
ATG4C	ENSG00000125703	9.46	0.22	9.65	0.14	9.63	0.05	9.66	0.10	9.32	0.25	9.68	0.22	9.56	0.32	9.50	0.18
BCL7A	ENSG00000110987	10.45	0.29	10.25	0.28	10.18	0.25	10.28	0.35	11.29	0.51	10.92	0.44	10.89	0.16	11.03	0.32
BRD8	ENSG00000112983	9.16	0.21	9.22	0.25	9.26	0.18	9.34	0.19	9.91	0.31	9.88	0.18	9.64	0.18	9.78	0.29
CAMK1D	ENSG00000183049	11.99	0.14	11.87	0.09	11.90	0.13	11.86	0.11	12.33	0.24	12.24	0.23	12.22	0.31	12.06	0.23
CBX4	ENSG00000141582	10.91	0.15	10.75	0.25	10.72	0.18	10.68	0.18	11.21	0.17	10.97	0.17	10.97	0.22	10.95	0.28
CCDC28A	ENSG00000024862	8.81	0.13	8.90	0.16	8.95	0.15	8.97	0.09	9.07	0.20	9.24	0.15	9.20	0.30	9.28	0.24
CCDC94	ENSG00000105248	9.79	0.23	9.68	0.21	9.59	0.23	9.69	0.22	10.14	0.25	9.96	0.15	9.94	0.24	10.02	0.15
CCR2	ENSG00000121807	10.87	0.59	11.25	0.46	11.56	0.33	11.44	0.71	10.65	0.51	11.59	0.29	11.46	0.27	11.29	0.33
CCR5	ENSG00000160791	10.51	0.30	10.78	0.18	10.87	0.13	10.81	0.37	9.88	0.30	10.28	0.24	10.17	0.10	10.13	0.26
CCT2	ENSG00000166226	11.32	0.11	11.25	0.09	11.21	0.10	11.27	0.08	11.42	0.25	11.10	0.28	11.51	0.23	11.24	0.37
CD180	ENSG00000134061	9.72	0.42	10.30	0.24	10.47	0.27	10.56	0.52	10.09	0.25	10.56	0.17	10.54	0.33	10.44	0.37
CD200R1	ENSG00000163606	10.00	0.42	10.25	0.17	10.31	0.21	10.33	0.38	9.40	0.30	9.88	0.25	9.83	0.29	9.73	0.19
CD36	ENSG00000135218	12.20	0.22	12.48	0.16	12.55	0.17	12.49	0.28	12.53	0.33	12.73	0.35	12.30	0.51	12.91	0.30
CPSF2	ENSG00000165934	10.29	0.26	10.46	0.17	10.52	0.16	10.49	0.25	10.36	0.23	10.54	0.20	10.64	0.14	10.76	0.15
CREM	ENSG00000095794	8.94	0.39	9.07	0.57	8.71	0.29	8.96	0.47	9.08	0.40	8.85	0.20	8.87	0.20	8.95	0.17

CRTC2	ENSG00000160741	11.62	0.15	11.56	0.06	11.54	0.06	11.52	0.10	11.69	0.23	11.59	0.16	11.51	0.29	11.51	0.13
CSGALNACT2	ENSG00000169826	11.96	0.18	11.95	0.18	11.83	0.11	11.95	0.13	12.15	0.25	12.04	0.20	11.86	0.38	12.08	0.27
CX3CR1	ENSG00000168329	10.51	0.57	11.02	0.38	11.21	0.30	11.24	0.57	10.05	0.48	10.86	0.41	10.78	0.37	10.85	0.27
CYBB	ENSG00000165168	13.04	0.25	13.28	0.14	13.42	0.14	13.31	0.20	13.37	0.26	13.51	0.27	13.56	0.14	13.63	0.23
DAB2	ENSG00000153071	9.85	0.33	9.97	0.18	10.12	0.27	10.12	0.34	9.58	0.20	9.78	0.18	9.70	0.33	9.89	0.23
DAZAP2	ENSG00000183283	13.89	0.12	13.81	0.11	13.82	0.12	13.79	0.10	13.98	0.15	13.73	0.11	13.73	0.08	13.78	0.14
DCAF12	ENSG00000198876	9.95	0.28	10.04	0.27	10.23	0.23	10.21	0.37	9.89	0.20	10.17	0.16	10.08	0.32	10.10	0.35
DHX57	ENSG00000163214	8.83	0.18	8.93	0.11	9.03	0.18	9.03	0.14	9.43	0.27	9.52	0.28	9.44	0.17	9.26	0.17
DNAJB6	ENSG00000105993	10.82	0.17	10.73	0.16	10.72	0.13	10.61	0.13	10.99	0.30	10.78	0.17	10.76	0.18	10.69	0.20
DTD2	ENSG00000129480	8.69	0.21	8.78	0.10	8.84	0.14	8.92	0.18	8.67	0.18	8.87	0.13	8.80	0.18	8.87	0.21
DUSP7	ENSG00000164086	10.09	0.32	10.19	0.22	10.33	0.12	10.37	0.33	10.13	0.22	10.34	0.15	10.22	0.20	10.34	0.16
ELMSAN1	ENSG00000156030	12.04	0.19	11.98	0.14	12.14	0.09	12.03	0.16	12.41	0.26	12.12	0.23	12.27	0.27	12.34	0.18
EXOC1	ENSG00000090989	10.01	0.13	10.12	0.12	10.20	0.11	10.23	0.11	9.75	0.25	9.87	0.13	9.88	0.12	9.99	0.24
EXOC6	ENSG00000138190	9.19	0.11	9.22	0.16	9.30	0.18	9.31	0.09	8.96	0.22	9.21	0.18	9.16	0.16	9.30	0.16
FAM53C	ENSG00000120709	11.23	0.21	11.12	0.22	11.05	0.17	11.07	0.23	11.51	0.24	11.25	0.22	11.32	0.21	11.31	0.27
FGL2	ENSG00000127951	14.55	0.32	14.70	0.30	14.81	0.13	14.72	0.23	14.21	0.34	14.62	0.27	14.61	0.45	14.63	0.27
FTH1P20	ENSG00000226564	9.45	0.19	9.39	0.26	9.26	0.21	9.40	0.21	8.09	0.13	7.98	0.05	8.00	0.09	8.02	0.08
FZD1	ENSG00000157240	10.14	0.16	10.25	0.23	10.42	0.19	10.40	0.35	9.85	0.32	10.18	0.24	9.75	0.12	9.88	0.24
GAPT	ENSG00000175857	10.14	0.41	10.58	0.19	10.61	0.27	10.61	0.41	10.75	0.47	11.25	0.29	11.48	0.20	11.09	0.28
GCA	ENSG00000115271	11.21	0.14	11.36	0.17	11.32	0.19	11.37	0.19	10.95	0.30	11.36	0.21	11.05	0.14	11.47	0.25
GIMAP2	ENSG00000106560	9.16	0.26	9.30	0.19	9.38	0.23	9.37	0.19	8.84	0.26	9.10	0.23	9.26	0.17	9.15	0.15
GNA12	ENSG00000146535	11.71	0.21	11.64	0.14	11.47	0.15	11.54	0.16	11.84	0.27	11.62	0.20	11.64	0.30	11.72	0.20
GNAS	ENSG00000087460	14.47	0.12	14.34	0.07	14.33	0.08	14.30	0.07	14.66	0.13	14.51	0.07	14.52	0.10	14.42	0.20
H3F3B	ENSG00000132475	15.04	0.15	14.94	0.13	14.88	0.12	14.87	0.08	15.28	0.18	15.11	0.13	15.09	0.19	15.12	0.12
HCP5	ENSG00000206337	9.69	0.24	9.92	0.22	10.02	0.27	10.02	0.16	10.02	0.21	10.30	0.18	10.31	0.19	10.29	0.30
HERPUD2	ENSG00000122557	10.07	0.12	10.01	0.10	10.13	0.08	10.08	0.09	10.13	0.24	10.36	0.24	10.03	0.23	10.32	0.27
HMGN1	ENSG00000205581	12.35	0.11	12.28	0.15	12.21	0.12	12.18	0.14	12.67	0.18	12.61	0.20	12.46	0.12	12.36	0.21
HNRNPA1	ENSG00000135486	13.13	0.16	13.03	0.13	12.97	0.11	12.97	0.18	13.92	0.20	13.82	0.14	13.85	0.35	13.71	0.15

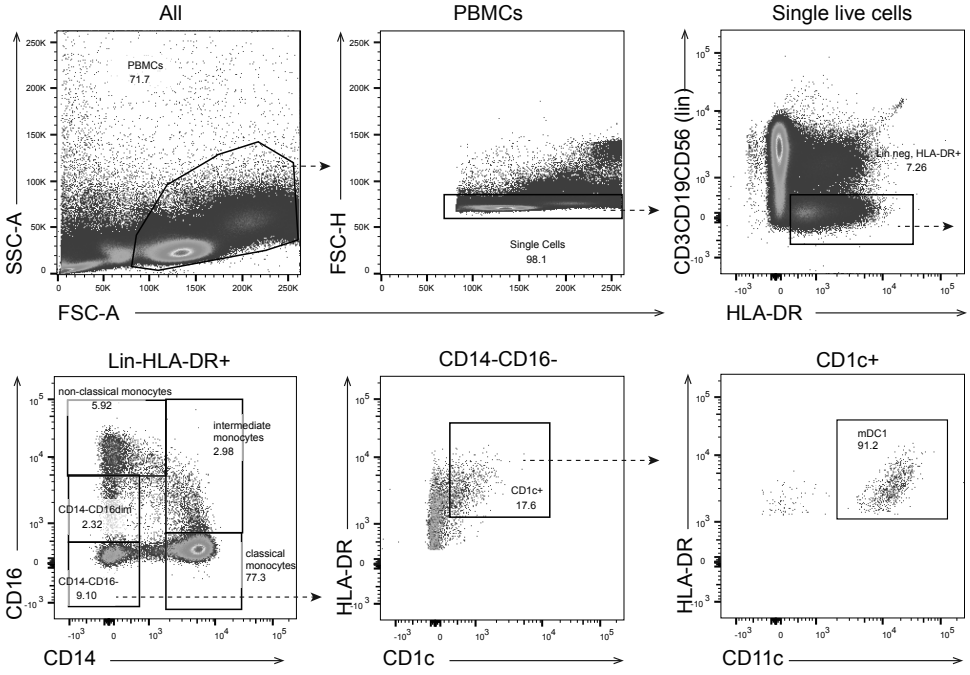
HNRNPDL	ENSG00000152795	13.23	0.13	13.10	0.11	13.08	0.12	13.07	0.11	13.52	0.12	13.38	0.11	13.30	0.19	13.36	0.08
HNRNPF	ENSG00000169813	12.23	0.12	12.26	0.13	12.35	0.13	12.37	0.09	12.28	0.23	12.51	0.26	12.49	0.18	12.46	0.22
ID3	ENSG00000117318	8.00	0.49	7.58	0.51	8.04	0.28	7.92	0.44	7.69	0.19	7.50	0.09	7.53	0.13	7.55	0.10
IFI16	ENSG00000163565	12.31	0.22	12.47	0.17	12.50	0.11	12.52	0.17	12.23	0.35	12.62	0.25	12.70	0.18	12.65	0.22
IFT52	ENSG00000101052	9.38	0.13	9.25	0.14	9.19	0.15	9.27	0.08	9.33	0.23	9.30	0.22	9.15	0.24	9.03	0.24
IGSF6	ENSG00000140749	10.96	0.38	11.18	0.20	11.29	0.20	11.35	0.34	11.41	0.54	12.01	0.30	11.69	0.62	11.86	0.26
IRF8	ENSG00000140968	12.75	0.14	12.62	0.19	12.73	0.10	12.60	0.14	13.55	0.36	13.31	0.29	13.52	0.22	13.37	0.19
JDP2	ENSG00000140044	10.17	0.34	10.00	0.29	9.92	0.18	9.87	0.31	10.18	0.32	9.95	0.17	10.09	0.22	9.95	0.17
KANSL2	ENSG00000139620	9.89	0.28	9.70	0.23	9.63	0.15	9.60	0.28	10.21	0.33	10.01	0.23	10.07	0.25	9.93	0.23
KCNQ1	ENSG00000053918	11.62	0.14	11.44	0.12	11.49	0.13	11.50	0.09	11.85	0.20	11.53	0.22	11.50	0.24	11.62	0.29
KIDINS220	ENSG00000134313	11.42	0.13	11.52	0.11	11.57	0.04	11.53	0.18	11.51	0.20	11.58	0.22	11.31	0.28	11.62	0.40
KLHL21	ENSG00000162413	10.18	0.18	9.99	0.18	10.07	0.09	9.93	0.13	10.64	0.41	10.31	0.15	10.36	0.10	10.22	0.23
KRCC1	ENSG00000172086	9.23	0.23	9.35	0.20	9.38	0.15	9.44	0.25	9.20	0.21	9.41	0.17	9.40	0.22	9.42	0.10
LGALS3BP	ENSG00000108679	9.26	0.42	8.79	0.49	9.20	0.40	9.01	0.47	8.27	0.21	8.35	0.21	8.62	0.45	8.35	0.12
LPXN	ENSG00000110031	11.97	0.13	12.11	0.13	12.10	0.09	12.07	0.09	11.60	0.34	11.94	0.22	11.77	0.42	11.99	0.11
LRRC8D	ENSG00000171492	10.61	0.20	10.76	0.12	10.79	0.12	10.73	0.19	10.70	0.31	10.95	0.18	10.70	0.41	11.02	0.30
MEF2D	ENSG00000116604	12.42	0.30	12.29	0.29	12.22	0.19	12.37	0.29	12.39	0.31	11.94	0.21	12.07	0.28	12.15	0.24
METTL7A	ENSG00000185432	10.62	0.24	11.00	0.25	11.05	0.10	11.03	0.29	11.89	0.35	12.15	0.34	12.43	0.16	12.08	0.42
MKNK2	ENSG00000099875	13.38	0.20	13.32	0.12	13.22	0.13	13.33	0.22	13.63	0.19	13.40	0.19	13.31	0.17	13.48	0.19
MPEG1	ENSG00000197629	15.05	0.19	15.23	0.15	15.34	0.20	15.28	0.18	14.97	0.33	15.21	0.17	15.22	0.31	15.37	0.16
MYLIP	ENSG00000007944	10.63	0.19	10.44	0.28	10.48	0.15	10.34	0.30	11.43	0.21	11.05	0.34	11.08	0.16	11.08	0.18
NASP	ENSG00000132780	10.83	0.07	10.77	0.07	10.74	0.10	10.67	0.11	10.84	0.37	11.11	0.17	11.07	0.32	11.16	0.35
NFKB1	ENSG00000109320	11.84	0.12	11.96	0.10	11.99	0.14	12.06	0.13	12.01	0.25	12.22	0.22	12.22	0.25	12.21	0.19
NGDN	ENSG00000129460	9.66	0.18	9.60	0.12	9.52	0.09	9.53	0.17	10.29	0.30	10.06	0.23	10.28	0.23	10.04	0.28
NPEPPS	ENSG00000141279	10.99	0.13	11.03	0.13	11.15	0.17	11.07	0.10	11.48	0.26	11.42	0.26	11.48	0.23	11.72	0.32
NR1D2	ENSG00000174738	10.46	0.41	10.25	0.44	10.13	0.29	10.13	0.43	10.31	0.27	9.94	0.30	10.30	0.13	10.01	0.31
NUP43	ENSG00000120253	8.59	0.26	8.70	0.20	8.84	0.16	8.81	0.24	9.10	0.18	9.35	0.17	9.36	0.25	9.34	0.27
OGFRL1	ENSG00000119900	13.45	0.14	13.70	0.21	13.68	0.18	13.66	0.18	13.07	0.29	13.39	0.22	13.15	0.36	13.42	0.24

OSBPL11	ENSG00000144909	11.04	0.17	11.19	0.08	11.22	0.10	11.23	0.16	11.05	0.30	11.31	0.24	11.10	0.42	11.36	0.19
PAFAH1B2	ENSG00000168092	10.74	0.22	10.61	0.17	10.64	0.05	10.59	0.14	11.03	0.23	10.81	0.29	10.86	0.16	10.73	0.28
PATZ1	ENSG00000100105	10.15	0.10	10.10	0.07	10.06	0.09	10.07	0.12	10.37	0.25	10.11	0.27	10.27	0.25	10.38	0.17
PDE4D	ENSG00000113448	7.67	0.66	7.55	0.60	7.18	0.37	7.42	0.67	8.51	0.33	8.14	0.17	8.24	0.15	8.28	0.20
PELI1	ENSG00000197329	10.31	0.30	10.38	0.36	10.15	0.23	10.12	0.25	11.13	0.50	10.63	0.28	10.64	0.30	10.70	0.21
POLR1C	ENSG00000171453	9.07	0.27	8.96	0.15	8.87	0.10	8.85	0.20	9.51	0.20	9.45	0.27	9.40	0.12	9.30	0.20
PPARD	ENSG00000112033	10.08	0.13	10.03	0.16	9.93	0.13	10.04	0.18	9.81	0.22	9.94	0.17	9.84	0.32	10.08	0.25
PPTC7	ENSG00000196850	12.07	0.17	12.00	0.16	11.94	0.14	11.96	0.10	12.11	0.28	11.99	0.19	11.75	0.26	11.89	0.21
PSMB4	ENSG00000159377	12.21	0.09	12.15	0.09	12.15	0.05	12.14	0.05	12.29	0.22	12.47	0.18	12.37	0.17	12.40	0.11
PTP4A1	ENSG00000112245	12.15	0.21	12.06	0.27	11.98	0.17	11.97	0.21	12.27	0.28	11.86	0.26	11.96	0.29	11.99	0.27
RAD51D	ENSG00000185379	8.21	0.15	8.31	0.13	8.29	0.06	8.33	0.13	8.62	0.18	8.81	0.16	8.85	0.17	8.72	0.16
RALGDS	ENSG00000160271	11.17	0.22	11.08	0.26	11.01	0.21	11.25	0.36	11.16	0.29	10.97	0.11	10.87	0.20	11.04	0.28
RBBP7	ENSG00000102054	10.96	0.11	10.91	0.10	10.87	0.07	10.86	0.12	11.28	0.22	11.06	0.19	11.28	0.13	11.14	0.23
RBM15	ENSG00000162775	9.56	0.19	9.48	0.13	9.46	0.07	9.42	0.19	10.20	0.31	10.03	0.16	10.08	0.41	9.96	0.17
RBM17	ENSG00000134453	11.07	0.13	11.00	0.07	10.96	0.09	10.95	0.10	11.38	0.17	11.23	0.21	11.31	0.35	11.13	0.24
RBSN	ENSG00000131381	9.80	0.12	9.71	0.16	9.67	0.10	9.67	0.10	10.08	0.37	9.92	0.19	10.07	0.20	9.77	0.30
REM2	ENSG00000139890	5.73	0.17	5.71	0.22	5.54	0.18	5.64	0.23	6.71	0.10	6.66	0.04	6.93	0.24	6.72	0.11
RGS18	ENSG00000150681	10.56	0.37	10.80	0.26	10.85	0.15	10.92	0.31	10.17	0.39	10.51	0.32	10.52	0.22	10.54	0.27
RNF168	ENSG00000163961	10.32	0.27	10.20	0.21	10.21	0.20	10.14	0.20	10.47	0.28	10.17	0.30	10.14	0.33	10.23	0.22
RNF4	ENSG00000063978	11.35	0.13	11.26	0.10	11.22	0.07	11.32	0.15	11.58	0.27	11.31	0.17	11.51	0.29	11.50	0.27
RNPS1	ENSG00000205937	11.77	0.08	11.67	0.11	11.64	0.07	11.65	0.07	12.13	0.21	12.03	0.13	12.12	0.06	12.28	0.24
RPAP2	ENSG00000122484	8.84	0.13	8.96	0.16	8.98	0.13	8.94	0.11	9.41	0.16	9.32	0.14	9.37	0.31	9.51	0.20
RPF1	ENSG00000117133	9.79	0.15	9.60	0.08	9.66	0.10	9.64	0.09	10.06	0.19	9.91	0.14	9.96	0.10	9.82	0.23
RPP30	ENSG00000148688	8.80	0.08	8.78	0.07	8.91	0.14	8.80	0.10	9.15	0.27	9.04	0.12	9.04	0.28	8.92	0.13
RUNX3	ENSG00000020633	12.48	0.18	12.20	0.21	12.27	0.15	12.24	0.33	12.38	0.19	12.15	0.31	12.20	0.32	12.16	0.24
SAMD9	ENSG00000205413	10.18	0.33	10.42	0.29	10.43	0.20	10.39	0.23	10.48	0.30	10.67	0.21	10.95	0.34	10.59	0.14
SCIMP	ENSG00000161929	11.76	0.27	11.83	0.14	11.97	0.11	11.90	0.18	11.54	0.34	11.90	0.10	11.91	0.23	11.84	0.21
SEC23IP	ENSG00000107651	10.04	0.20	10.14	0.15	10.23	0.13	10.17	0.19	10.16	0.23	10.24	0.38	10.54	0.27	10.44	0.34

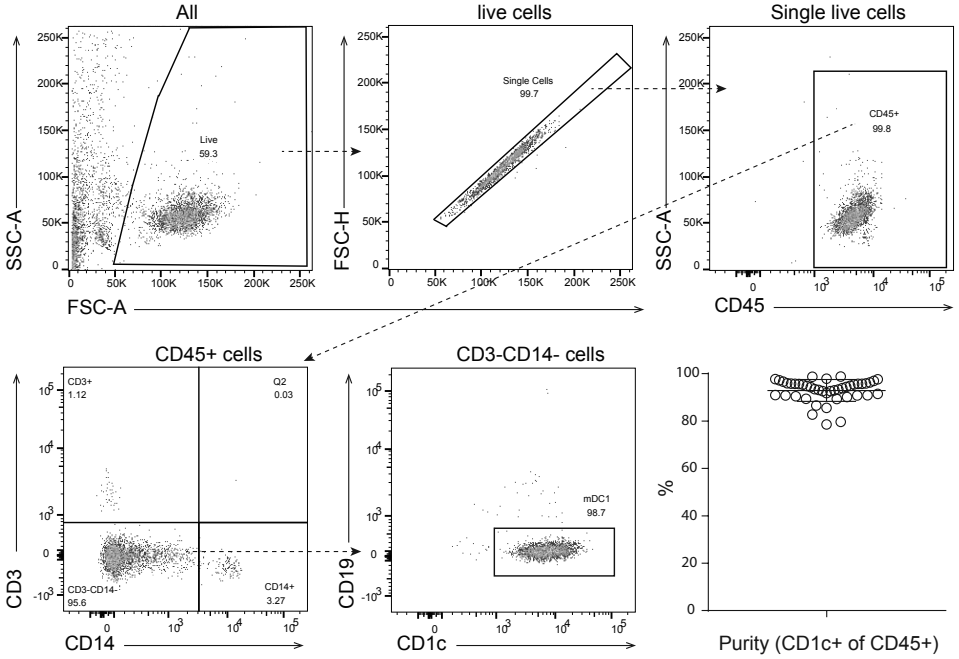
SH3BP5	ENSG00000131370	11.67	0.19	11.48	0.11	11.34	0.24	11.31	0.23	12.48	0.37	12.04	0.36	12.10	0.40	11.91	0.30
SH3TC1	ENSG00000125089	12.65	0.26	12.59	0.17	12.36	0.18	12.48	0.26	12.97	0.33	12.55	0.26	12.41	0.26	12.60	0.27
SLC20A1	ENSG00000144136	10.98	0.23	10.96	0.19	10.90	0.16	10.75	0.17	10.74	0.39	10.60	0.15	10.35	0.40	10.58	0.31
SLC25A20	ENSG00000178537	9.60	0.23	9.87	0.17	9.88	0.19	9.96	0.35	9.94	0.26	10.26	0.19	9.99	0.22	10.01	0.22
SLC2A1	ENSG00000117394	11.33	0.31	11.05	0.33	11.01	0.18	10.94	0.37	11.73	0.42	11.43	0.26	11.57	0.27	11.48	0.23
SLC7A5	ENSG00000103257	10.40	0.64	10.23	0.65	9.85	0.51	10.03	0.66	10.57	0.59	9.96	0.25	10.06	0.12	10.01	0.44
SLC9A3R1	ENSG00000109062	11.13	0.18	10.88	0.25	10.93	0.24	10.88	0.21	11.56	0.39	11.21	0.26	11.34	0.22	11.42	0.29
SNX20	ENSG00000167208	11.71	0.13	11.76	0.16	11.79	0.06	11.85	0.11	11.59	0.16	11.73	0.12	11.76	0.25	11.57	0.19
SPATA2	ENSG00000158480	8.87	0.26	8.63	0.26	8.65	0.15	8.72	0.10	8.96	0.21	8.68	0.22	8.68	0.11	8.65	0.19
SPG11	ENSG00000104133	11.67	0.13	11.77	0.15	11.81	0.10	11.81	0.08	11.84	0.24	11.98	0.17	11.76	0.24	12.03	0.11
SRP54	ENSG00000100883	9.66	0.11	9.71	0.12	9.78	0.15	9.76	0.15	9.60	0.26	9.79	0.30	9.80	0.22	9.96	0.24
SRSF2	ENSG00000161547	12.99	0.12	12.92	0.17	12.85	0.09	12.93	0.18	12.98	0.19	12.97	0.07	12.92	0.14	12.81	0.20
SSBP1	ENSG00000106028	10.59	0.12	10.56	0.10	10.52	0.07	10.48	0.08	10.86	0.26	10.79	0.17	10.74	0.37	10.56	0.35
STK38L	ENSG00000211455	11.46	0.15	11.65	0.14	11.69	0.09	11.73	0.16	10.97	0.40	11.34	0.23	11.33	0.45	11.25	0.27
SUCLG2	ENSG00000172340	9.26	0.22	9.18	0.20	9.41	0.21	9.29	0.27	9.81	0.30	9.63	0.16	9.81	0.31	9.95	0.25
TEP1	ENSG00000129566	12.19	0.18	12.31	0.12	12.37	0.07	12.33	0.09	12.39	0.35	12.62	0.21	12.57	0.06	12.70	0.11
TERF2	ENSG00000132604	9.15	0.10	9.23	0.12	9.29	0.11	9.31	0.13	9.44	0.20	9.63	0.17	9.71	0.34	9.66	0.25
TEX261	ENSG00000144043	10.55	0.09	10.59	0.07	10.65	0.04	10.60	0.12	10.66	0.30	10.74	0.33	10.53	0.29	10.46	0.17
THEMIS2	ENSG00000130775	13.46	0.17	13.44	0.16	13.28	0.12	13.35	0.25	13.37	0.38	13.04	0.47	13.16	0.09	13.22	0.39
THRAP3	ENSG00000054118	11.24	0.12	11.31	0.19	11.42	0.14	11.48	0.12	11.74	0.24	11.80	0.23	11.99	0.16	11.94	0.14
TLE4	ENSG00000106829	10.45	0.11	10.49	0.08	10.57	0.12	10.61	0.12	10.46	0.29	10.71	0.20	10.59	0.33	10.40	0.27
TLR5	ENSG00000187554	9.40	0.27	9.63	0.18	9.47	0.15	9.43	0.33	9.10	0.20	9.24	0.29	9.45	0.29	9.09	0.24
TLR6	ENSG00000174130	9.95	0.17	10.18	0.17	10.28	0.15	10.22	0.19	10.13	0.29	10.34	0.25	10.47	0.16	10.22	0.28
TLR7	ENSG00000196664	9.03	0.38	9.46	0.25	9.72	0.23	9.67	0.39	9.05	0.18	9.46	0.22	9.54	0.27	9.40	0.25
TLR8	ENSG00000101916	10.79	0.39	11.03	0.19	11.26	0.20	11.26	0.35	10.44	0.56	10.99	0.23	10.98	0.37	11.01	0.32
TMEM2	ENSG00000135048	10.00	0.20	10.01	0.27	9.66	0.21	9.81	0.32	10.82	0.40	10.34	0.28	10.68	0.30	10.50	0.24
TMEM229B	ENSG00000198133	9.80	0.52	9.70	0.44	10.12	0.28	9.66	0.40	9.75	0.28	10.00	0.50	9.71	0.19	9.93	0.26
TNFAIP8L2	ENSG00000163154	10.18	0.43	10.35	0.32	10.53	0.15	10.53	0.36	9.60	0.38	10.06	0.17	9.71	0.22	9.97	0.20

TNFRSF1A	ENSG00000067182	11.21	0.18	11.30	0.23	11.36	0.14	11.42	0.22	11.09	0.36	11.27	0.15	11.23	0.29	11.38	0.25
TRAF6	ENSG00000175104	9.45	0.21	9.36	0.25	9.35	0.14	9.28	0.14	9.55	0.22	9.26	0.15	9.23	0.27	9.43	0.17
TRAFD1	ENSG00000135148	9.84	0.22	9.94	0.25	10.13	0.15	10.03	0.19	10.01	0.30	10.13	0.20	10.43	0.22	10.21	0.22
TSNAX	ENSG00000116918	9.62	0.15	9.73	0.11	9.78	0.11	9.78	0.16	10.03	0.17	10.17	0.23	10.35	0.44	10.12	0.15
TWISTNB	ENSG00000105849	9.18	0.26	9.15	0.21	9.09	0.10	9.03	0.18	9.57	0.28	9.34	0.20	9.52	0.25	9.43	0.13
UBC	ENSG00000150991	14.94	0.20	14.76	0.19	14.78	0.19	14.75	0.14	15.10	0.18	14.90	0.21	14.90	0.20	14.96	0.16
USP3	ENSG00000140455	11.84	0.17	11.74	0.10	11.67	0.11	11.72	0.17	12.05	0.17	11.72	0.25	11.71	0.36	11.77	0.20
VMP1	ENSG00000062716	10.40	0.17	10.61	0.13	10.54	0.09	10.64	0.27	10.49	0.24	10.67	0.18	10.92	0.22	10.61	0.22
WHAMM	ENSG00000156232	9.67	0.29	9.55	0.22	9.55	0.13	9.47	0.20	10.05	0.22	9.88	0.20	9.71	0.15	10.05	0.21
ZNF326	ENSG00000162664	10.11	0.22	10.04	0.17	10.04	0.06	9.94	0.12	10.31	0.19	10.14	0.10	10.05	0.19	10.12	0.26
ZNF639	ENSG00000121864	9.90	0.12	9.76	0.05	9.66	0.07	9.65	0.09	10.15	0.24	9.89	0.21	9.96	0.14	9.88	0.22
AC015813.1	ENSG00000264112	9.36	0.25	9.26	0.12	9.15	0.16	9.10	0.18	9.38	0.24	9.21	0.12	9.20	0.24	9.10	0.20
SIK1B	ENSG00000275993	10.75	0.43	10.80	0.56	10.27	0.30	10.50	0.41	11.67	0.28	11.11	0.31	11.47	0.43	11.28	0.60

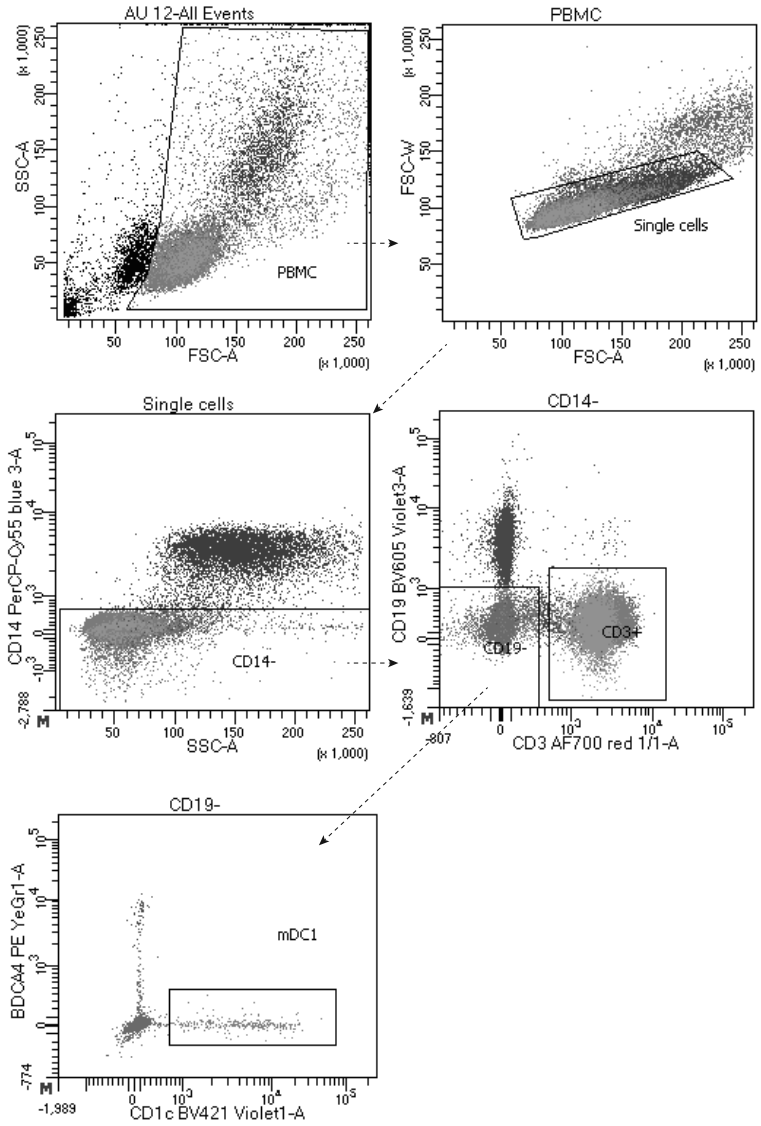
Abbreviations: AU: HLA-B27-associated Anterior Uveitis, BU; Birdshot Uveitis, HC: Healthy Control, IU: Idiopathic Intermediate Uveitis, SD: standard deviation.



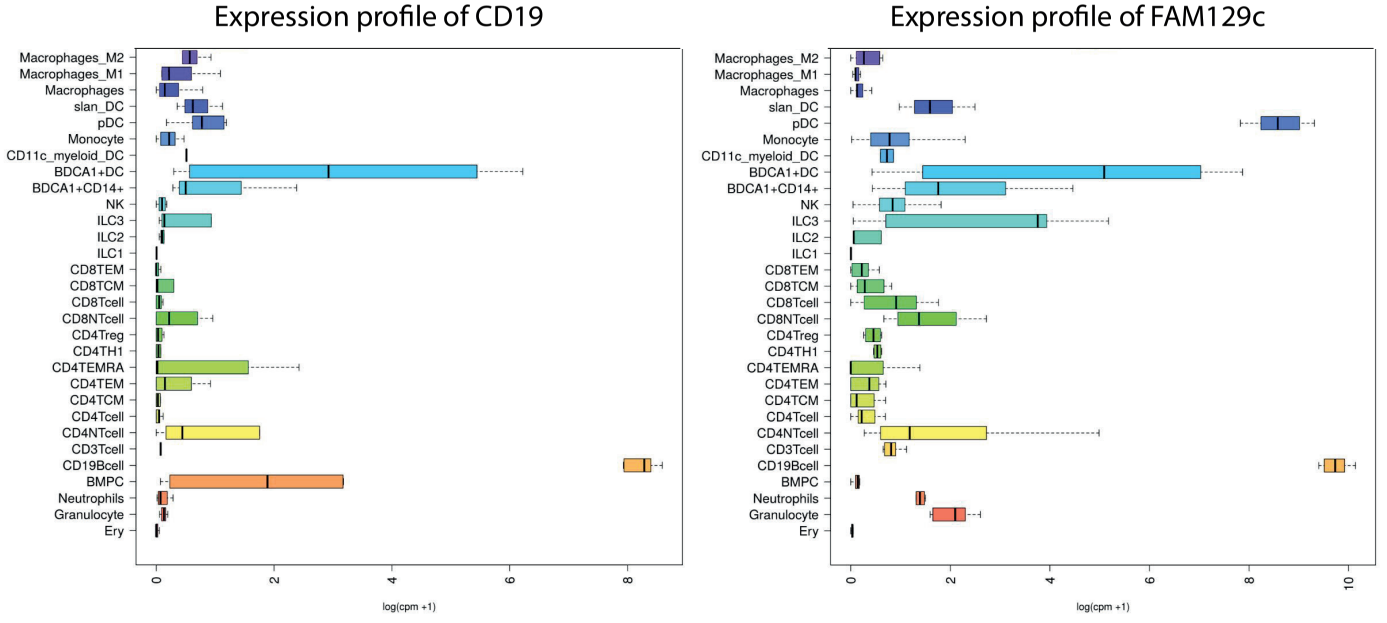
Supplementary Figure 1. Gating strategy used to identify CD1+CD11+ myeloid dendritic cells by flow cytometry of peripheral blood mononuclear cells



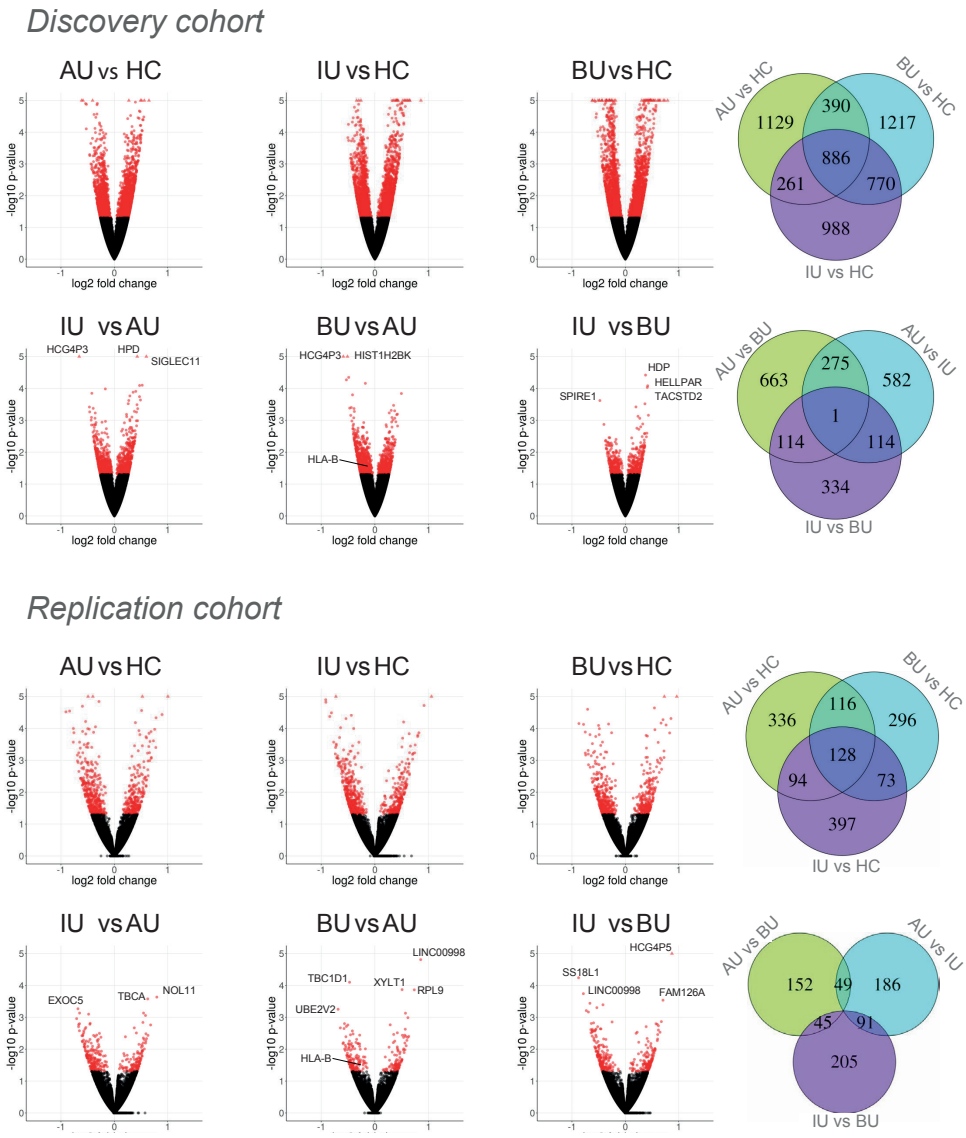
Supplementary Figure 2. Purity check MACS sorted CD1c+ dendritic cells. Gating strategy for purity check of CD1c$^+$ sorted cells that were sorted using Magnetic-activated cell sorting (MACS). Purity is here defined as CD1c$^+$ as % of CD45$^+$.



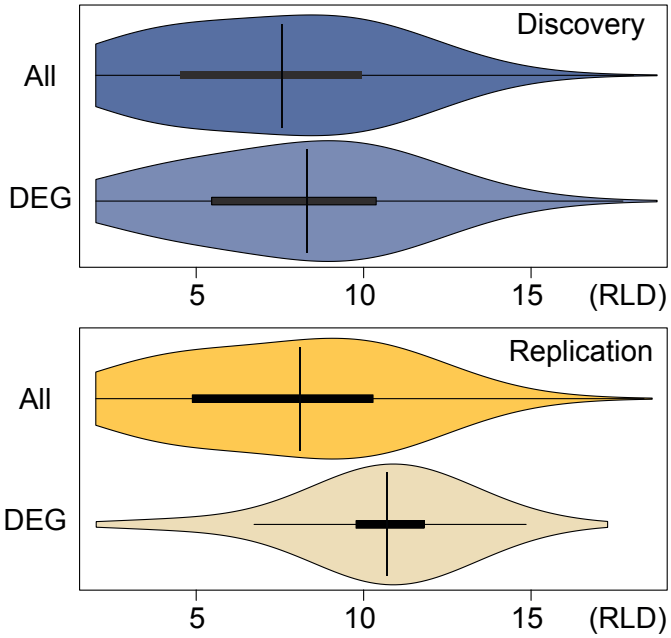
Supplementary Figure 3. Gating strategy used for FACS sorting of CD1c⁺ myeloid dendritic cells for the replication cohort.



Supplementary Figure 4. CD19 and FAM129c expression in CD1c+ dendritic cells. Gene expression data for CD19 and FAM129C in leukocyte cell subsets was obtained from publically available transcriptomic profiling studies. Gene expression is plotted as the logarithm of the counts per million (plus 1). These data are available in a (GUI) tool developed by our laboratory (***Devaprasad et al - manuscript in preparation***).



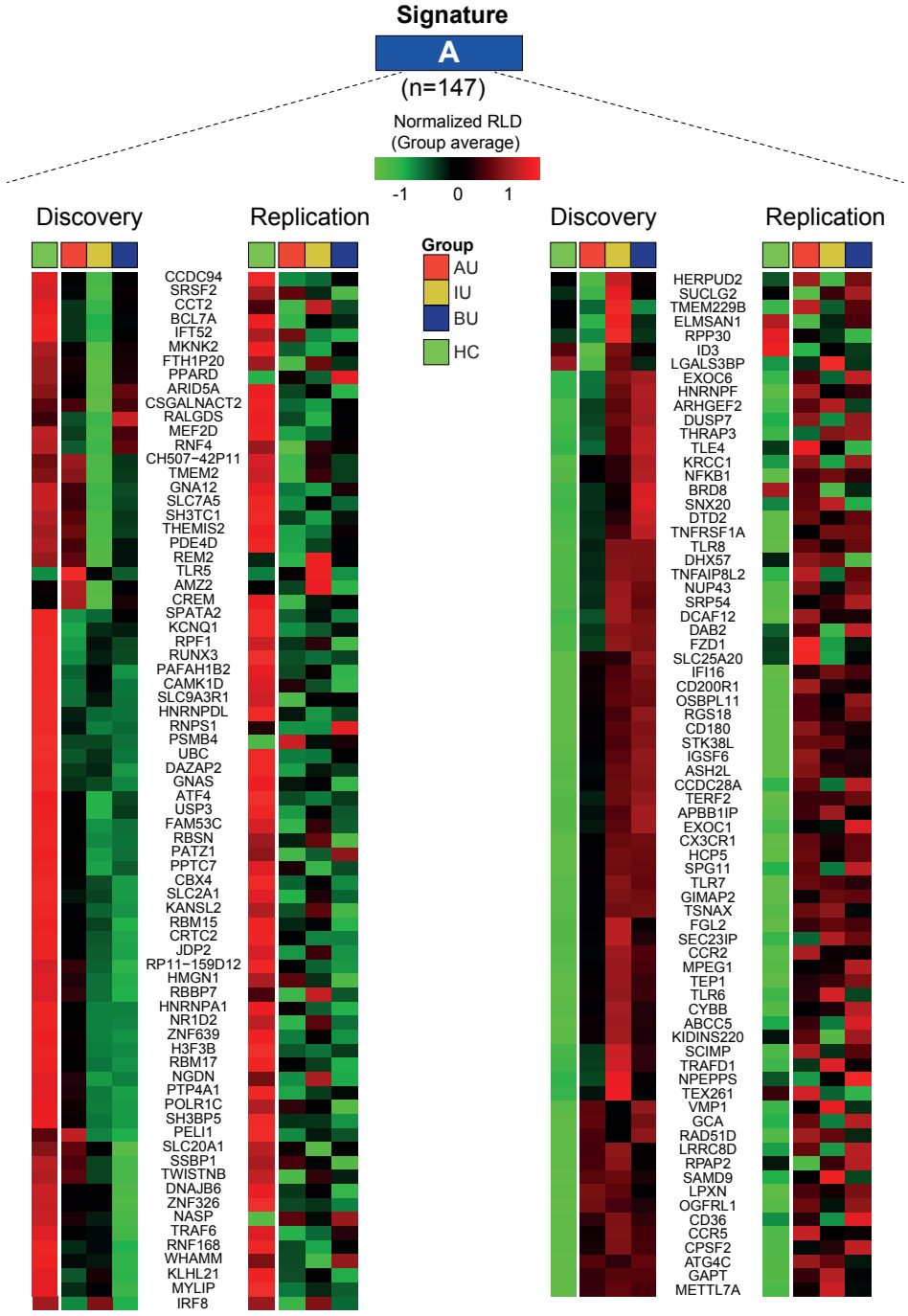
Supplementary Figure 5. Volcano plots of differentially expressed genes between the disease groups for the discovery and replication cohort. In red: differentially expressed genes, in black: non-differentially expressed genes. On the x-axis is the log2 fold change in RLD (expression level). On the y-axis the Log10 of the p-value. On the right are Venn diagrams depicting the DEGs for each comparison. **Abbreviations:** AU: HLA-B27-associated Anterior Uveitis, BU; Birdshot Uveitis, DEG: differentially expressed gene, HC: Healthy Control, IU: Idiopathic Intermediate Uveitis.



Supplementary Figure 6. Mean expression values (RLD) of the differentially expressed genes are higher in the replication cohort. Violin plots represent the distribution of RLD of all or DEGs in the discovery and replication cohort. The median RLD, interquartile range are indicated in black. The median RLD for all DEGs in the discovery cohort is <10 and the median RLD in median >10. **Abbreviation:** DEG: differentially expressed gene.

Supplementary Figure 7. The average gene expression (RLD) for the 147 replicated DEGs from ***Signature A*** of CD1c+ myeloid dendritic cells (mDC1s). **A.** The mean relative expression for relatively downregulated and upregulated genes is provided for each investigated group of the discovery and replication cohort (HC, AU, IU, BU). The expression data is scaled and color coded from red - high expression, green - low expression. **B.** A selection of replicated transcription factors from within the connectivity plot of **Figure 5B** (***NFKB1, ATF4, JDP2, PATZ1, RUNX3, IRF8***), and 9 of the top 10 genes from 147 DEGs (for combined uveitis versus controls): CD180, TLR7, CX3CR1, CCR5, HCP5, IFI16, GNAS, SPATA2, and SH3BP5 from the discovery and replication cohort. The relative expression for these genes is also shown for expression data obtained from Mathan et al. 2017[1] in unstimulated CD1c+ myeloid dendritic cells or cells exposed to GM-CSF or TLR7/8 ligand. **Abbreviations:** AU: HLA-B27-associated Anterior Uveitis, ATF4: activating transcription factor 4, BU; Birdshot Uveitis, CCR: CC cytokine subfamily receptor, CX3CR1: CX3C cytokine subfamily receptor, GNAS: GNAS complex locus, HC: Healthy Control, HCP5: HLA complex P5, IFI: Interferon Gamma Inducible Protein 16, IRF8: interferon regulatory factor 8, IU: Idiopathic Intermediate Uveitis, JDP2: Jun dimerization protein 2, HMGN1: High-mobility group nucleosome binding domain 1, NFKB1: nuclear factor kappa B subunit 1, PATZ1: POZ/BTB and AT hook containing zinc finger 1, RUNX3: runt related transcription factor 3, SPATA2: Spermatogenesis Associated 2, SH3BP5: SH3 omain binding protein 5, TFAP2A: transcription factor AP-2 alpha, TLR: Toll-like receptor

1. Mathan TSM, Textor J, Sköld AE, et al. Harnessing RNA sequencing for global, unbiased evaluation of two new adjuvants for dendritic-cell immunotherapy. ***Oncotarget***. 2017;8(12):19879-19893.



Supplementary Figure 7. *Legend on previous page*


10

Chapter 10

SUMMARY, GENERAL DISCUSSION & FUTURE PERSPECTIVES

SUMMARY AND GENERAL DISCUSSION

Non-infectious uveitis (NIU) refers to a group of intraocular inflammatory conditions with great variability in disease course, visual prognosis, and treatment response. This inter-patient variability poses a challenge to find the right therapeutic approach for an individual patient. Furthermore, the treatment strategy is also dependent on personal factors, such as pregnancy or the presence of comorbidities (i.e., diabetes), that make a certain therapy more or less desirable for a specific patient. Also, as current therapies come with heavy side effects and fail to control NIU in up to 40% of all patients (see **chapter 1**, '***treatment of non-infectious uveitis***'), there is a high need for new and more effective therapies.[1–3] Ideally, such strategies will become more patient tailored (i.e. 'personalized medicine' or 'precision medicine'), taking clinical, personal, and molecular factors into account (see **chapter 1**). Therefore, we studied NIU using a systems approach[4], including clinical and molecular aspects as well as studying the personal impact of the disease on quality of life.

OCULAR PAIN AND QUALITY OF LIFE IN NON-INFECTIOUS UVEITIS PATIENTS

Ocular pain in non-infectious uveitis (NIU) is typically linked to acute anterior uveitis. Despite evidence that ocular pain might be more common among NIU patients, reports in non-anterior uveitis subtypes (e.g., intermediate or posterior) are scarce.[5–10] Since only a fraction of patients with non-anterior uveitis experience ocular pain it may not be well recognized by the uveitis specialist. Consequently, the Dutch uveitis patient society requested us to conduct a study on the burden of ocular pain in NIU (**chapter 2**). We performed a cross-sectional study of 147 patients that were seen at the outbound clinic of the department of Ophthalmology of the University Medical Center Utrecht. These patients received three questionnaires: the National Eye Institute Visual Functioning Questionnaire (NEI VFQ-25), which measures vision-related quality of life (QoL), the 36-Item Short-Form Health Survey (SF-36), which measures general health related QoL, and the Dutch version of the McGill Pain Questionnaire (MPQ-DLV), dedicated to monitor subjective pain.[11–16]

NIU patients had generally more ocular pain than an ocular disease free reference group[11], as was shown by a lower mean VFQ Ocular Pain score. Although anterior uveitis patients scored worse than non-anterior uveitis patients, non-anterior uveitis patients still had more ocular pain compared to the reference group, indicating that ocular pain affects patients with various forms of uveitis including non-anterior uveitis. Curiously, these ocular pain scores of non-anterior uveitis patients were not entirely new as they fell within the range of other reports in literature.[5–10] This was the first study, however, to investigate (the nature of) ocular pain and its effect on quality of life in more detail.

Ocular pain was associated with several other aspects of quality of life, but mostly affected work (summarized in the VFQ-25 subscale 'role difficulties'). The negative effect of NIU on work has hitherto been attributed to the combined effect of the recurrent or chronic course with often unpredictable relapses that require multiple hospital visits and intensive treatment.[17] Our results indicate that the experience of ocular pain can be added to this list. Ocular pain furthermore negatively influenced general and mental health, but most importantly, patients feared for their eye-sight more when they felt ocular pain. As fear in itself also negatively influences quality of life[18,19], a vicious circle might develop, and recognition of these emotions is therefore highly important.

Although both the VFQ and the MPQ questionnaires are able to provide more insight into the nature, severity and impact of ocular pain (see for details **chapter 2**), unfortunately, these questionnaires are not able to identify the etiology of the pain. Pain in the case of anterior uveitis is in most cases caused by ciliary muscle contractions or stimulation of the nociceptor fibers of the ophthalmic nerve by inflammatory mediators, and can - in part - be treated with topical mydriatics.[20,21] In contrast, the etiology of pain in the posterior part of the eye is as yet unidentified. Interestingly, patients describing ocular pain also frequently (38%) described coinciding headache or involvement of the ipsilateral temple, brow, neck or shoulder (12-21%). This is most likely the result of referred pain since the eyes share innervation by trigeminal nerve (of which the ophthalmic nerve is a branch) with adjacent tissues.[22]

To summarize, our results show that (peri) ocular pain is a relevant complaint of NIU patients, including non-anterior uveitis patients, and that ocular pain has an impact on quality of life. Further effort should be put into uncovering the etiology of the pain in non-anterior uveitis. For the present, it is important for the treating physician to recognize the complaint as well as the possible impact it might have.

NEW INSIGHTS IN THE CLINICAL COURSE OF HLA-B27 ASSOCIATED ACUTE ANTERIOR UVEITIS

Incidence of visual impairment and blindness in HLA-B27 associated acute anterior uveitis

In **chapter 3** we investigated retrospectively the prevalence, causes and risk factors for permanent vision loss in 338 eyes of 212 HLA-B27 associated acute anterior uveitis patients. In our study 9% of all patients suffered from irreversible visual impairment (defined as visual acuity ≤20/50 Snellen equivalent) or blindness (≤20/200) in at least one eye, after a median of 10 years of follow-up. In 1% this vision loss was bilateral. These results are in line with literature and confirm an outcome that is often referred to as "relatively

good".[23–29] However, temporary visual impairment or even blindness occurred in as much as two thirds of all patients at least once during follow-up. As patients present with uveitis between the age of 20-40[30] and on average experience 1-2 episodes a year[24,25], they are subjected to decades of uveitis exacerbations during which their vision is repeatedly affected. This vision loss - despite being temporary - adds greatly to the personal and socioeconomic impact.[7,31] Also, it is important to notice that visual impairment in this patient group developed after 10 years, with one patient in this study meeting the criteria for blindness after even 21 years of disease. This means that, despite a relatively long follow-up time of 10 years, the incidence of permanent vision loss caused by HLA-B27-associated uveitis might still be underestimated.[23–29]

Risk factors for permanent vision loss in HLA-B27 associate acute anterior uveitis

We found secondary glaucoma to be the most common cause for permanent vision loss in HLA-B27 associated acute anterior uveitis (**chapter 3**). This is a novel finding and differs from the most frequent causes of vision loss in non-anterior uveitis, where cystoid macular edema (CME), or macular atrophy due to chronic CME, are the most frequently reported causes of vision loss.[32,33] Risk factors for permanent visual impairment or blindness in our study were ocular hypertension, hypotony, and panuveitis. Interestingly, not the number of uveitis attacks, but the severity of the eye inflammation (progress to panuveitis), was related to vision loss. This is of particular interest because from this it might be hypothesized that the pain-related fear of vision loss (see **chapter 2**) is perhaps not wholly without foundation, as progression of uveitis to pan uveitis will involve more tissue damage. At the time that this study was conducted the reported risk factors included posterior synechiae, poorly-controlled inflammation, chronic uveitis, male gender, and corticosteroid injections or systemic therapy.[28] It is important to note, however, that these risk factors were studied in relation to temporary - not permanent - vision loss. After publication of our results in 2016, the negative relationship between secondary glaucoma and the risk for blindness in anterior uveitis patients has been confirmed by others.[34] Despite being related to the development of ocular hypertension, local steroid therapy is the mainstay of treatment for this type of uveitis.[35] Because the treatment strategies in our series were highly diverse, it remains unclear from our data whether development of secondary glaucoma was caused by the inflammation or by corticosteroid treatment. However, considering that up to 19% patients with HLA-B27 associated uveitis develop secondary glaucoma[24,26,29], we strongly recommend to closely monitor intraocular pressure, especially after commencing treatment with topical or periocular steroids.

Relapse rate of HLA-B27 associated acute anterior uveitis during pregnancy

In **chapter 4** we studied the course of HLA-B27 associated acute anterior uveitis during pregnancy, a time in life during which the immune system changes to facilitate allogenic tolerance. Treatment with systemic IMT during pregnancy is controversial due to the potential adverse effects on the unborn child.[36] More knowledge about the relapse rate could be used to guide treatment strategies. Our results show that the risk of recurrence decreases during pregnancy to around 20% of the risk outside pregnancy. These results are in line with previous reports about relapse rate during (other subtypes of) non-infectious uveitis[37–40] and support the theory that during pregnancy the survival of the fetus requires tolerance of the maternal immune system, resulting in a temporary milder disease course for several inflammatory diseases.[41,42] Although the size of our study population hampered investigation of this effect between the separate trimesters, it is important to note that all uveitis episodes in our study occurred in the first two trimesters, which is in line with the hypothesis that the tolerogenic state increases during pregnancy.[37,38,40] As described in other studies, the effect was temporary and the relapse rate returned to pre-pregnancy frequencies.[37–39]

METABOLIC DIFFERENCES BETWEEN HLA-B27 POSITIVE AND NEGATIVE ACUTE ANTERIOR UVEITIS

Although HLA-B27-associated anterior uveitis and HLA-B27-negative idiopathic acute anterior uveitis share most of their clinical features, HLA-B27 positive uveitis generally has a younger age of onset, a more severe and recurrent disease course, and is linked to a less favorable visual outcome.[26,30]

In **chapter 5**, we studied the metabolic profiles of the aqueous humor (AqH) of patients with HLA-B27 positive and negative acute anterior uveitis using two independent cohorts, totaling 30 HLA-B27 positive, 16 HLA-B27 negative anterior uveitis patients, and 20 cataract patients. The AqH samples were subjected to two rounds of Direct Infusion Mass Spectrometry (DIMS) and an independent round of Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). Our results confirm that these clinically similar diseases also have greatly overlapping molecular profiles.[43,44] However, we were able to identify - and replicate - several changes in the metabolic profile of the aqueous humor that differentiated HLA-B27 positive from HLA-B27 negative acute anterior uveitis patients. These differences included branched-chain amino acid (BCAA) metabolism (higher in HLA-B27+ patients), ascorbate (vitamin C) and aldarate metabolism (lower in HLA-B27+ patients) and a disruption (i.e., fragmentation) of the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle or Krebs cycle).

More specifically, we identified ketoleucine to be consistently upregulated in HLA-B27 positive anterior uveitis, and this metabolite correlated with disease activity. Ketoleucine (also known as 4-methyl-2-oxopentanoate, α-ketoisocaproic acid, or 2-ketoisocaproate) is a deaminated derivate of leucine and is a key metabolite in BCAA metabolism.[45] About half of the HLA-B27 positive anterior uveitis patients will eventually develop ankylosing spondylitis (AS)[46], a rheumatic condition which shares a risk factor for disease development in HLA-B27 and is intimately linked to (subclinical) intestinal inflammation and ***gut microbiome dysbiosis.***[47] Interestingly, the gut microbiome contributes to altered levels of BCAAs[48] and fecal matter of AS patients show changes in BCAAs valine, leucine, and ketoleucine that correlate with erythrocyte sedimentation rate (BSE) - a sign often used in the clinics to assess inflammation.[49] The microbiome might be of importance for the pathogenesis of NIU as well.[50,51] Uveitis patients show a changed gut microbial composition compared to healthy controls and even the intraocular space has a personalized microbiome.[52,53] Importantly, there are indications that the microbiome might be dependent on the HLA haplotype.[54] As BCAA levels are also changed in the plasma of AS patients but not in rheumatoid arthritis patients it is tempting to speculate that BCAA metabolism may be a feature of HLA-B27 linked pathology.[55,56] In support of this hypothesis is the observation that the correlation between ketoleucine and disease activity was predominantly observed in HLA-B27 positive uveitis patients. It would interesting to investigate this metabolite as a biomarker in relation to HLA-B27 associated acute anterior uveitis related complications such as secondary glaucoma, the leading cause of vision loss in this uveitis subtype (**chapter 3**).

IMMUNE PROFILING TO BETTER UNDERSTAND DISEASE - AN EXAMPLE

The development of novel sensitive, high-throughput, multiplexed experimental techniques make it possible to extensively phenotype the immune system.[57] In **chapter 6** we describe the immune profiling of a patient with ***CRB1*** related retinal dystrophy that was initially diagnosed and treated as intermediate uveitis. Because the intraocular inflammation was persistent, despite having tried multiple therapeutic strategies, additional genetic testing was done and revealed a retinal dystrophy masquerading as uveitis. Although therapy non-response is an important issue which happens in ~40% (see *'treatment of non-infectious uveitis'* in **chapter 1**), this case also illustrates the importance of excluding a masquerade syndrome[58] in case of therapy non-response.

Despite being a degenerative disease of the retina[59] we found many changes in immune mediators, and observed that the frequency of myeloid dendritic cells (see also **chapter 9**) correlated with disease activity in this patient. Interestingly, since publishing these findings, other studies have reported inflammatory perturbations (cells in the vitreous or CME) in

up to half of the patients with ***CRB1*** related retinal dystrophies.[60] The inflammatory activity is most likely the direct result of retinal damage[61], but the production of pro-inflammatory mediators by activated microglia in the retina may further complicate (or masquerade) this retinal condition.[62,63]

NEW INSIGHTS IN THE PATHOGENESIS OF ADULT NON-INFECTIOUS UVEITIS

In order to better understand pathways underlying NIU and find molecular clues to serve as biomarkers for clinically relevant outcomes or potential therapeutic targets, we applied several high-throughput techniques to immunophenotype the blood of patients with one of three subtypes of non-infectious uveitis: HLA-B27 associated acute anterior uveitis (in this thesis referred to as ***AU***), idiopathic intermediate uveitis (in this thesis referred to as ***IU***), and Birdshot uveitis (in this thesis referred to as ***BU***), each representing a different primary site of inflammation (e.g., anterior uveitis, intermediate uveitis and posterior uveitis). Over the years we assembled two independent cohorts including a total of 55 patients and 29 healthy volunteers. All patients had active uveitis at the time of participating in this research and did not receive systemic immunosuppressive therapy for at least three months. The combined results of the **chapters 7-9** support a strong involvement of multiple layers of the immune system, including regulatory RNAs (**chapter 7**), T cells (**chapter 8**) and CD1c+ mDCs (**chapter 9**) in the pathogenesis of adult non-infectious uveitis.[64–69]

Serum MicroRNAs in uveitis

NIU is considered a 'complex' condition driven by environmental factors, epigenetic changes and genetic susceptibility. Genetic studies have revealed association with immune genes (see '*pathogenesis - what do we know*' in **chapter 1**). However, it is unclear what epigenetic factors, such as DNA methylation, histone modifications, or non-coding RNAs (e.g., microRNAs), might contribute to NIU.[53,70,71]

In **chapter 7** we describe the systematic identification and replication of a cluster of microRNAs (miRNAs) that was elevated in the serum of non-infectious uveitis patients. The robust cluster consisted out of miR-193-5p, miR-223-3p, miR-223-5p, miR-140-5p, miR-29a-3p, miR491-5p, and the small nuclear RNA U6. Understanding the downstream effects of serum miRNA perturbations is challenging, because individual miRNAs regulate numerous genes while a single gene may be regulated by multiple miRNAs.[72,73] Yet, most biological processes are considered to be tuned by the concerted action of multiple miRNAs (i.e., clusters) targeting functionally related pathways.[74–76] In support of this is the observation that all microRNAs within the identified 'uveitis' cluster strongly correlated and that there was an extensive overlap in both predicted and experimentally validated target pathways.

As MiRNAs can target multiple genes, changes in their expression can result in pro-inflammatory as well as regulatory signaling, depending on expression levels and context. A good example is one of the uveitis-associated miRNAs miR-223. This miRNA interacts with the IL-23 pathway during inflammation and inhibition of miR-223 has been shown to improve disease in an intestinal inflammation model in mice suggesting a highly pro-inflammatory role.[77] In contrast, a recent study of miR-223 knockout mice revealed that total loss of expression of this miRNA exacerbated intestinal inflammation[78], which was accompanied by a pro-inflammatory dendritic cell phenotype.[79] These results suggest that ubiquitous expression of miR-223 may be necessary for normal tissue homeostasis. Therefore, the increase in the serum levels of the miRNA cluster may drive inflammation, but it is also possible that (part of) this miRNA cluster acts in an attempt to control damage that is caused by the inflammation. The observation that the pathways targeted by some of these miRNAs are relevant for ocular biology, including VEGF signaling[80–82], photoreceptor development and retina homeostasis[83,84] support this view.

Furthermore, the uveitis-associated cluster correlated with a reduction in the proportion of a cell population distinguished by high CD16 expression. Based upon other cell surface makers ($CD11c^{+}HLA\text{-}DR^{-}CD14^{-}lin^{dim}$), this cluster most likely represents a subset of cytotoxic $CD16^{+}CD56^{dim}$ Natural Killer (NK) cells, a cell type that is gaining more interest in studies into the pathophysiology of uveitis (see '***NK cells in non-infectious uveitis***' below).

T cells in non-infectious uveitis

In **chapter 8** we conducted an immune-phenotyping study of mononuclear blood cells of the first cohort of non-infectious uveitis patients using flow cytometry.[85] The overall changes in relative abundance of leukocyte subsets were modest, which is typically reported in flow cytometry studies of non-infectious uveitis.[68,86,87]

The most profound changes were found in the T cell compartment. In humans, multiple lines of evidence have linked type 17 immune responses and IL-17-producing T cells ('Th17' cells) to NIU.[87–93] In line with this, we observed an increase of ($CCR6^{+}CXCR3^{-}$) Th17 cells[94,95] within memory ($CD45RO^{+}$) CD4 T cells (CD4 T_M) that was noticeable for all uveitis types and confirms previous observations in human uveitis and murine models.[96,97] Strikingly, we observed a relatively large uveitis-subtype specific difference in CD4 T_M cell proportions with Birdshot patients having the highest number of CD4 T_M cells. This discrepancy in CD4 T_M proportion contributed to relatively higher relative abundance of Th17 cells in Birdshot patients compared to the other uveitis subtypes, especially HLA-B27 associated anterior uveitis. To date the quantitative differences in Th17 cells in blood between uveitis entities in human NIU has remained an underappreciated feature. It may

be possible that this difference has implications for treatment response as resistance to steroid treatment in non-infectious uveitis is reported to be Th17 mediated.[98] Evidence that quantitative Th17 differences may have clinical impact was provided by their close association with the future requirement of IMT: using follow-up data of the cohort of patients, we discovered that the proportion of ($CD3^+CD4^+CXCR3^-$) $CCR6^+$ T(h17) cells was higher in patients that later needed IMT and thus, that this T cell subset may harbor predictive capacity for the (future) need of IMT.[99]

Manual or user-defined gating is inherently prone to human error and introduces potentially misleading or irreproducible results. With the technological improvement of cytometry comes an increase in number of markers (and thus the amount of 2D scatter plots that have to be examined manually) which may lead scientists to overlook relevant cell subsets.[100] To alleviate some of these constraints, we performed automatic and unsupervised clustering of flow cytometry data from the T helper panel by FlowSOM[101] which characterizes and clusters cell populations considering the expression of ***all*** markers in the panel. This analysis confirmed the elevation of Th17 cells (and decrease of NK cells) in uveitis patients as well as the specific elevation of Th17 cells in patients that needed prolonged systemic IMT before commencing therapy.

Conventional flow cytometry, moreover, remains relatively limited in number of markers that can be measured simultaneously (~12-16-colors per panel). This prevents deep phenotyping of cell status (e.g., activation status, intra-cellular signaling, etc.). Future detailed phenotyping focusing on specific subsets (like the T and NK populations) may benefit from using mass cytometry (CyTOF), a mass spectrometry based technology that may measure up to 40-50 markers simultaneously, to obtain more in-depth information on the cell signatures that can predict disease outcome of NIU patients.[102] Addition of other surface markers and intracellular cytokines would lead to better definition of Th17 cells. This would improve between-study comparability, but is also crucial to assess expression patterns in more detail to determine the boundaries of Th17 plasticity.[103–105]

NK cells in non-infectious uveitis

High expression of the uveitis associated microRNA cluster was associated with lower frequencies of CD16 expressing Natural Killer (NK) cells (**chapter 7**). In **chapter 8** we saw a similar decrease of circulating $CD56^+$ NK cells in uveitis patients. Natural Killer cells are specialized immune cells that play a critical role in the first line of defense against infected, cancerous and auto-reactive cells. Overall, NK cells are divided in two groups: $CD16^{hi}CD56^{dim}$ and $CD16^-CD56^{hi}$.[106] It is worth noting that NK receptors (KIRs) have

been genetically linked to uveitis and might influence disease in an HLA dependent manner.[107,108] Also, NK cells interact with dendritic cells and influence Th17 skewing through this interaction.[109] In experimental autoimmune uveitis (EAU), intraocular NK cells are increased and treatment with soluble NK marker (s)CD83 can attenuate EAU.[110] Taken together, these data support a relatively unexplored role for NK cells in the pathogenesis of NIU and further effort should be put into studying their function in the pathology of NIU.

CD1c+ myeloid dendritic cells in non-infectious uveitis

Despite the existence of many studies investigating the role of T cells in the pathogenesis of NIU, it is as yet unknown where and through which signals these T cells are activated. The HLA and ERAP associations suggest a central role for antigen processing and presentation in the pathogenesis of NIU, but the role of antigen presenting cells (APCs) in human NIU is not well understood. Recently, elevated proportions of circulating CD1+ expressing myeloid dendritic cells (also called mDC1s) have been associated with uveitis activity, providing rationale for the role of this dendritic cell subset in human uveitis.[68] In **chapter 9** we investigated the gene expression profile of $CD1c^{+}$ myeloid dendritic cells in 51 NIU patients and 29 healthy controls divided over two cohorts. Although were unable to ascertain a significant shift in relative abundance the CD1c+ mDC population in blood described before[68,111] (**chapter 8**), the gene expression profile proved to exhibit changes that aid in finding the role of this cell in the disease pathogenesis of non-infectious uveitis.

We used a combination of strategies to identify the most relevant disease related signals. Differentially expressed genes (DEGs) were first selected based upon statistical thresholds. These DEGs were then used as input for weighted gene co-expression network analysis (WGCNA) to find clusters of co-expressed genes (called ***gene modules***) under the asumption that co-expressed genes are likely to act together in the same pathways.[112] We then challenged this concept by deeming a gene module relevant if we were able to replicate it in a second - independent - cohort of patients. In other words, we considered genes robustly linked to NIU if they consistently were differently expressed and displayed relative similar 'behavior' in the two cohorts. Using this approach, we identified a gene network of 147 genes (that we called ***Signature A***). This gene cluster distinguished uveitis patients from healthy controls and was mainly involved in Toll Like Receptor (TLR) signaling, cytokine production, and regulation of gene expression and translation. More specifically, we observed higher expression levels of several toll like receptor family members (including ***CD180,*** and ***TLR-5, -6, -7, -8)*** and chemokine receptors (including ***CX3CR1, CCR5,*** and ***CCR2)***. Furthermore, signature A contained six transcription factors

with high module membership (e.g., their high interconnectivity with other module-members) which can be considered 'master regulators' of this molecular network: there was increased expression of transcription factor ***NFKB1*** (encoding for two subunits of the NF-κB complex depending on co-translational processing[113]) and decreased expression of ***IRF8***, ***ATF4***, ***JDP2***, ***PATZ1*** and ***RUNX3***.

One of the main pathways activated in these CD1c+ mDCs was TLR-mediated NF-κB signaling. Interestingly, it was recently discovered that activation of dendritic cells through TLR-7 resulted in enhanced autoreactive IRBP specific Th17 responses.[114] From our data it seems that TLR induced activation of the NF-κB pathway might be mediated via adaptor protein 'TNF receptor-associated factor 6' (***TRAF6***). In support of this hypothesis is the finding that TRAF6 was required for TLR-7 induced NF-κB signaling leading to TNF-α and IL-6 production.[115] The ligands that initiate binding and activation of TLRs in human NIU are unknown, but it is worth noting that recently miR-29a - one of the uveitis associated miRNAs (**chapter 7**) - has been shown to be able to activate dendritic cells through specific binding to TLR-7.[116]

Intriguingly, many genes of ***Signature A*** that we found to be upregulated in uveitis patients are usually promptly down-regulated upon in vitro activation of mDCs.[118] Also, we observed relatively unchanged expression levels of genes classically linked to antigen presentation or T cell signaling (e.g., ***HLA***, ***CD80***, ***CD83, CD86***). Therefore, we hypothesize that these CD1c+ mDCs are not fully activated. Neither do these CD1c+ mDCs resemble the typical 'semi-mature' phenotype, which is hallmarked by high expression of MHC molecules and costimulatory molecules combined with low (protein) expression of inflammatory cytokines such as IL-12, IL-6 and TNF-α.[119,120] The elevated expression levels of pattern (or 'danger') recognition receptors (i.e., ***TLRs***, ***CD180*** etc), several chemokine receptors that are implicated in the recruitment DCs from the blood to inflamed tissues[121] and other genes linked to migration might render the CD1c+ mDCs of NIU patients hypersensitive to stimuli and most likely represent a so called 'primed' state.[122–125] We hypothesis that this may result in an enhanced immune reaction after stimulation. For example, a similar hypersensitiveness has recently been described in peripheral blood monocytes from HLA-B27 associated anterior uveitis patients, which exhibited a more prominent inflammatory response to stimulation by (bacterial) lipopolysaccharide (LPS).[126] In general, these findings (summarized in **Figure 1**) support the concept of a vital role for CD1c+ mDCs in the pathogenesis of non-infectious uveitis. Future efforts should be made to functionally validate the exact function of the here identified key-genes in this process, to see whether targeting (transcription of) these genes might hold therapeutic potential.

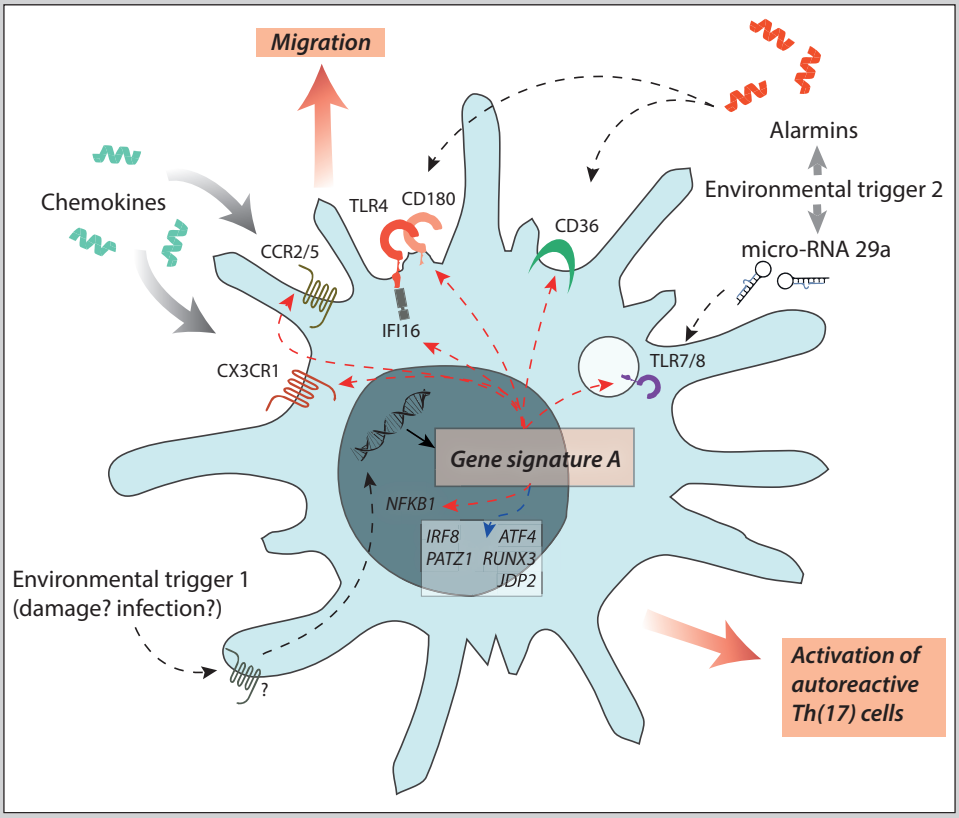


Figure 1. Circulating CD1c+ myeloid dendritic cells of non-infectious uveitis patients exhibit gene transcription profiles reminiscent of a 'hypersensitive' state. Replicated 'gene signature A' consists of 147 genes that have altered expression levels in uveitis patients. These include higher expression levels of several 'pattern recognition receptors' (e.g., TLR5 -6 -7 and -8, CD180) and other scavenging receptors (e.g., CD36) that make these cells highly perceptive for secondary stimuli. These (activating) stimuli could be secondary to an environmental trigger (e.g., infection) or (ocular) tissue damage that results in the productions of several 'alarmins' or (some of the) microRNAs from **chapter 7**. Once activated, these mDCs can activate T cells and skew a T cell reaction towards a Th17 phenotype. Several chemokine receptors (e.g., CCR2, CCR5, CX3CR1) also showed higher gene expression levels. These chemokine receptors are involved in chemotaxis and - after binding with their respective chemokines - promote migration of the mDCs to secondary (i.e., lymph nodes) or tertiary (i.e., in the eye[117]) lymphoid structures. Key transcription factors involved in gene signature A are given in italics: red arrow indicating higher expression levels and blue arrows indicating lower expression levels. The reason that these blood CD1c+ mDCs of uveitis patients show this hypersensitive profile is still unknown, but most likely involves an environmental trigger.

CLOSING REMARKS & FUTURE PERSPECTIVES

The studies presented in this thesis provide new and valuable information about non-infectious uveitis. Some insights might already be applied in the daily clinics, others demand additional research.

From a clinical point of view, we saw that ocular pain is a common complaint of NIU patients that can also affect patients with non-anterior uveitis and that has a great impact on quality of life, - more specifically - on working life (**chapter 2**). Recognition of the validity of ocular pain complaints, and addressing the fears of vision loss that accompany it, is highly recommendable for the treating ophthalmologist. Furthermore, ophthalmologists should closely monitor intraocular pressure of HLA-B27 associated uveitis patients as our results show secondary glaucoma to be the primary cause of permanent vision loss in this patient group (**chapter 3**). Because topical and periocular steroids can also cause glaucoma, alternatives (conversion to systemic treatment or glaucoma surgery) should be discussed early on with patients who develop intraocular hypertension. However, systemic treatment has a great impact and clues as to which patients will benefit from it most would be highly useful.

Markers for personalized medicine

Insight in the disturbances of the immune system will help uncover ('***signatures***' of) molecular markers that point towards specific and/or individual treatment strategies or that can serve as a biomarker for clinical outcomes (i.e. 'patient tailored medicine' or 'precision medicine'). We discovered that patients who required future systemic immunosuppressive treatment (IMT) display higher proportions of $CCR6^+$ T(h17) cells in the blood. Future studies with larger cohorts are needed to validate this finding and to enable proper stratification criteria (i.e., cut-off values) to prospectively select patients that need IMT in daily clinical care. Proof of concept for such an approach was recently provided in psoriatic arthritis, where stratification of patients based on Th17 cells to better match distinct IMT regimes may have outperformed standard care.[99] It is also imaginable that this technique might be further developed to identify other subgroups within NIU patients such as (specific) therapy (non-) responders or patients that are prone to side-effects. Also, it might aid in the - thus far unanswered - question when it is safe to stop therapy. In this line, a recent study found that the existence of a specific 'immunoregulatory' circulating cell phenotype was indicative for clinical remission in uveitis patients further substantiating the utility for molecular signatures in clinical practice[127]

Sometimes there are personal - rather than molecular - reasons why a certain therapy might be more or less desirable for a certain patient. For instance, the existence of comorbidities like pregnancy or diabetes, which makes systemic treatment with corticosteroids less desirable. Our research showed that full-term pregnancy significantly reduces the relapse rate of HLA-B27-associated uveitis (**chapter 4**). Although relapses can still occur during pregnancy, especially in the first trimesters, clinicians should take

these results into consideration when adjusting treatment to a treatment that is less toxic for the unborn child. Immune profiling showed subtle but undeniable perturbations that were specific for HLA-B27 associated anterior uveitis (**chapters 5 and 7-9**), especially on the miRNA level (where we found a spatio-temporal connection between the amount of attacks and the level of serum miRNAs) and the transcriptomic profiles of circulating CD1c+ mDCs. Future immune profiling studies with more patients and longitudinal follow-up (i.e. immune profiling at different time points during follow-up) are needed to provide robust and reproducible evidence for the relationship between molecular changes and the relapse rate, especially during pregnancy, and potentially find molecular clues to predict or suppress relapses.

Potential therapeutic targets

Treatment with systemic immunosuppressing therapy (IMT) is associated with many side effects. The high number of patients that fail IMT, as well as a limited number of (registered) alternatives, further illustrate the pressing need for new therapeutic targets. These new therapeutics preferably target only a small part of the immune system resulting in fewer side effects. With this in mind we set out to broadly phenotype several layers of the immune system of non-infectious uveitis patients.

We identified a uveitis related microRNA cluster in **chapter 7**. MicroRNAs in general, and miR-223-3p specifically, have been proposed as a therapeutic target for inflammatory diseases, like NIU, and demand further investigation.[128] Therapeutic targeting of miRNAs can be aimed at inhibition or promotion of a specific miRNA (for example by synthetically produced microRNAs that respectively bind or mimic the target miRNA), depending on the role of the microRNA in the disease pathogenesis (i.e., pathogenic or protective). Therefore, the exact role of the miRNA cluster that we found should be first established (see '***serum miRNAs in non-infectious uveitis***' above). Interestingly, it is lately been shown that pain might be also influenced by miRNAs.[129,130] Although none of the miRNAs that we found in **chapter 7** have thus far been linked to pain, further investigations (perhaps including other miRNAs) might lead to new insights as to the pathogenesis and/or treatment of ocular pain (**chapter 2**).

Our findings in **chapter 8** support a central role for IL-17 producing Th17 cells. However, clinical trials using the monoclonal anti-IL-17 antibody ***Secukinumab*** for the treatment of NIU have shown conflicting results.[131,132] These conflicting results might be related to differences in the administration method (intravenous versus subcutaneous), or suggest that targeting the ***product*** of Th17 cells (IL-17) is not sufficient to stop the pathogenicity of

Th17 cells in some patients. The observation of heterogeneity in the abundance of Th17 cells between patients with anatomically distinct forms of NIU (**chapter 8**) are in support of this. Th17 cells could also be inhibited by targeting molecules that are upstream of Th17 cells, namely, IL-23 (e.g., ***Ustekinumab***) and the Th17-related JAK-STAT-pathways.[133–135] In general, our findings from **chapter 8** support the line of thought that Th17 cells play a key role in the pathogenesis of NIU and that specifically targeting this cell subset via (one of) the routes described above might prove an effective therapeutic strategy. Since Th17 numbers were associated with treatment upcoming trials might benefit from stratifying patients based on the immune profiling of their T cell populations.

In **chapter 9** we discovered that circulating CD1c+ mDCs - antigen presenting cells that have an important role in providing the activating signal to T cells - are in a 'primed' or 'hypersensitive' state. As these cells are at the basis of an immune response it is tempting to speculate that reversing this state might attenuate uveitis. We used several computational analyses to identify 'key' genes that are involved in this process, including several transcription factors. The role of most molecules highlighted in this thesis, but specifically transcription factors, is very much tissue and cell-type specific, and can vary from highly pro-inflammatory to highly regulatory or protective. Therefore, in vitro experiments and functional studies are needed to assess their exact role and unravel if interfering with (one of) these signals might abrogate uveitis activity. A first step would be to identify the signals that cause the hypersensitive state (including the observed gene ***Signature A***) to be able to mimic circumstances in in-vitro models using cultured cells. With this model it could be investigated how the hypersensitive state could be reversed.

Perspective

Our molecular profiling studies robustly identified several perturbations in the immune system and suggest some molecular markers that perform a key role in these processes. However, many of the observations need to be further tested in ***in-vitro*** or ***functional*** experiments before they can be applied in daily clinics (for reasons described above). That being said, our homogeneous cohorts with patients that all had active uveitis as well as the thorough approaches - including technical and/or biological validation - means that the markers that are presented in this thesis may serve as a reliable starting point for future experiments. At the same time, our stringent selection criteria also limited the number of patients that met the inclusion criteria, which hampered the discovery of more subtle molecular disturbances, such as the molecular differences between the three uveitis subtypes. Future studies - ideally with more patients and/or more material - are therefore likely to discover additional molecular routes that are not described in this

thesis. Larger studies would also be able to unravel the pathways that cause the clinically different phenotypes. We furthermore propose a longitudinal follow-up with multiple time-points of sampling as this might hold the key to finding molecular signatures that predict subtype specific clinically relevant (i.e., the most visually threatening) outcomes, such as glaucoma for HLA-B27 associated uveitis, CME for idiopathic intermediate uveitis and retinal dystrophy for Birdshot uveitis.

Our research confirms that uveitis related changes can be detected in the peripheral blood (**chapters 7-9**). This paves the way for the further development of clinical tests in blood instead of eye tissue. . It is important to realize, however, that the immune changes that are found in the peripheral blood do not necessarily mirror the changes at the site of inflammation (i.e., the eye). This does not mean that observations made in the blood are irrelevant, it rather means that there is another layer of complexity to fully understand the observations.

The broad immune profiling of the peripheral blood of NIU patients presented in this thesis is the first step towards a ***systems level*** understanding of this family of eye conditions. However, the full systems view is not yet complete as it needs the addition of more data-layers (i.e., more insight in the behavior of different cell types) and more (refined) integration of these layers. From the observations made in this thesis, we may prioritize further studies to several cell subsets that might be most relevant: our observations in circulating miRNAs (**chapter 7**) and the perturbations in blood immune cells (**chapter 8**) point towards a role for NK cells (see '***NK cells in non-infectious uveitis***' above). Also, the profound decrease in plasmacytoid (p)DCs, that we (**chapter 8**) as well as others[68,111] found, invites further research into this cell type. As we have collected these cells - and others - from the patients studied in these cohorts we will be able to continue with these proposed studies in the near future. The integration of these data layers is complex and requires state-of-the-art computational modeling. Although we have presented the data 'building blocks' to build a systems view of uveitis, and have presented some first steps towards the integration of these layers, full integration is yet to be completed. This integration may hold the key to better understand the context in which molecular networks operate and how these contribute to disease or clinical outcome. These techniques, however, are laborious and require a large amount of data (i.e., 'big data') and years of investigation to develop. Our team is determined to continue to study the molecular signatures of patients, and follow-up on the integration with clinical data, which will guide us to the realization of personalized medicine for non-infectious uveitis patients.

KEY MESSAGES FROM THIS THESIS

- ✓ Peri-ocular pain is more prevalent in non-anterior uveitis patients than previously assumed and influences quality of life, most importantly, work related quality of life (**chapter 2**).
- ✓ After a median follow-up of 10 years 9% of HLA-B27 associated anterior uveitis patients suffered from permanent visual impairment (≤20/50) or blindness (≤20/200) in at least one eye, as a direct result of uveitis. In 1% of all patients this vision loss was bilateral. Temporary vision loss ≤20/50 occurred in as much as two third of all patients at least once during follow-up (**chapter 3**).
- ✓ Secondary glaucoma is the most common cause of permanent vision loss in HLA-B27 associated anterior uveitis. Shifts in intraocular pressure (e.g., >21mmHg or <6mmHg) as well as extension of inflammation to pan uveitis (rather than number of episodes) are predictive for the development of permanent vision loss (**chapter 3**).
- ✓ The chance of an exacerbation of HLA-B27 associated anterior uveitis during pregnancy is reduced to ~20% of the recurrence rate outside pregnancy. After pregnancy the relapse rate returns to pre-pregnancy frequencies (**chapter 4**).
- ✓ The metabolic profile of the aqueous humor of HLA-B27 associated acute anterior uveitis is characterized by higher branched-chain amino acid metabolism, lower ascorbate and aldarate metabolism and fragmentation of the TCA cycle (**chapter 5**).
- ✓ Ketoleucine might be indicative for disease activity that is specifically related to the presence of HLA-B27 (**chapter 5**).
- ✓ We identified and replicated 7 microRNAs that are elevated in the blood of NIU patients: U6-rRNA, miR-193-5p, miR-223-3p, miR-223-5p, miR-140-5p, miR-29a-3p, miR491-5p. These microRNAs act as a cluster (**chapter 7**).
- ✓ High expression of the uveitis associated microRNA cluster was associated with lower frequencies of $CD16^+$ natural killer cells (**chapter 7**).
- ✓ Patients with anatomically distinct types of non-infectious uveitis exhibit shared and unique changes in blood immune cell composition, in particular heterogeneity in Th17 cell abundance (**chapter 8**).
- ✓ Circulating CCR6 expressing T helper cells (e.g., Th17 cells) might be predictive of future need for systemic immunosuppressive therapy in NIU patients (**chapter 8**).
- ✓ The proportion of circulating CD1c+ myeloid dendritic cells (mDCs) is unchanged (**chapter 8**), but in uveitis patients these cells show a substantial change in gene expression levels of genes involved in TLR cascades, NF-κB1- and cytokine signalling (**chapter 9**), suggestive of an 'hypersensitive' state.
- ✓ This hypersensitive state is characterized by a uveitis related 'gene signature' consisting of 147 co-expressed genes that show altered expression levels in CD1c+ mDCs. These genes include transcriptional regulators such as *RUNX3*, *NFKB1*, *ATF4*, *GNAS*, *JDP2* and *IRF8,* innate immune receptors such as *TLR5 to -8, IFI16, CD180,* and chemokine receptors *CCR5, CCR2* and *CX3CR1* (**chapter 9**).

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Appendices

Samentvatting (summary in Dutch)
Dankwoord (Acknowledgements)
About the author
List of publications

NEDERLANDSE SAMENVATTING

Niet-infectieuze uveitis is een verzamelnaam voor ontstekingen binnen in het oog die niet door een infectie (bacterie, virus of schimmel), maar door het eigen immuunsysteem worden veroorzaakt. Als groep is het één van de belangrijkste oorzaken voor blindheid van mensen vóór pensioensleeftijd in de westerse wereld. Binnen deze groep bevinden zich meerdere uveitis vormen, die in eerste instantie worden ingedeeld naar de primaire plek van de ontsteking: anterieure, intermediaire of posterieure uveitis. Als de ontsteking meerdere segmenten van het oog treft wordt dit pan uveitis genoemd. De uveitis kan samengaan met een andere aandoening (zoals psoriasis of de reumatische ziekte morbus Bechterew) of op zichzelf staan (zoals Birdshot).
Al deze vormen van niet-infectieuze uveitis hebben elk een eigen presentatie, klinisch beloop en prognose. Maar zelfs binnen één diagnose kunnen patiënten een verschillend beloop hebben of andere behandelingen nodig hebben. Waar sommige patiënten genoeg hebben aan plaatselijke medicijnen (in de vorm van oogdruppels of plaatselijke medicijndepots door middel van injecties naast het oog) hebben andere patiënten langdurige (vaak jarenlange) onderdrukking van het immuunsysteem nodig met systemische medicijnen. Deze medicijnen zijn ingrijpend voor de patiënt vanwege het langdurige gebruik en de bijwerkingen, waaronder een verminderde afweer. Daarnaast spelen ook persoonlijke (patiëntgerelateerde) factoren mee bij de therapiekeuze: een bijkomende aandoening (zoals diabetes) of levensgebeurtenis (zoals zwangerschap) kan de keuze voor een therapie beïnvloeden. Ook reageert ~40% niet op systemische medicijnen waardoor na de inwerktijd van 3 maanden alsnog geswitcht moet worden naar een ander medicijn terwijl de oogontsteking nog niet onder controle is.

Als we beter zouden kunnen voorspellen hoe het verloop zou zijn (ernstig verloop of niet? Wat is de kans op blindheid? Moet er met systemische medicatie worden ingegrepen of zijn lokale medicijnen voldoende?) zouden we sneller een juiste behandeling in kunnen zetten. Kortom, er is behoefte aan meer patiënt-afhankelijke therapie, ook wel 'personalized medicine' genoemd. Personalized medicine houdt in dat een therapeutische strategie (frequentie van polibezoeken, medicatiekeuze etc.) niet alleen gebaseerd is op de diagnose en de ziekteactiviteit, maar ook op (meerdere) markers in het bloed. Voor het ontwikkelen van personalized medicine is een beter inzicht in het ziekteproces cruciaal.
Daarom zijn we in 2014 in het UMC Utrecht een onderzoek gestart waarbij we niet-infectieuze uveitis hebben bestudeerd vanuit verschillende invalshoeken (de zogenaamde 'systems approach'). We hebben hierbij het klinische beloop van niet-infectieuze uveitis bestudeerd, de impact van niet-infectieuze uveitis op de kwaliteit van leven, alsmede (de moleculaire routes van) het onderliggende ziekteproces. Omdat het immuunsysteem de

hoofdrol speelt in het ziekteproces hebben we de veranderingen in het immuunsysteem bij patiënten met niet-infectieuze uveitis in kaart gebracht. Deze bevindingen hebben we vervolgens gekoppeld aan klinische data (bijvoorbeeld het wel of niet moeten gebruiken van systemische medicatie) met als doel de basis te leggen voor personalized medicine, maar ook het vinden van potentiele nieuwe aangrijpingspunten voor therapie.

PIJN IN OF RONDOM HET OOG EN KWALITEIT VAN LEVEN BIJ NIET-INFECTIEUZE UVEITIS

In **hoofdstuk 2** hebben we, op verzoek van de patiëntengroep Uveitis van de Oogvereniging Nederland, een onderzoek uitgevoerd naar pijn in en rondom de ogen en het effect hiervan op de kwaliteit van leven. Pijn is een typische en bekende klacht bij patiënten met anterieure uveitis. Er zijn echter ook patiënten met intermediaire of posterieure uveitis die pijn ervaren, al is hier veel minder over bekend. Omdat lang niet elke patiënt met deze vorm van uveitis hier last van heeft wordt het soms minder goed (h)erkend. We hebben voor dit onderzoek de gegevens verzameld van 147 niet-infectieuze uveitis patiënten die op dat moment een poli afspraak hadden bij het UMC Utrecht. Deze patiënten vulden 3 gevalideerde vragenlijsten in: een vragenlijst die de visus-gerelateerde kwaliteit van leven meet (NEI VFQ-25), een vragenlijst die de algehele gezondheid gerelateerde kwaliteit van leven meet (SF-36) en een vragenlijst die verschillende (subjectieve) aspecten van pijn meet (de MPQ-DLV). Uit dit onderzoek bleek dat niet-infectieuze uveitis patiënten gemiddeld meer pijn in of rondom de ogen ervaren dan mensen die geen oogaandoening hadden. Hoewel patiënten met anterieure uveitis logischerwijs meer pijn aangaven, hadden ook patiënten met een niet-anterieure vorm van uveitis meer pijn dan mensen zonder oogaandoening. Patiënten met oogpijn hadden ook vaak (38%) hoofdpijn, of pijn elders in de hoofd/schouder streek (slaap, wenkbrauw, nek en schouder, 12-21%). Deze pijn komt waarschijnlijk niet direct van dat gebied, maar wordt daar gevoeld doordat de nervus trigeminus (waarvan de nervus ophthalmicus een aftakking is) deze gebieden innerveert.

Pijn in en rondom de ogen was van grote invloed op de kwaliteit van leven, maar had vooral een negatieve invloed op werk. Daarnaast hing pijn in of rondom de ogen samen met de algehele gezondheidsbeleving en de mentale gezondheid. Verrassend genoeg was er geen verband tussen pijn (beleving) in het oog en pijn (beleving) elders in het lichaam. Belangrijk om te vermelden is dat er een sterk verband was tussen het hebben van oogpijn en de zorgen die een patiënt ervaart over zijn of haar zichtvermogen. Aangezien angst zelf ook weer een negatief effect heeft op de kwaliteit van leven is belangrijk voor de oogarts om dit verband te herkennen en bespreken. In het geval van anterieure uveitis wordt de pijn voornamelijk veroorzaakt door een spierspasme van de musculus

ciliaris naast de iris of door prikkeling van de pijngevoelige zenuwvezels van de nervus ophthalmicus door stoffen die vrijkomen bij een ontsteking. Waar de pijn bij een niet-anterieure uveitis vandaan komt is een stuk lastiger om te zeggen omdat achter in het oog maar weinig pijnzenuwen aanwezig zijn. Hoewel er meer onderzoek nodig is naar de oorzaak van de pijn in het geval van niet-anterieure uveitis kunnen we op basis van de bevindingen in dit hoofdstuk concluderen dat pijn in en rondom de ogen een grote invloed heeft op de kwaliteit van leven en dat het bij een grotere groep patiënten voorkomt dan eerder werd aangenomen.

NIEUWE INZICHTEN IN HET BELOOP EN DE PATHOGENESE VAN HLA-B27 GEASSOCIEERDE ACUTE ANTERIEURE UVEITIS

In **hoofdstuk 3** hebben we onderzocht hoe vaak permanente slechtziendheid en blindheid voorkwamen als gevolg van HLA-B27 geassocieerde acute anterieure uveitis (AAU). Hiervoor hebben we retrospectief de gegevens van 212 patiënten (in totaal 338 ogen) bekeken. Hieruit bleek dat, na een gemiddelde follow-up van 10 jaar, 9% van alle patiënten slechtziend (Snellen visus ≤20/50) of blind (Snellen visus ≤20/200) waren geworden aan ten minste één oog. In 1% van alle patiënten betrof dit beide ogen. Dit komt overeen met eerdere studies en bevestigt de relatief goede visuele prognose van deze vorm van uveitis, in vergelijking met andere vormen. Echter, dit wil niet zeggen dat het beloop als mild mag worden bestempeld: bijna 2/3^{e} van alle patiënten was ten tijde van een uveitis episode tijdelijk slechtziend of zelfs blind. Hoewel de visus na behandeling herstelde mag de persoonlijke en socio-economische impact hiervan niet onderschat worden aangezien een patiënt gemiddeld 1 tot 2 uveitis episodes per jaar heeft en dat voor tientallen jaren. Ook is het belangrijk om op te merken dat een oog pas de criteria voor slechtziendheid of blindheid bereikte na gemiddeld 10 jaar, met uitschieters tot 21 jaar. Dit betekent dat de daadwerkelijke prognose, ondanks een relatief lange follow-up van 10 jaar in onze studie, nog steeds onderschat wordt.

De meest voorkomende oorzaak voor blindheid was uveitis gerelateerd (secundair) glaucoom. Dit was ten tijde van de publicatie in 2016 een nieuwe bevinding en verschilt van de meest voorkomende oorzaak van andere vormen van uveitis, waar (langdurig) cystoid macula oedeem veelal de voornaamste oorzaak van blindheid is. Inmiddels zijn deze bevindingen bevestigd door andere studies. Risicofactoren voor het ontwikkelen van slechtziendheid of blindheid waren in onze studie een te hoge (>21mmHg) of te lage (<6mmHg) oogdruk of progressie van anterieure uveitis naar panuveitis (uitbreiding naar achter in het oog). Op basis van deze bevindingen raden wij aan om de oogdruk binnen deze patiëntengroep goed in de gaten te houden en op tijd de therapie aan te passen.

In **hoofdstuk 4** hebben we het beloop van HLA-B27 geassocieerde AAU tijdens de zwangerschap onderzocht. Aangezien deze vorm van uveitis relatief jonge mensen raakt komt zwangerschap in deze uveitis groep relatief vaak voor. Tijdens de zwangerschap verandert het immuunsysteem: om geen reactie op de foetus te ontwikkelen wordt het immuunsysteem tijdelijk toleranter. Het is tevens een periode waarin therapie – en met name systemische medicatie – aangepast moet worden omdat deze schadelijk kan zijn voor het ongeboren kind. Uit onze studie bleek dat het risico op een episode tijdens de zwangerschap vermindert tot ~20% van het risico buiten de zwangerschap. Het effect was tijdelijk en na de bevalling kwamen uveitis episodes weer net zo vaak voor als voor de zwangerschap.
Hoewel beslissingen rondom het aanpassen van de therapie in verband met de zwangerschap voor iedere patiënt en situatie persoonlijk gemaakt zal moet worden kunnen de bevindingen uit dit hoofdstuk in het achterhoofd worden gehouden bij het maken van deze aanpassingen.

Hoewel HLA-B27 positieve AAU en (HLA-B27 negatieve) idiopathische AAU veel klinische kenmerken delen, zijn er ook klinische verschillen: zo presenteert de ziekte zich bij HLA-B27 positieve patiënten vaker op jongere leeftijd en komen recidieven en een heftigere ontsteking vaker voor bij HLA-B27 positieve patiënten. Dit kan duiden op (subtiele) verschillen in het onderliggende ziektemechanisme. In **hoofdstuk 5** hebben we het metabole profiel van het voorste oogkamervocht van HLA-B27 geassocieerde uveitis patiënten bestudeerd en vergeleken met (HLA-B27 negatieve) idiopathische anterieure uveitis patiënten. We maakten daarbij gebruik van twee cohorten, met in totaal 30 HLA-B27 positieve AAU en idiopathische AAU patiënten. Het oogvocht werd geanalyseerd door tweemaal een Direct Infusion Mass Spectrometry (DIMS) toe te passen en op het overgebleven oogvocht een Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS).

Hoewel het metabole profiel grotendeels overlapte waren er ook verschillen tussen deze uveitis groepen. Zo hadden HLA-B27 positieve AAU patiënten een verhoogd branched-chain aminozuur (BCAA) metabolisme, een verlaagd vitamine C metabolisme en veranderingen in de citroenzuurcyclus (de energie huishouding). Binnen het BCAA metabolisme was met name ketoleucine consequent verhoogd in het oogvocht van HLA-B27 positieve AAU patiënten. Dit metaboliet was ook gerelateerd aan uveitis activiteit bij HLA-B27 positieve AAU patiënten, maar niet bij HLA-B27 negatieve patiënten. De veranderingen van het BCAA metabolisme (en specifiek ketoleucine waarden) zouden dus wel eens HLA-B27 gerelateerd kunnen zijn. Hier hebben we in de literatuur ook meerdere aanwijzingen voor gevonden. Ongeveer de helft van HLA-B27 positieve AAU patiënten

ontwikkelt tevens m. Bechterew, een reumatische aandoening die ook HLA-B27 gerelateerd is. De levels van BCAA's zijn ook veranderd in m. Bechterew (maar niet in andere reumatische aandoeningen!) en correleren ook in deze ziekte met parameters voor ziekteactiviteit, zoals bezinking. Daarnaast blijkt uit recente studies dat zowel m. Bechterew, als uveitis, gerelateerd zijn aan veranderingen in de darmflora. De darmflora blijkt ook afhankelijk te zijn van HLA-type. Interessant genoeg heeft de darmflora ook direct invloed op de levels van BCAA's. Dit alles ondersteunt de hypothese dat BCAA metabolisme – en ketoleucine – HLA-B27 gerelateerd kunnen zijn. Deze bevinding geeft meer inzicht in het onderliggende ziektemechanisme, maar zou in de toekomst ook verder ontwikkeld kunnen worden tot een objectieve maat van ziekteactiviteit. Met de opkomst van Raman spectroscopie (een techniek waarin specifieke metabolieten kunnen worden gemeten door de cornea heen) zou dit op een non-invasieve manier kunnen gebeuren.

HET ONDERZOEKEN VAN HET IMMUUNSYSTEEM IN HET BLOED

De recente ontwikkeling van nieuwe, gevoelige, 'high-throughput' technieken heeft een nieuw tijdperk voor onderzoek ingeluid. Deze technieken maken het mogelijk om niet één, maar een grote hoeveelheid markers in het bloed te onderzoeken en zo een moleculair 'profiel' te scheppen, hetgeen meer waarde heeft dan één marker als het gaat om testen. Dit kan voor één patiënt worden ingezet, om zo meer inzicht in het ziekteproces te krijgen, maar ook op grotere schaal: om het immuunsysteem van mensen met een aandoening in kaart te brengen en inzicht te krijgen in hoe specifieke takken van het immuunsysteem zich gedragen en/of samenwerken binnen een bepaald ziekteproces.

In **hoofdstuk 6** volgen we de veranderingen in het immuunsysteem van een patiënt met CRB1 gerelateerde retinale dystrofie die zich in eerste instantie presenteerde als moeilijk behandelbare intermediaire uveitis (een zogenaamd 'uveitis masquerade syndroom'). We hebben klinische data onder andere gekoppeld aan immuuncellen (middels flow cytometrie) en signaalstoffen (cytokinen, middels Luminex) in het bloed. Hieruit volgde dat er vele veranderingen in het immuunsysteem te zien waren. Het meest opvallende was dat de relatieve hoeveelheid myeloide dendritische cellen (zie tevens hoofdstuk 9) leek samen te hangen met de ziekte activiteit (mate van cystoid macula oedeem). Dit is opvallend aangezien deze genetische aandoening degeneratief van aard zou zijn en niet inflammatoir (samenhangend met ontsteking). Sinds dat onze bevindingen zijn gepubliceerd in 2016 zijn er andere studies geweest die tevens melding maken van ontstekingsactiviteit in tot wel de helft van deze patiënten. Toekomstige studies zijn nodig om uit te zoeken of deze ontstekingsactiviteit simpelweg het gevolg is van retinale (degeneratieve) schade of dat de ontsteking een actief proces is dat deel uitmaakt van de aandoening (bij sommige patiënten).

VERANDERINGEN IN HET IMMUUNSYSTEEM BIJ PATIENTEN MET NIET-INFECTIEUZE UVEITIS

Om patiëntgerichte behandeling te kunnen verwerkelijken hebben we moleculaire (bio-) markers – of nog beter, moleculaire profielen - nodig die we kunnen koppelen aan klinische uitgangspunten, zoals prognose en therapie. Hiervoor is meer kennis van het immuunsysteem essentieel. Om de verstoringen van het immuunsysteem bij niet-infectieuze uveitis beter te begrijpen, en daardoor aanwijzingen (markers) voor personalized medicine en / of aangrijpingspunten voor (nieuwe) therapieën te vinden, hebben we de afgelopen jaren bloed verzameld van patiënten met één van drie vormen van niet-infectieuze uveitis: HLA-B27 geassocieerde anterieure uveitis, idiopathische intermediaire uveitis of Birdshot uveitis. In totaal hebben we twee cohorten van in totaal 55 uveitis patiënten geïncludeerd en deze vergeleken met elkaar en met 29 gezonde controles (vrijwilligers). Alle patiënten hadden actieve uveitis op het moment dat bloed werd afgenomen en kregen geen systemische medicatie die het immuunsysteem onderdrukte voor ten minste 3 maanden. In het laboratorium hebben we verschillende takken van het immuunsysteem onderzocht en beschreven in **hoofdstuk 7-9.**

Samenvattend zien we dat er meerdere onderdelen van het immuunsysteem grote veranderingen laten zien bij uveitis patiënten. De grootste veranderingen gelden voor alle drie de uveitis subtypen, maar er zijn ook veranderingen die specifiek zijn voor een bepaald subtype, al zijn deze subtieler.

MicroRNA's in niet-infectieuze uveitis

Niet-infectieuze uveitis is een complexe aandoening waarbij zowel genetische factoren als omgevingsfactoren een rol spelen. Genetische studies hebben verschillende genen die een rol spelen in het immuunsysteem gevonden die het risico op het ontwikkelen van uveitis vergroten. Het HLA type is hier de bekendste van. Echter, het hebben van een bepaalde genetische variatie wil nog niet zeggen dat een persoon uveitis ontwikkeld: hiervoor zijn meerdere genetische variaties in combinatie met (nog onbekende) omgevingsfactoren nodig. Omgevingsfactoren kunnen (het aflezen van) genen beïnvloeden. Eén van de manieren waarop ze dat kunnen doen is door microRNA's. In **hoofdstuk 7** hebben we een cluster van 7 samenwerkende microRNA's gevonden die verhoogd aanwezig zijn in het bloed van uveitis patiënten. Eén microRNA kan meerdere genen beïnvloeden en tegelijkertijd kan één gen beïnvloed worden door meerdere microRNA's. De precieze rol van deze microRNA's moet daarom in toekomstige (functionele) studies worden uitgezocht. Wel weten we al dat deze microRNA's samen genen kunnen beïnvloeden die een rol spelen in inflammatie, maar ook in de ontwikkeling van fotoreceptoren, die een belangrijke rol spelen in het netvlies. Dit roept de vraag op of dit cluster een pro-in-

flammatoir effect heeft, of dat het een (beschermende) reactie op de uveitis is. Daarnaast bleek dit cluster van microRNA's samen te hangen met veranderingen in de samenstelling van immuuncellen in het bloed, met name natural killer (NK) cellen (zie ook '***Natural Killer cellen in niet-infectieuze uveitis***'). Doordat we deze microRNA's niet in één, maar in twee groepen hebben gevonden, en met twee verschillende technieken, vormt dit onderzoek een solide basis voor verder onderzoek naar de rol van deze microRNA's alsmede hun potentie als therapeutisch aangrijpingspunt.

T cellen in niet-infectieuze uveitis

In **hoofdstuk 8** hebben we de relatieve hoeveelheden van immuuncellen in het bloed van uveitis patiënten in kaart gebracht. De meest uitgesproken veranderingen waren zichtbaar binnen een groep immuuncellen die T-helper cellen (Th) worden genoemd. T helper 1 en 17 cellen zijn de drijvende kracht achter het ontstekingsproces van niet-infectieuze uveitis in muismodellen en ook in onze studie zagen we een relatieve toename van CCR6 positieve T(h17) cellen. Deze toename was te zien in alle drie de uveitis subgroepen, maar aangezien er in het bloed van Birdshot patiënten al meer T cellen waren (waaruit Th17 cellen ontstaan), waren er relatief meer Th17 cellen in Birdshot patiënten. Het is belangrijk deze verschillen te onderkennen, met name omdat dit ook consequenties voor therapie (respons) kan hebben. Ongeacht de uveitis subgroep bleek een hoge relatieve hoeveelheid Th17 cellen voorspellend te zijn voor het nodig hebben van systemische immunosuppressieve therapie. Deze kennis kan helpen met het maken van therapeutische beslissingen. Om een goed afkappunt te vinden waar in de dagelijkse kliniek mee gewerkt kan worden moet dit onderzoek eerst herhaald worden in een groter cohort. Desalniettemin vormt deze bevinding een interessante basis voor de ontwikkeling van een laboratoriumtest die voorspelt wie wel en niet baat heeft bij systemische therapie.

Natural Killer cellen in niet-infectieuze uveitis

Hoge expressie van het uveitis-microRNA cluster (**hoofdstuk 7**) was geassocieerd met lagere expressie van natural killer (NK) cellen. Ook in **hoofdstuk 8** vonden we lagere hoeveelheden van NK cellen in het bloed van niet-infectieuze uveitis patiënten. NK cellen zijn gespecialiseerde immuuncellen die een rol hebben in de eerste reactie tegen cellen die een mogelijk gevaar vormen (geïnfecteerde cellen, kankercellen en auto reactieve cellen). Er is nog relatief weinig onderzoek gedaan naar de rol van NK cellen in het ziekteproces van niet-infectieuze uveitis. Wel is bekend dat variaties in het gen voor NK receptoren (KIR) geassocieerd zijn met uveitis. Ook zijn er relatief veel NK cellen aanwezig in de ogen van muizen met uveitis. De verlaagde aantallen die we zagen in het bloed zou wel eens het gevolg kunnen zijn van migratie naar het oog. Behandeling van deze muizen

met een NK-marker (sCD83) zorgde voor vermindering van de uveitis. Op basis van onze bevindingen lijkt het zinvol om dit type immuuncel en zijn rol in niet-infectieuze uveitis verder te onderzoeken in mensen.

CD1c+ myeloide cellen in niet-infectieuze uveitis

Hoewel de rol van T cellen steeds duidelijker wordt, is het nog onduidelijk hoe deze T cellen geactiveerd worden: T cellen hebben een activerend signaal nodig van antigeen presenterende cellen, zoals dendritische cellen (DC's), voordat ze actief worden en bij kunnen dragen aan de uveitis. In **hoofdstuk 9** onderzochten we CD1c positieve myeloide dendritische cellen (CD1c+ mCD's): de meest vóórkomende dendritische cel in het bloed waarvan recent enkele wetenschappelijke bevindingen een mogelijk verband met niet-infectieuze uveitis hadden aangetoond. Door middel van RNA-sequencing hebben we het gen expressie profiel van deze cellen in kaart gebracht. Hierdoor weten we welke genen worden "afgelezen" en dus hoe deze cellen zich gedragen. De CD1c+ mDC's van uveitis patiënten lieten een veranderd gen expressie profiel zien in vergelijking met gezonde controles. Met behulp van verschillende statistische analyses hebben we een cluster van 147 samenwerkende genen gevonden waarvan het genexpressie profiel binnen de CD1c+ mDC cellen veranderd was in uveitis. Deze genen waren betrokken bij het oppikken en doorgeven van signalen van buitenaf. De combinatie van deze receptoren maken deze cellen erg vatbaar voor signalen. Met andere woorden, ze zijn 'hypersensitief' en zullen veel sneller reageren op prikkels en een ontstekingsproces in gang zetten. We hebben enkele 'sleutelgenen' binnen dit cluster geïdentificeerd. Op dit moment zijn we bezig te onderzoeken of het beïnvloeden van deze sleutel moleculen invloed heeft op uveitis activiteit en eventueel als toekomstig therapeutisch aangrijpingspunt zou kunnen dienen.

Samenvattend ondersteunen de bevindingen van **hoofdstuk 7-9** het idee van een ontstekingsproces waarbij autoreactieve Th17 cellen een centrale rol spelen. Het activerende signaal voor deze Th17 cellen zou onder andere van CD1c+ mDC's afkomstig kunnen zijn, die een hypersensitief profiel vertonen in uveitis patiënten. Deze, en de andere bevindingen die gepresenteerd zijn in dit proefschrift, zijn een stap vooruit in ons inzicht in het complexe ziekteproces van niet-infectieuze uveitis. Sommige nieuwe inzichten kunnen direct worden toegepast, andere inzichten vormen een veelbelovende basis voor de ontwikkeling van nieuwe behandelstrategieën en meer patiëntgerichte zorg.

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Fleurieke Verhagen was born on the 14th of October, 1988, in Culemborg, the Netherlands. During high school she participated in a pre-university course in philosophy at Leiden University. After finishing high school (pre-university level) at Beekvliet (Sint-Michielsgestel) in 2007 she started her medical training at Utrecht University. During this time she was an active member of the society for medical students (MSFU Sams), she was part of the committee for the annual symposium for medical students (2009) and member of the sorority for female medical students (Najade). In her final year she was a graduate medical intern at the ophthalmology department of the University Medical Center Utrecht and participated in research under the guidance of Joke de Boer and Ymkje Hettinga. Her master thesis focussed on the prevalence and causes of blindness caused by childhood uveitis in the Netherlands. In December 2013 she obtained her medical degree and subsequently started her PhD under the supervision of prof. dr. Joke de Boer, prof. dr. Tim Radstake and dr. Jonas Kuiper at the department of Ophthalmology and the Laboratory of Translational Immunology at the University Medical Center Utrecht. The results of this research are described in this thesis. She presented her work at several national and international scientific meetings, including the annual meeting of the Dutch Ophthalmological Society (NOG), the Dutch Uveitis Working Group, the International Uveitis Study Group (IUSG, Dublin, August 2016), the International Ocular inflammation Society (IOIS, Lausanne, October 2017), and the Association for Research in Vision and Ophthalmology (ARVO, Honolulu Hawaii, May 2018). She received a travel grant to present her work at the ARVO from the Nelly Reef fund and the de Girard de Mielet-van Coehoorn Stichting. In October 2018 she started her residency in ophthalmology at the University Medical Center Utrecht. Fleurieke is married to Pieter and together they welcomed their daughter Emma in 2016.

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- **F.H. Verhagen**, E.C.A. Stigter, M.L. Pras-Raves, B.M.T. Burgering, S.M. Imhof, T.R.D.J. Radstake, J.H. de Boer, J.J.W. Kuiper. Aqueous humor analysis identifies higher branched chain amino acid metabolism as a marker for HLA-B27 acute anterior uveitis and disease activity. Accepted for publication in the ***Am J Ophthalmol.*** 2018

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