LYMPHOCYTE SUBPOPULATIONS AND CYTOKINE EXPRESSION IN UVEITIS

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Tese para obtenção do grau de Doutor em Medicina

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À Joana e à Sofia

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The results presented and discussed in this thesis were published in the following scientific peer-reviewed journals:

1- Guedes MC, Borrego LM, Proença RD. Roles of Interleukin-17 in Uveitis. Indian J Ophtalmol. 2016 Sep; 64(9): 628-634.

2- Guedes MCE, Arroz MJ, Martins C, Angelo-Dias M, Proença RD, Borrego LM. Regulatory T cells and IL-17A levels in noninfectious uveitis. Graefes Arch Clin Exp Ophthalmol. 2020 Jun; 258(6):1269-1278.

3- Guedes MC, Arroz MJ, Martins C, Angelo-Dias M, Borrego LM, Proença RD. T-Lymphocyte Regulatory Subsets and Inflammatory Cytokine Levels after Treatment of Non-Infectious Uveitis. (Submitted).

4- Guedes MC, Martins C, Arroz MJ, Angelo-Dias M, Borrego LM, Proença RD. Cytokine Profiles in the Peripheral Blood and Aqueous Humor of Patients with Herpetic Uveitis. (Accepted for publication in Journal of Ophthalmic Inflammation and Infection.)

The full articles are available in **APPENDIX I.**

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ABBREVIATIONS

AAU - Acute anterior uveitis

ACAID - Anterior Chamber-Associated Immune Deviation

Act - Activins

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

APCs - Antigen-presenting cells

APMPPE - Acute Posterior Multifocal Placoid Pigment Epitheliopathy

APRIL - A proliferation inducing ligand

AqH - Aqueous humour

ARN - Acute retinal necrosis

ATD - Autoimmune thyroid disease

ATP - Adenosine triphosphate

BAB - Blood-Aqueous Barrier

BAFF - B-cell activating factor

BD - Behçet Disease

BMP - Bone morphogenetic protein

BRB - Blood-Retina Barrier

BSCR - Birdshot Corioretinopathy

CD - Cluster of differentiation

CD - Crohn's disease

CME - Cystoid macular edema

CMV - Cytomegalovirus

CNTF - Ciliary neurotrophic factor

Col-Treg - Collagen-II specific type1 Treg

CSIF - Cytokine synthesis inhibitory factor

CT-1 - Cardiotrophin-1

CTLA-2α - cytotoxic T-lymphocyte antigen-*2 α*

DCs - Dendritic cells

EAU - Experimental autoimmune uveitis

EBV - EpsteinBarr virus

EIU - Endotoxin-induced uveitis

FasL - FasLigand

FOXP3 - Forkhead boxP3

GDF - Growth differentiation factor

GM-CSF - Granulocyte macrophage colony-stimulating

HLA - Human Leukocyte Antigen

HSV - Herpes Simplex Virus

HZV - Herpes Zoster Virus

IBD - Inflammatory Bowel Disease

IFN-ɣ - interferon gamma

IFNɣR - IFN-ɣ receptor

IL - Interleukin

IL-10R - 10 receptor

IL-6R - IL-6 receptor

IOP - Intraocular pressure

IU - Infectious uveitis

JIA - Juvenile Idiopathic Arthritis

KPs - Keratic precipitates

LIF - Leukaemia inhibitory factor

MAPK - Mitogen-activated protein kinase

MCP-1 - Monocyte chemoattractant protein-1

MEWDS - Multiple Evanescent White-Dot Syndrome

MHC - Major histocompatibility complex

MS - Multiple Sclerosis

MTX - Methotrexate

NIU - Non-infectious uveitis

NK - Natural killer

NTPDase - Ecto-nucleoside triphosphate diphosphohydrolase

OSM - Oncostatin M

Ova-Tregs - Ovalbumin-specific Treg

PCR - Polymerase Chain Reaction

PDL1 - Programmed death-ligand 1

pTreg - peripheral Treg cells

RA - Rheumatoid Arthritis

RPE - Retinal Pigment Epithelial

SjS - Sjögren's syndrome

SLE - Systemic lupus erythematosus

SNP - Single nucleotide polymorphisms

SUN - Standardization of Uveitis Nomenclature

T1D - Type 1 diabetes

TGF-β - Transforming Growth Factor-beta

Th - T helper

TIGIT - T-cell immunoreceptor with Ig and ITIM domains

TNFR-1 - TNF Receptor -1

TNF-α - *Tumor necrosis factor α*

Treg - Regulatory T cell

tTreg - thymus Treg cells

VEGF - Vascular endothelial growth factor

VKH - Vogt–Koyanagi–Harada

VZV - Varicella zoster virus

α-MSH - Neuropeptide Alpha-Melanocyte-Stimulating Hormone

SUMMARY

Lymphocyte subpopulations, particularly regulatory T-cells (Tregs), have been extensively studied in the last few years. Tregs are T-cells with an immunosuppressive role, responsible for the expression of regulatory cytokines like IL-10 and TGF-β. A reduction of circulating Treg levels has been associated with various auto-immune diseases, especially in active disease. As for non-infectious uveitis (NIU), results have been conflicting, and further studies are needed to better understand the role of the frequency and function of total Treg and subsets in NIU.

The present thesis aimed to analyze the lymphocyte subpopulations, especially Tregs, as well as inflammatory and anti-inflammatory cytokines in patients with active NIU and compare them with normal controls. One subgroup of NIU patients was further evaluated after treatment and uveitis resolution. Later on, in infectious uveitis (IU) patients, aqueous humour (AqH) samples were also analyzed for cytokine characterization.

In active NIU, we found no significant differences in Treg levels (including naïve and memory subsets) between patients and controls. In NIU patients evaluated over time, our results showed an increased percentage of both total and memory Tregs in patients with active inflammation, without significant difference from controls after treatment. Nevertheless, it is interesting that this initial increase in total and memory Treg percentages was not associated with an increased CD39 expression, which may lead us to speculate whether these cells maintained their normal suppressive function.

When comparing serum cytokine levels in NIU patients and controls, our results showed a tendency for IL-17A elevation in the NIU group and positive correlations between the inflammatory (TNF-α + IFN-ɣ + IL-17A) /anti-inflammatory (IL-10 + TGF-β) cytokine ratio and the IL-17/IL-10 ratio with the absolute counts of memory Tregs. We also found that higher IL-17A levels were associated with higher serum concentrations of memory and naïve Tregs as well as higher TNF-α and IFN-ɣ levels. After treatment, lower levels of IL-17A and TNF-α were present in patients in uveitis remission. Furthermore, the inflammatory/anti-inflammatory ratio also showed a significant reduction between evaluations.

As for cytokine levels in IU patients, our results showed that while there were no significant differences between patients and controls regarding serum cytokine profiles, increased concentrations of IL-10, TNF-α, and IFN-ɣ in the AqH samples were found in patients diagnosed with varicella-zoster virus (VZV)-associated uveitis.

Our results do not support the role of total Treg frequency alone as a uveitis biomarker, and since, to our knowledge, these are the first studies addressing the role of Treg naïve and memory subsets and their respective CD39 expression in the peripheral blood of NIU patients, further studies should be addressed to elucidate the possible role of each subset in NIU pathogenesis and treatment.

As for cytokine profiles in infectious and non-infectious uveitis, our results highlight the importance of IL-17 in active NIU and the possible association between elevated intraocular IL-10 levels and VZV-associated infection.

We hope that this work may contribute to finding new biomarkers for active intraocular inflammation and new therapeutic targets for future research.

KEYWORDS: Non-infectious uveitis; Infectious uveitis; Interleukin-10; Interleukin-17; Regulatory T cells.

SUMÁRIO

As subpopulações linfocitárias, e em particular as células T reguladoras (Tregs), têm sido alvo de intensa investigação nos últimos anos. As Tregs são células reconhecidas pelo seu papel imunorregulador e responsáveis pela expressão de citocinas anti-inflamatórias como, por exemplo, a IL-10 e o TGF-β. A redução de níveis circulantes de Tregs tem sido associada a várias doenças auto-imunes, particularmente em casos de doença activa. No caso da uveíte não-infecciosa (NIU), os resultados têm sido contraditórios, sendo necessários estudos adicionais para que se possa entender o papel definitivo da frequência e função das subpopulações de células Tregs na sua patogénese.

O presente estudo tem como objectivo analisar as subpopulações linfocitárias, em especial a subpopulação Treg, bem como os perfis de citocinas inflamatórias e anti-inflamatórias circulantes de doentes com NIU activa, comparando-os com os de controlos normais. Há ainda a realçar a análise adicional de um subgrupo de doentes com NIU após tratamento e resolução da uveíte. Em doentes com uveíte infecciosa (IU) foram ainda analisadas amostras de humor aquoso (HAq), tendo em vista a caracterização de um perfil de citocinas.

Em doentes com NIU activa não foram encontradas diferenças significativas nos níveis periféricos de Tregs (incluindo *naïve* e de memória) relativamente aos controlos. Em doentes avaliados ao longo do tempo, os nossos resultados mostraram um aumento das percentagens de Tregs totais e de memória, numa primeira avaliação, mas novamente sem diferença relativamente aos controlos após o tratamento. Ainda assim é interessante observar que este aumento inicial nas percentagens de Tregs totais e de memória não foi acompanhado de um aumento significativo na expressão de CD39, o que pode significar a perda da normal função supressora destas células.

Relativamente aos níveis séricos de citocinas em doentes com NIU e controlos, os nossos resultados mostraram uma tendência para elevação da IL-17 no grupo com uveíte, bem como correlações positivas entre o *ratio* de citocinas inflamatórias (TNF-α + IFN-ɣ + IL-17) /anti-inflamatórias (IL-10 + TGF-β) e o *ratio* IL-17/IL-10 com as contagens absolutas de Tregs de memória. Para além disso, níveis séricos mais elevados de IL-17 mostraram uma associação com concentrações mais elevadas de Tregs *naïve* e de memória e ainda com níveis séricos mais elevados de TNF-α e de IFN-ɣ.

Após tratamento, houve uma diminuição significativa nos níveis circulantes de IL-17 e de TNF-α no grupo de doentes com NIU em remissão, bem como uma redução significativa, entre avaliações, no *ratio* de citocinas inflamatórias/anti-inflamatórias. Por fim, no grupo de doentes com IU, os nossos resultados mostraram um aumento das concentrações de IL-10, TNF-α e IFN-ɣ no humor aquoso de doentes com infecção intraocular comprovada por vírus varicella zoster (VVZ). Já no sangue periférico, não se encontraram diferenças significativas entre perfis de citocinas de doentes e controlos saudáveis.

Os nossos resultados não apoiam a utilização isolada da frequência periférica total de Tregs como um biomarcador de uveíte activa. Pensamos que este estudo é pioneiro na avaliação de subpopulações Treg *naïve* e de memória, e sua respectiva expressão CD39, no sangue periférico de doentes com NIU. No entanto, são necessários mais estudos para que o papel individual de cada uma destas subpopulações Treg na patogénese e eventual tratamento da NIU possa ser esclarecido.

Relativamente aos perfis de citocinas em doentes com uveíte de causa infecciosa e não-infecciosa, este trabalho de investigação permite sublinhar a importância da citocina IL-17 na NIU activa e uma eventual associação entre níveis intraoculares elevados de IL-10 e a uveíte associada a infecção por VVZ.

Estes resultados podem vir a contribuir para a identificação de biomarcadores de inflamação intraocular activa e na investigação de novos alvos terapêuticos.

PALAVRAS-CHAVE: Uveíte não-infecciosa; Uveíte infecciosa; Interleucina-10; Interleucina-17; Células T reguladoras.

I. INTRODUCTION

1. Ocular Immune Privilege

The eye is well known for favoring an immunosuppressive environment in an attempt to protect vision from a possible inflammatory reaction that may damage intraocular structures. Several mechanisms contribute to this immune privilege: (1) Physical barriers; (2) Anterior Chamber-Associated Immune Deviation (ACAID); and (3) Inhibitory Ocular Microenvironment.

1.1 Physical barriers

The eye is a complex organ with distinct anatomic structures and blood supplies that form blood–ocular barriers. The blood-ocular barriers are highly specialized and selectively control the inward/toward crossing of compounds into the eye. There are two main blood-ocular barriers, the Blood-Aqueous Barrier (BAB) and the Blood-Retinal Barrier (BRB).

The Blood-Aqueous Barrier is formed by endothelial cells of the iris's blood vessels and the ciliary epithelium's non-pigmented cell layer. Under normal conditions, only small molecules can penetrate the aqueous humor through the ciliary processes' fenestrated capillaries. A disruption of the zonula occludens between the non-pigmented ciliary cells causes a breakdown of the BAB and can occur in inflammatory conditions, allowing immune factors and leucocytes into the eye's anterior segment.

The Blood-Retina Barrier consists of an inner and an outer component. The inner BRB is formed of tight junctions between retinal capillary endothelial cells and the outer BRB of tight junctions between Retinal Pigment Epithelial (RPE) cells (Fig. 4). Retinal edema, a frequent manifestation of retinal disease, is directly associated with a breakdown of the BRB and vision loss and can occur in retinal diseases from various causes (vasogenic or inflammatory).

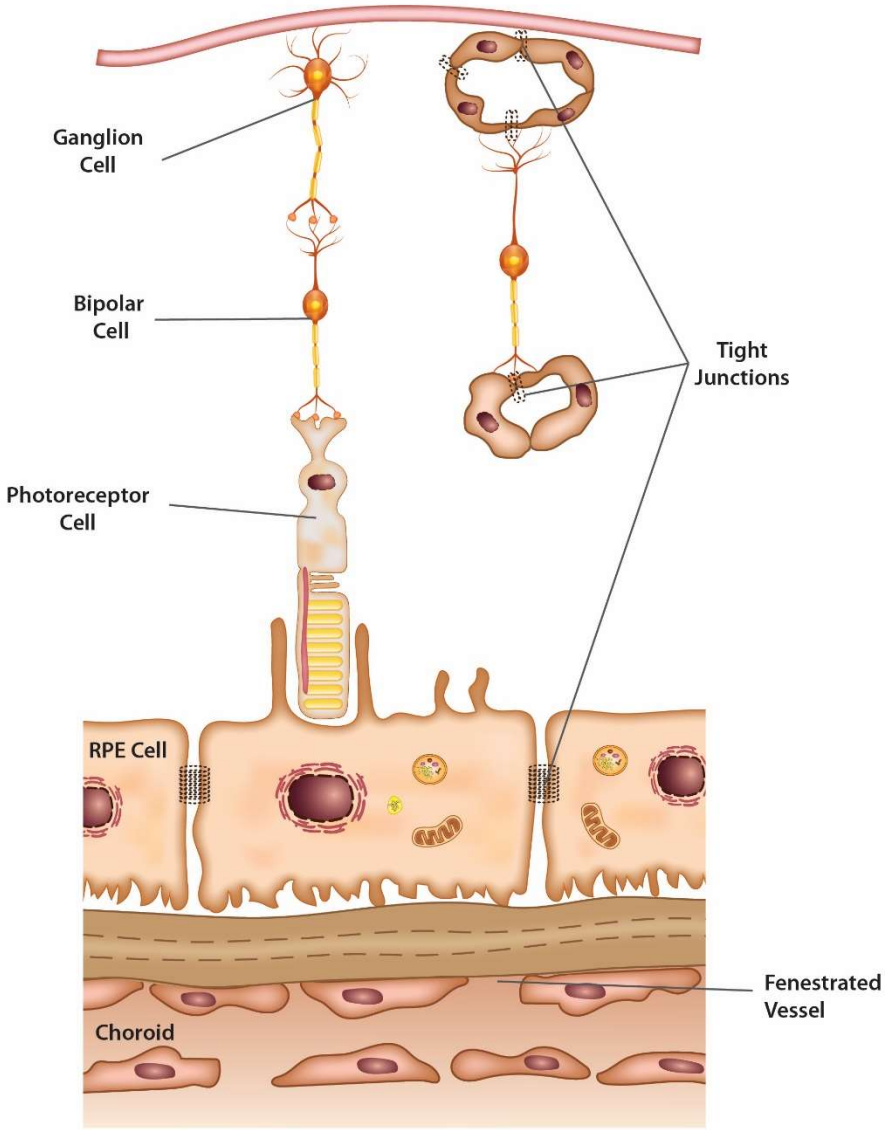

Fig. 1 - Tight junctions form the BRB at two sites: between endothelial cells of retinal vessels, that supply the inner retina (ganglion cells and bipolar cells), and the retinal pigment epithelium (RPE) cell, which filters blood from the fenestrated leaky choroidal vessels. (Author's drawing)

1.2 Anterior Chamber-Associated Immune Deviation (ACAID)

ACAID is a down-regulation of delayed-type cellular immunity. Antigens released into the aqueous humour are presumably recognized by the iris and ciliary body's dendritic cells. Since there is a lack of efferent lymphatics in the eye, these antigen-presenting cells (APCs) can then enter the venous circulation and induce antigen-specific regulatory T cells in the spleen, bypassing the lymphatic system. Successful ACAID induction requires the antigen-bearing eye and an intact spleen to be in place for at least an initial 4- to 5-day interval, implying that a camero-splenic axis exists for the transfer of immunologically relevant information (1). The injection of an antigen into the anterior chamber induces a rise in Tumor Necrosis Factor α (TNF-α) and Monocyte Chemoattractant Protein-1 (MCP-1) in aqueous humour and an infiltration of circulating $F4/80^{+}$ monocytes that home to the iris. The induction of ACAID is dependent on this infiltration of circulating monocytes that eventually emigrate to the thymus and spleen, where they induce regulatory T cells that inhibit the inductive or effector phases of a cell-mediated immune response (2) (Fig. 2).

1.3 Inhibitory Ocular Microenvironment

Suppression of immunogenic inflammation has been associated with immunosuppressive factors found in aqueous humour, which are produced by ocular tissues:

Transforming Growth Factor-beta (TGF-β)

Aqueous humour is capable of altering the functional program of TCR-ligand-activated, primed T-cells, converting these cells into TGF-beta-producing regulatory T-cells (Tregs) (3);

Neuropeptide Alpha-Melanocyte-Stimulating Hormone (α-MSH)

Several immunomodulating neuropeptides are found in the aqueous humour and are produced by the RPE. The neuropeptide α-MSH has been demonstrated to have a critical central role in modulating immune activity within the eye (4), and the effects of aqueous humour on immune cells are very similar to the effects of α-MSH peptide treatment on immune cells (5).

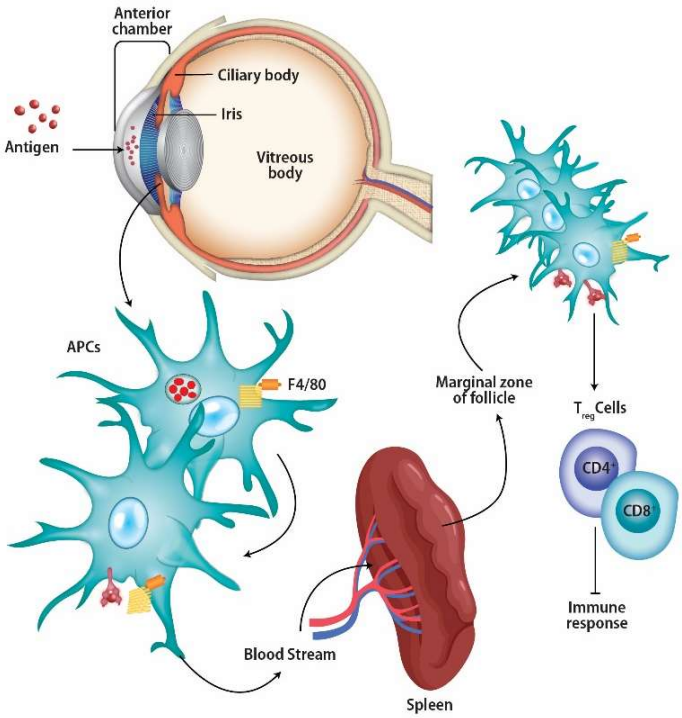

Fig. 2 - In the ACAID model, foreign antigens injected into the anterior chamber of the eye are captured by F4/80+ antigen-presenting cells (APCs) of the iris and ciliary body and travel via the blood to the spleen, where they promote the development of antigen-specific CD8+ Treg cells capable of suppressing delayed-type hypersensitivity responses toward eye-derived antigens. (Author's drawing)

Membrane-bound negative regulators

T cells express regulatory activity when they come in contact with cultured RPE (6). Besides TGF-β and α-MSH, RPE cells express soluble cytotoxic T-lymphocyte antigen-2 α (CTLA-2α) and retinoic acid that promote regulatory activity (7). Besides, under conditions of inflammation, RPE cells express enhanced levels of Programmed Death-Ligand 1 (PDL1) and possibly major histocompatibility complex (MHC) class II (8), which also promote Treg cell activation. Moreover, cells throughout the ocular microenvironment express FasLigand (FasL), which has been suggested to be a significant contributor to immune privilege because its expression is necessary for graft survival within the eye (9). The expression of PDL1 and FasL, along with the physical barrier created by the RPE and endothelial cells' tight junctions, form the immune barrier of the ocular microenvironment.

Cellular negative regulators of inflammation

The negative regulators of inflammation expressed within the eye suppress the production of proinflammatory cytokines and program immune cells to suppress inflammation and promote tolerance.

The effects of aqueous humour on macrophages, the ACAID phenomena, and Treg cells' activation by retinal dendritic-like cells (10) demonstrate that antigen presentation within the immune-privileged eye does not follow conventional pathways that lead to effector T cell activation. The retina's microglial cells are potential cellular regulators of inflammation (11) and prevent T cell activation, and their immunosuppressive functions seem to be induced by the negative regulators produced by the RPE.

Even though the eye is an immune-privileged tissue that has adapted several negative regulators to prevent the activation of inflammation within its tissue microenvironment, there are conditions in which this immune privilege fails. One of those conditions occurs in the presence of the intraocular inflammation caused by uveitis. The ocular tissues contain many potential autoantigenic targets, especially the retina, apparently sequestered from the immune system (12). The retina (containing retinal antigens) is formed early during embryogenesis, and the optic cup is closed by weeks 6 to 7. Lymphocytes of the B series develop in the liver by 9 weeks of gestation and are present in the blood and spleen by 12 weeks. T lymphocytes start to leave the thymus from about 14 weeks of gestation, and subsequently, cells with helper and suppressor phenotypes are present in the spleen. Sequestered retinal antigens are recognized as not-self and escape to the bloodstream after damage (trauma, infection, inherited degenerations), activating peripheral autoreactive T cells (13). Once activated, antigen-specific T cells enter the retina and eye tissues and attract pro-inflammatory macrophages, which cause more tissue damage (14).

In experimental autoimmune-uveitis, inflammation declines inversely with an increase in Tregs, which accumulate and promote resolution. Immune privilege appears not to afford much protection against the damaging effects of uveitis, possibly because it works at the level of tissue homeostasis, mainly keeping healthy tissue free of random migrants, which might provoke inflammation (15). However, in uncontrolled uveitis, both infection and the immune response to infection can cause permanent structural damage.

2. What is uveitis?

Uveitis is the inflammation of the uvea, the pigmented vascular layer that lies between the inner retina and the outer fibrous layer composed of the sclera and cornea. The uvea consists of the middle layer of pigmented vascular structures of the eye and includes the iris, ciliary body, and choroid (Fig. 3). These distinct parts of the uvea can be affected separately: iris (Iritis), ciliary body (Cyclitis, Iridocyclitis), choroid (Choroiditis), or the entire uvea (Panuveitis).

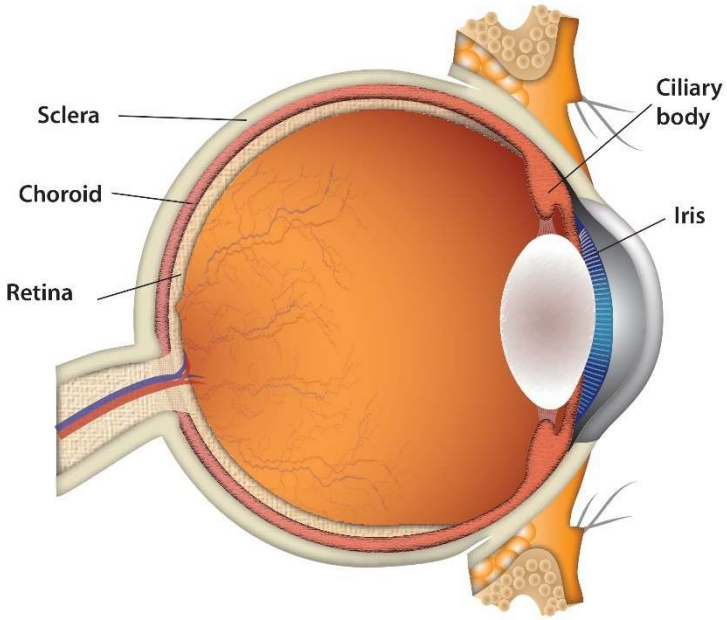

Fig. 3 - The uvea is the middle layer of the eye beneath the sclera. It is made up of the iris, ciliary body, and choroid. (Author's drawing)

Accordingly, uveitis is also sub-classified as Anterior Uveitis when it affects the anterior segment of the eye in which it is restricted to the anterior chamber, iris, and ciliary body; Intermediate Uveitis when it affects the pars plana region of the ciliary body (Pars Planitis) and the vitreous gel (Vitritis); Posterior Uveitis when inflammation affects the posterior segment of the eye including the retina (Retinitis), the retinal vessels (Vasculitis), the choroid (Choroiditis), or the optic nerve (papillitis, optic neuritis) and finally Panuveitis when both the anterior and posterior segments of the eye are affected. Table I summarizes the uveitis anatomical classification according to the Standardization of Uveitis Nomenclature (SUN) (16).

Table I. Anatomical Classification of Uveitis

Type	Primary Site of Inflammation	Includes
Anterior uveitis	Anterior Chamber	Iritis Iridocyclitis
Intermediate	Vitreous	*Pars Planitis*
Posterior Uveitis	Retina and/or Choroid	Focal, multifocal or diffuse choroiditis Chorioretinitis Retinochoroiditis Retinitis Neuroretinitis
Panuveitis	Anterior chamber, Vitreous and Retina and/or Choroid	

2.1. Clinical Features and Diagnosis

Managing uveitis is often challenging and developing a differential diagnosis is the key to success. Classification of the uveitis and developing a list of potential diagnoses will help determine appropriate diagnostic testing, guide therapy, and help determine prognosis. Associated symptoms and signs are essential sources of information and require a systemic review of symptoms and a detailed physical examination. Referral to an internist, primary care physician, rheumatologist, or other specialist is often required in the work-up of uveitis.

Ocular inflammation may be the initial presentation of an underlying systemic disease that, if undiagnosed and untreated, may lead to significant morbidity and even death.

Diagnosis is mainly based on recognizing patterns that include findings from the medical history, clinical examination, laboratorial testing, and medical imaging. The first step in developing a differential diagnosis for patients with inflammatory eye disease is to classify the uveitis in as detailed a fashion as possible.

2.1.1 Onset, duration, and course

Uveitis must be classified in terms of onset, duration, and course (16) (Table II). The onset of uveitis should be described either as sudden or insidious. The duration of an attack of uveitis should be described as either limited (up to 3 months) or as persistent (more than 3 months). The term acute is used to describe the course of specific uveitic syndromes characterized by a sudden onset and limited duration, such as HLA-B27-associated uveitis. The term recurrent is used to describe repeated episodes of uveitis separated by periods of inactivity without treatment for at least 3 months. The term chronic is used to describe persistent uveitis characterized by prompt relapse (in less than 3 months) after therapy discontinuation.

Table II. Uveitis Classification regarding disease Onset, Duration and Course (SUN* Working Group)

Category	Descriptor	Comment
Onset	Sudden	
	Insidious	
Duration	Limited	≤ 3 months duration
	Persistent	> 3 months duration
Course	**Acute**	Episode characterized by a sudden onset and limited duration
	Recurrent	Repeated episodes separated by periods of inactivity without treatment for at least 3 months
	Chronic	Persistent uveitis with relapse up to 3 months after discontinuing treatment

*SUN=Standardization of Uveitis Nomenclature (16).

Most occurrences of anterior uveitis, such as HLAB27-associated uveitis and idiopathic anterior uveitis, fall into this category. Other diseases that typically can cause acute uveitis include the Vogt–Koyanagi–Harada (VKH) syndrome, Toxoplasmosis, Acute Posterior Multifocal Placoid Pigment Epitheliopathy (APMPPE), Multiple Evanescent White-Dot Syndrome (MEWDS), and traumatic iritis. Causes of chronic uveitis include Juvenile Idiopathic Arthritis, Birdshot Choroidopathy, Serpiginous Choroidopathy, Tuberculous uveitis, Sympathetic Ophthalmia, and Sarcoidosis.

2.1.2 Granulomatous/Non-Granulomatous

In anterior uveitis, inflammatory cells attach to the corneal endothelium in conglomerates called keratic precipitates (KPs). The appearance of KPs has been used to classify the inflammatory process as granulomatous or non-granulomatous. The more common non-granulomatous type of KP is characterized by fine white collections of lymphocytes, plasma cells, and pigment. Granulomatous KPs are large, greasy-appearing collections of lymphocytes, plasma cells, and giant cells. Patients with granulomatous KPs usually have a history of chronic disease with an insidious onset and frequently have posterior segment disease in addition to their anterior segment inflammation. Other ocular findings suggestive of granulomatous inflammation are iris nodules and choroidal granulomas. Causes of granulomatous uveitis are listed in Table III.

Table III. Causes of Granulomatous Uveitis (17)

Sarcoidosis
Sympathetic Ophthalmia
Multiple Sclerosis
Lens-induced Uveitis
Vogt–Koyanagi–Harada Syndrome
Syphilis
Tuberculosis

2.1.3 Anterior Uveitis

Anterior uveitis is much more common than intermediate, posterior, or panuveitis and accounts for 85% of uveitis instances (18). It describes a disease limited predominantly to the anterior segment of the eye. The inflammation is characterized by conjunctival hyperemia, anterior chamber cell and flare, KPs, posterior synechiae, and peripheral anterior synechiae. A mild cellular inflammatory response in the anterior vitreous is often seen.

The anterior chamber is easily examined with the slit lamp for signs of ocular inflammation. Since inflammatory cells do not arise in the aqueous, the presence of cells or increased protein (flare) in the anterior chamber is evidence of a spillover from the inflamed iris or ciliary body. Anterior chamber cells are best seen by directing the slit lamp beam obliquely across the eye and focusing posterior to the cornea. Although the cells' presence represents an index of activity, there is considerable variation among physicians on the grading of the number of cells. Nevertheless, the gradings for anterior chamber inflammation, anterior chamber cells and flare, according to the Standardization of Uveitis Nomenclature (SUN) (16) are summarized in Table IV and Table V.

Table IV. The SUN* Working Group Grading Scheme for Anterior Chamber Cells

Grade	Cells in Field **
0	<1
0,5+	1-5
1+	6-15
2+	16-25
3	26-50
4+	>50

* SUN=Standardization of Uveitis Nomenclature; **Field size is a 1mm by 1mm slit beam.

Table V. The SUN* Working Group Grading Scheme for Anterior Chamber Flare

Grade	Description
0	None
1+	Faint
2+	Moderate (iris and lens details clear)
3+	Marked (iris and lens details hazy)
4+	Intense (fibrin or plastic aqueous)

* SUN=Standardization of Uveitis Nomenclature

The common causes of anterior uveitis are listed in Table VI

Table VI. Causes of Anterior Uveitis (17)

Idiopathic
HLA-B27-associated Uveitis
Ankylosing Spondylitis
Reiter's Syndrome
Inflammatory Bowel Disease
Psoriatic Arthritis
Behçet's Disease
Juvenile Idiopathic Arthritis
Fuchs' Heterochromic Iridocyclitis
Sarcoidosis
Syphilis
Posner-Schlossman Syndrome (glaucomatocyclitic crisis)
Herpes Virus
Masquerade Syndrome

2.1.3.1 HLA-B27-Associated Uveitis

Sudden onset anterior uveitis is the phenotype of uveitis typically associated with HLA-B27 (19). The frequency of uveitis varies with different forms of spondylarthritis. It will affect up to 50% of patients with ankylosing spondylitis (AS) in a lifetime (20), and it is the most common clinically significant co-morbidity recognized among these patients (21). In Psoriatic Arthritis, the likelihood of uveitis association is 7 to 19% (22). This likelihood is lower still in association with either Crohn's disease or Ulcerative Colitis (23).

The connection between HLA-B27 and spondylarthritis has not yet been fully elucidated, although it is widely accepted that the entire intracellular process of HLA-B27 formation needs to be considered. There are some prevailing theories regarding the mechanism, including the hypothesis of molecular mimicry and the hypothesis of the cell-surface HLA-B27 homodimer. The molecular mimicry hypothesis posits that the antigenic components of infectious bacterial pathogens partially resembling or cross-reacting with HLA molecules can stimulate CD8+T lymphocytes, followed by responding to one HLA-B27 relevant self-peptide or the peptides directly produced by HLA-B27 (24). This hypothesis is primarily based on previously identified amino acid structures of homologous origin between the HLA structure and specific sequences of some bacterial antigens (25). The cell-surface HLA-B27 homodimer formation hypothesis is based on the fact that HLA-B27 heavy chains tend to form homodimers without beta-2 microglobulin via the disulfide bonds of the cysteine at C67 (26). The dimeric HLA-B27 complexes, mostly found in patients' gut and synovium, may contribute to the genesis of AS and some other spondyloarthropathies. These HLA-B27 dimers could occur on APCs, thus stimulating the IL-23 receptor on T-lymphocytes leading to the production of IL-17 (27). Moreover, HLA-B27 dimers might contribute to AS's development since they have been linked to receptors expressed on natural killer immunocytes, myelomonocytes, and lymphocytes. The binding is realized via killer cell immunoglobulin-like receptors and leucocyte immunoglobulin-like-receptors, thus acting in processes related to autoimmune disorders (28, 29).

In HLA-B27 positive individuals, recurrent episodes of acute anterior uveitis (AAU) have been associated with increased ocular vascular permeability, which normalizes between episodes. This indicates that a temporary breakdown of the selectively permeable blood-aqueous barrier occurs during AAU episodes (30).

HLA-B27 positive patients with systemic disease have a higher probability of recurrent uveitis episodes than those without systemic symptoms (31). However, uveitic activity may not always be related to the systemic disease activity (32), and the inflammatory rheumatologic manifestations seem to precede uveitis (33). The frequency of extraocular B27-associated disease is within the 30% to 90% range (34, 35). Males seem to be 2 to 5 times more affected than females (36), and the mean age for the appearance of uveitis is in the range of 32 to 35.6 years in the literature (37, 38).

The diagnosis of HLA-B27-associated uveitis is based on clinical features and positivity to HLA-B27 antigen after the exclusion of other infectious or inflammatory diseases.

Clinically, HLA-B27-positive AAU has an acute onset and is a unilateral alternating, non-granulomatous uveitis with a high tendency for recurrences with a mean number of 0.6 - 3.3 attacks per patient and per year of follow-up, and a mean duration of each episode of 4-6 weeks (39, 40). It is also characterized by sudden redness, blurring vision, pain, and photophobia (41). A relevant exudation in the anterior chamber is also present, hypopyon and fibrin can occur, and posterior synechiae may be observed.

Ocular complications are common and are present in over 65% of HLA-B27-positive AAU patients (39). Frequent complications are listed in Table VII.

Inflammation in HLA-B27-associated uveitis generally responds well to topical therapy with corticosteroids and cycloplegics. However, patients who present with hypopyon, a fibrinous reaction in the anterior chamber, pupillary seclusion, and/or macular edema may need additional periocular therapy with a subconjunctival or sub-tenon steroid injection or even oral corticosteroids. The need for immunosuppressive therapy is often associated with the incidence and gravity of extraocular manifestations or increased recurrence rate.

Anterior uveitis is generally considered to be associated with a better visual prognosis than other uveitis forms such as panuveitis and posterior uveitis. In a study on the causes of visual impairment and blindness in intraocular inflammatory diseases, 10% of HLA-B27-associated anterior uveitis patients suffered legal blindness or severe visual impairment (42). However, a more recent study reported that, among a large case series of 175 patients with HLA-B27-associated uveitis, only 7% of patients demonstrated significantly decreased final visual acuity (2 lines) (40).

Table VII. Ocular Complications in HLA-B27-Associated Uveitis

Complication	Frequency (31, 39, 40)
Posterior synechiae	13-91%
CataractA<SWXDCA<SWXDC\	7-28%
Ocular Hypertension/Glaucoma	7-20% / 2-12%
Cystoid Macular Edema	6-13%
Vitritis	10-21%
Papilitis	2-18%

2.1.3.2 Herpetic Uveitis (and Keratouveitis)

Herpetic keratitis is probably the most common ocular disease associated with uveitis, and patients with stromal keratitis often have concurrent anterior uveitis. Although uveitis occurs during the initial onset of epithelial disease in some patients, most patients have uveitis with stromal involvement (43). Recurrent episodes are common and can severely damage the eye. Anterior uveitis may also occur in patients with a history of previous epithelial herpetic infection without currently active corneal disease. Herpetic keratouveitis manifests as a red, photophobic, and painful eye with decreased vision.

This infection should be suspected in patients with a significant corneal opacity accompanied by synechiae, anterior chamber cells, large KPs, and ocular hypertension. Sectorial and patchy iris atrophy consistent with ischemic damage is characteristic of Herpes Zoster Virus infection. Hypopyon, as well as hyphema, may also be seen in more severe cases. The use of aqueous humor Polymerase Chain Reaction (PCR) may help identify the Herpes Simplex or Herpes Zoster Virus as the causative agents in patients with uveitis of unknown etiology (44).

According to the Herpetic Eye Disease Study Group, patients with herpetic keratouveitis or uveitis are often treated with oral antivirals (acyclovir or valaciclovir) as well as with topical corticosteroids, cycloplegics, and intraocular pressure (IOP)-lowering agents (45). Long term use of oral antiviral agents is also beneficial in reducing the recurrence rate of stromal disease and uveitis (46).

2.1.4 Intermediate Uveitis

Intermediate uveitis is characterized by inflammation that primarily affects the *pars plana*, vitreous, and peripheral retina. Aggregates of inflammatory cells are frequently seen in the inferior vitreous and have been termed vitreous snowballs. Similarly, the accumulation of inflammatory cells and debris along the *pars plana* and *ora serrata* has been called snowbanks. Mild anterior uveitis often coexists, and cystoid macular edema is a frequent finding. Common conditions associated with intermediate uveitis are listed in Table VIII.

Table VIII. Causes of Intermediate Uveitis (17)

Sarcoidosis
Inflammatory Bowel Disease
Multiple Sclerosis
Lyme Disease
Pars Planitis (idiopathic)

2.1.5 Posterior Uveitis

Inflammatory disease limited to the posterior segment of the eye, particularly to the retina and choroid, is termed posterior uveitis. For vitreous haze involving obstruction of fundus details by vitreous cells and protein exudation, an original scale of clinical photographs (47) illustrating six grades of vitreous haze was adopted by the SUN working group (16), but with the extra grade of 0.5+ for trace added to the grading scheme. Vitreous haze grading is listed in Table IX.

Table IX. Grading Scheme for Vitreous Haze (47)

Score	Description	Clinical Findings
0	Nil	None
0,5+	Trace	
1+	Minimal	Posterior pole clearly visible
2+	Mild	Posterior pole details slightly hazy
3+	Moderate	Posterior pole details very hazy
4+	Marked	Posterior pole barely visible
5+	Severe	Fundal details not visible

Many diseases cause posterior uveitis, so it is useful to further subdivide these disorders as predominantly retinitis or choroiditis and as a focal or multifocal disease. Frequent disorders causing focal and multifocal retinitis as well as choroiditis are listed in Table X.

Table X. Causes of Posterior Uveitis (17)

Focal Retinitis	Multifocal Retinitis	Focal Choroiditis	Multifocal Choroiditis
Toxoplasmosis	Syphilis	Toxocariasis	Histoplasmosis
Onchocerciasis	Herpes Virus	Tuberculosis	Sympathetic Ophthalmia
Cysticercosis	Cytomegalovirus	Nocardiosis	**Vogt–Koyanagi–Harada Syndrome**
Masquerade syndromes	Sarcoidosis	Masquerade syndromes	Sarcoidosis
	Masquerade syndromes		Serpiginous choroidopathy
	Candidiasis		Birdshot choroidopathy
			Masquerade syndromes

2.1.5.1 Vogt–Koyanagi–Harada Disease

Vogt-Koyanagi-Harada (VKH) disease is a severe bilateral granulomatous posterior or panuveitis associated with serous retinal detachments, disk edema, and vitritis (48). Systemically, it may be associated with tinnitus, hearing loss, vertigo, meningismus, poliosis, and vitiligo, although not all patients present with the complete constellation of these extraocular findings.

The prevalence of VKH varies in different populations in the world, being more common in Asia, Latin America, and the Middle East (48).

VKH seems to be an autoimmune inflammatory condition mediated by CD4+T cells that target melanocytes (49). The trigger that induces altered tolerance to melanocytes is unknown but genetic susceptibility of patients expressing HLA DRB1*0405, combined with a viral infection, may play a role in initiating the autoimmune process (50).

VKH presents clinically in 4 different phases: prodromal, acute uveitic, convalescent, and chronic recurrent. In the prodromal phase, before ocular involvement, clinical manifestations include headache (82%), meningismus (55%), fever (18%), nausea (9%), vertigo (9%), orbital pain, and auditory disturbances (51).

Cerebrospinal fluid may show pleocytosis in more than 80% of patients (52). In the acute uveitic phase, there is a sudden onset of bilateral granulomatous uveitis with pockets of subretinal fluid and choroidal thickening, blurring of vision, and conjunctival injection. Signs also include swelling and hyperemia of the optic nerve head and retinal edema (48). The initial posterior uveitis, if untreated, spreads to both the vitreous and anterior uvea, although vitritis and anterior uveitis are not necessary for the diagnosis.

Several weeks to months after the acute uveitic phase, depigmentation of the choroid, vitiligo, and poliosis occurs. This convalescent-phase usually lasts for months. Depigmentation of the choroid usually results in a "sunset glow" bright orange-red appearance of the fundus. Finally, chronic recurrent intraocular inflammation develops in some patients and is characterized by exacerbations of granulomatous anterior uveitis that is usually resistant to systemic steroid therapy. This chronic recurrent phase usually develops 6 to 9 months after the initial presentation and is marked by complications such as RPE proliferation, subretinal fibrosis, subretinal neovascular membranes, posterior subcapsular cataract, posterior synechiae, glaucoma, and band keratopathy (53, 54).

The revised diagnostic criteria for Vogt-Koyanagi-Harada syndrome (55) are listed in Table XI.

Table XI. Revised diagnostic criteria for Vogt-Koyanagi-Harada syndrome (55)

Complete Vogt-Koyanagi-Harada Syndrome
1. No history of penetrating ocular trauma or surgery preceding the initial onset of uveitis.
2. No clinical or laboratory evidence suggestive of other ocular disease entities.
3. Bilateral ocular involvement [(a) or (b) must be met, depending on stage of disease] a) Early manifestations 1. Diffuse choroiditis, focal areas of subretinal fluid, bullous serous retinal detachments; 2. If equivocal fundus findings, then the following must be present: - Focal areas of delays in choroidal perfusion, multifocal areas of pinpoint leakage, large placoid areas of hyperfluorescence, pooling within subretinal fluid, and optic nerve staining; - Diffuse choroidal thickening, without evidence of posterior scleritis by ultrasonography. b) Late manifestations 1. History suggestive of prior disease based on findings in the following: 2. Ocular depigmentation: sunset glow fundus or Sugiura's sign; 3. Other signs: nummular chorioretinal depigmented scars, RPE clumping or migration, or recurrent or chronic anterior uveitis.

4. Neurological findings (may have resolved): meningismus, tinnitus, or CSF pleocytosis.
5. Integumentary findings (not preceding the onset of uveitis): alopecia, poliosis, or vitiligo.
Incomplete Vogt-Koyanagi-Harada syndrome
Criteria 1 and 3 and either 4 or 5 must be present.
Probable Vogt-Koyanagi-Harada syndrome
Criteria 1 and 3 must be present.

Systemic high-dose corticosteroids are the gold standard of initial treatment for VKH. A previous study showed that patients receiving systemic corticosteroids at a 200 mg/day dose within 13 days of disease onset required a shorter duration of steroid use (10.9 months vs. 24.2 months) with an equal final visual acuity (56). Treatment with high-dose systemic steroids has also been shown to be associated with a decreased incidence of sunset glow fundus (57). The treatment duration must not be inferior to 6 months based on studies that show a decrease in further ocular symptoms (58), a decrease in recurrence, and an improvement in final visual acuity compared to those treated for shorter periods (59). Immunosuppressive agents such as methotrexate, azathioprine, cyclosporine A, mycophenolate mofetil, and alkylating agents have been used successfully to treat VKH (60-62) and the International Uveitis Study Group, thus, have recommended such immunosuppressive agents as mandatory in the treatment of VKH to prevent recurrences (63).

2.1.6 Panuveitis

The term Panuveitis is reserved for diseases involving all eye segments, typically with a severe sight-reducing inflammatory response. The common causes of Panuveitis are listed in Table XII.

Table XII. Causes of Panuveitis (17)

Syphilis

Sarcoidosis
Vogt-Koyanagi-Harada Syndrome
Sympathethic uveitis
Behçet Disease
Herpes Virus- Acute Retinal Necrosis
Infectious Endophthalmitis
Masquerade syndrome

2.1.6.1 Behçet Disease

Behçet disease (BD) is a systemic inflammatory condition of unknown pathogenesis, particularly prevalent in the Mediterranean, Middle Eastern, and Far Eastern countries (64). Its histopathologic hallmark is an occlusive vasculitis, affecting vessels of various sizes, especially veins (65). It is characterized by oral and genital ulcers, ocular inflammatory disease, skin lesions, vasculitis, and the involvement of several other organs (66). The prevalence of ocular involvement in patients with BD is variable, ranging from 40 to 70% (67, 68).

Extraocular manifestations include skin and mucosal manifestations (buccal and genital aphthous ulcers, erythema nodosum, pseudofolliculitis, acneiform nodules, superficial migratory thrombophlebitis) (65), joint manifestations (arthralgia or arthritis), neurologic manifestations (meningitis or meningoencephalitis (69), central venous thrombosis, cerebral arteritis), vascular involvement (deep venous thromboses, superficial thrombophlebitis, arterial aneurysms or arterial thromboses) and other manifestations (gastrointestinal tract involvement, renal involvement, cardiac involvement, and testicular and epididymal involvement). As there is a lack of a universally recognized pathognomonic test, BD diagnosis is primarily based on clinical criteria. Diagnostic Criteria for BD (point score system) revised by an International Team Group (70) are listed in Table XIII.

Table XIII. International Criteria for BD - point score system: scoring>= 4 indicates diagnosis.

Sign/Symptom	Points
Ocular lesions	2

Sign/Symptom	Points
Genital aphthosis	2
Oral aphthosis	2
Skin lesions	1
Neurological manifestations	1
Vascular manifestations	1
Positive pathergy test*	1

*Pathergy test is optional, and the primary scoring system does not include pathergy testing. However, where pathergy testing is conducted, one extra point may be assigned for a positive result.

The eye is a frequently affected organ in BD, and uveitis, which is potentially blinding, is one of the disease's major manifestations. Other rarer ocular manifestations have been described, including episcleritis, scleritis, conjunctival ulcers, keratitis, orbital inflammatory disease, isolated optic neuritis, and oculomotor palsies (71-73).

Uveitis occurs in 60 to 80% of cases, on average 4 years after the onset of the disease's systemic manifestations, notably buccal aphthous ulcers (65, 66). It may be the presenting sign in 10 to 20% of patients (17).

The intraocular inflammation associated with BD is typically acute and recurrent. It presents in the form of bilateral, symmetric or asymmetric, non-granulomatous panuveitis, associated with retinal vasculitis (74).

Anterior uveitis is most often associated with posterior segment inflammation, although it can be isolated in 5 to 10% of cases (71). The appearance of hypopyon reflects particularly severe anterior chamber inflammation and is found in 12 to 30% of cases (75).

Diffuse vitritis of variable intensity is common and may be found in over 85% of cases (76). Occlusive or non-occlusive retinal vasculitis is found in more than 90% of patients. It may involve vessels of all sizes, mainly veins (73). Branch retinal vein occlusion occurs in about 6% of cases, while central vein occlusions and branch retinal arterial occlusions are rarely seen (77). Moreover, foci of retinitis are found in over 50% of cases of ocular BD. They present as superficial retinal infiltrates and are variable in number, location, and size (75).

Ocular complications of BD are frequent and include macular edema, posterior synechiae, cataract, optic atrophy, and glaucoma. Diffuse non-cystoid or cystoid macular edema appears in 20 to 75% of cases and affects the long-term visual prognosis (71).

In BD treatment, systemic corticosteroids allow for rapid and effective control of acute ocular inflammatory manifestations while awaiting immunosuppressors' full effect. The systematic and early use of immunosuppressors in the treatment of BD-associated uveitis has contributed significantly to improved visual prognosis while also facilitating discontinuation of steroids (78).

The most used conventional immuno-suppressants in BD are azathioprine and cyclosporine A. Biologic agents like anti-tumor necrosis factor-alpha (anti-TNF-α) are indicated for refractory cases and have been increasingly recommended as first-line therapy (79, 80).

2.1.6.2 Acute Retinal Necrosis

Acute Retinal Necrosis (ARN) is an acute panuveitis secondary to herpetic infection characterized by retinal periarteritis progressing to diffuse necrotizing retinitis. The American Uveitis Society defines it as having: 1) One or more foci of retinal necrosis with discrete borders located in the peripheral retina; 2) rapid progression in the absence of antiviral therapy; 3) circumferential spread; 4) evidence of occlusive vasculopathy with arterial involvement and 5) a prominent inflammatory reaction in the vitreous and anterior chamber (81).

Serious ocular complications can develop if there is a significant delay in diagnosis and treatment. These can cause irreversible visual impairment and include cataract, band keratopathy, glaucoma, macular edema, and retinal detachment (82). Since anti-viral treatment regimens and ocular prognosis are different for HSV, VZV, or CMV related infections, it is vital to find the causative agent. PCR analysis of intraocular fluids for HSV, VZV, and CMV in this setting has a sensitivity of 80.9–84.0% and a specificity of 97.4–100.0% (83).

3. Cytokines in Uveitis

T cells expressing the surface marker cluster of differentiation 4 (CD4) are known as T helper (Th) cells and play essential roles in the pathogenesis of autoimmune diseases, including uveitis (84). Two major classes of Th cells were defined (85): Th1 cells, defined by the expression of the transcription factor T-bet and secretion of interferon-gamma (IFN-ɣ), important in cellular immunity; and Th2 cells, defined by the transcription factor GATA binding protein 3 and secretion of interleukin (IL)-4 and IL-5, which are essential for humoral immunity (86).

Th1 cells have been previously implicated in the induction of uveitis in experimental autoimmune uveitis (EAU), but several authors have also reported the crucial role of IL-17-producing Th17 cells in the genesis of EAU in mice (87). In animal models, this interplay between the Th1 and Th17 responses in driving uveitis is complex, as both can drive intraocular inflammation (88).

Th17 cells develop from naive $CD4^{+}$T cells in the presence of IL-1β, IL-6, and TGF-β produced by antigen-presenting cells (APCs). In addition to IL-17A, Th17 cells express IL-22, IL-17F, IL-21, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IFN- ɣ (89). Another T-cell subpopulation associated with the development of autoimmune disease is regulatory T cells (Tregs). Treg cells are important in maintaining tolerance and preventing autoimmunity and can be identified by their expression of the forkhead boxP3 (FOXP3) transcription factor and their ability to produce transforming growth factor-beta (TGFβ), IL-10, and IL-35 (90). The role of Treg cells in the pathogenesis of non-infectious uveitis (NIU) has been accessed by several studies showing these cells may be decreased in patients with active uveitis (91), BD (92), and VKH disease (93).

Regarding the importance of cytokines in the development of infectious and NIU, previous studies have demonstrated elevated plasma levels of several cytokines in active intraocular inflammation. However, the inflammatory processes occurring within the eyes and the regulatory role of cytokines in the ocular micro-environment are still subject of an ongoing investigation.

IL-17 pathway cytokines (IL-17A, IL-6, and IL-23) seem to be particularly crucial in NIU's pathogenesis and may be useful in a clinical setting either as biomarkers in the diagnosis of specific uveitic disorders or as future therapeutic targets.

3.1 The IL-17 Pathway

Th17 cells are a subset of CD4+ T-cells responsible for the production of interleukin (IL)-17. This T-cell subset is responsible for the production of IL-17A, a proinflammatory cytokine involved in several autoimmune diseases. Both IL-6 and transforming growth factor (TGF)-β are necessary for Th17 differentiation and IL-17 expression; they feature complimentary roles since the sole presence of TGF-β induces T-regulatory (Treg) cell production from CD4+ T-cells (94-96). IL-6 needs to be present in order to promote the differentiation of a Th17 cell subset in addition to concurrent Treg cell inhibition (97). Although IL-6 and TGF-β have synergistic roles in Th17 cell differentiation, IL-23 is also important for Th17 cell expansion and activation (94). On the other end of this spectrum are interferon (IFN)-ɣ and IL-27, which play a regulatory role in uveitis induction by suppressing Th17 differentiation (87, 98).

IL-17 induces the production of other inflammatory cytokines such as IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, TGF-β, and TNF-α; chemokines such as monocyte chemoattractant protein- 1 (MCP-1), cytokine-induced neutrophil chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2); prostaglandin E2; intercellular adhesion molecule-1; and matrix metalloproteinases. It is also involved in the recruitment of neutrophils, monocytes, and Th1 cells, acting with other inflammatory cytokines to induce inflammation in target tissues (99).

Th1 cells have previously been implicated in the induction of uveitis in experimental autoimmune uveitis (EAU), but since then, several authors have reported the crucial role of IL-17-producing Th17 cells in the genesis of experimental uveitis in mice. They have indicated that treatment with anti-IL-17 antibodies reduces the induction and severity of uveitis in EAU. Moreover, the degree of intraocular inflammation was reduced in IL-17 -/- mice (100), and although both Th1 and Th17 cells are activated during EAU (87, 100), it has been proposed that Th17 cells are responsible for retinal inflammation in the early stages of uveitis, whereas Th1 expression is increased during the late phases and resolution of the disease (87). It has thus been shown that Th1 and Th17 cells are implicated in the genesis of EAU and may be synergistic with each other, having definitive functions at different stages of the disease.

In addition to its obvious proinflammatory role in the induction of EAU, IL-17 expression has also been shown to be increased in the human peripheral blood of patients suffering from autoimmune and infectious uveitis.

3.1.1 Experimental Autoimmune Uveitis

IL-17 is crucial in the development of EAU in mice and other models of experimental autoimmune disorders (87, 100). In EAU, the intraocular expression of IL-17 is elevated in mice with uveitis, and it promotes the release of inflammatory mediators from ARPE-19 cells, disrupting the retinal pigment epithelium barrier function (101).

There is abundant information regarding the role of Th17 cells and IL-17, its main proinflammatory cytokine, in the induction and clinical severity of EAU, although some studies have inconsistently determined the specific timing of increased IL-17 expression in this condition. It has been proposed that Th17 cells play a more critical role in the early stages of the disease, while more abundant Th1 cells are evident in the late resolutive phases (87). Conversely, other investigations have shown that Th1 cells dominate the early inflammatory stages and that a Th17 response occurs later in the clinical course (100).

The Th1 cytokine, IFN-ɣ, seems to serve a protective role in EAU, suggesting that IL-17 has an important inflammatory effect given that IFN-ɣ inhibits IL-17 expression through Th17 suppression. In a study addressing the different functions of Th1 and Th17 cells in EAU, IL-17-/- mice showed no difference in terms of uveitis severity concerning the early stages of the disease and, after anti-IFN-ɣ and anti-IL-4 antibody treatment and the concomitant increase in Th17 expression, only the late stages of the disease were affected, showing the aforementioned differential response of Th1 and Th17 cell subsets during the clinical course of EAU (100).

In another study concerning the monophasic and relapsing phases of EAU, the authors concluded that IFN-ɣ-producing cells might be responsible for initiating recurrence in the relapsing form of EAU. Conversely, IL-17-producing cells might be implicated in intraocular inflammation's primary mechanisms, thus exhibiting different roles in the monophasic and relapsing forms of the disease (102).

Previous data have also shown that even though a Th1- or Th17-driven response can initiate uveitis in an animal model, IL-17 plays a critical role in the induction of EAU; moreover, anti-IL-17 treatment can reduce the severity of antigen-induced autoimmune uveitis in mice. The same authors suggested that IL-23 may be a key element in the early stages of intraocular inflammation and that its function may even surpass the expansion and activation of Th17 cells.

Furthermore, there seemed to be an exacerbation of the Th17 response and increased disease severity with inhibition of the IL-12–IFN-ɣ pathway, reinforcing the previously described regulatory activities of these Th1 cytokines in Th17 differentiation (88).

3.1.2 Non-infectious Uveitis

Increased IL-17 expression has been proven in several studies measuring its aqueous humour or peripheral blood concentration in non-infectious uveitic disorders (Table XIV) and other autoimmune diseases as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, systemic lupus erythematosus, and psoriasis.

In a study measuring IL-15, IL-17, IFN-ɣ, TNF-α, and IL-10 levels in the aqueous humour of patients with active autoimmune uveitis from different etiologies, including Behçet's disease, Vogt–Koyanagi–Harada (VKH) disease, and HLA-B27-associated-uveitis, IL-17 levels were found to be higher in these individuals than in control subjects, and these levels were correlated with disease activity (103).

These results were confirmed by another study that measured IL-17 levels in the peripheral blood of a large group of patients with autoimmune uveitis with and without associated systemic disease.

IL-17 levels were elevated in the serum of uveitis patients compared to controls, and they also served as a marker for disease activity (104). Both studies suggest that IL-17 may be used as a possible biomarker in autoimmune uveitis.

3.1.3 Infectious Uveitis

IL-17 has been implicated in the development of toxoplasmic encephalitis in an animal model, and since then, there have been studies that have analyzed its role in the induction and maintenance of intraocular inflammation caused by an infectious agent. In a previous study, upregulation of IL-17 levels in mice chronically infected with *Toxoplasma gondii* that lacked the IL-27 receptor was observed, and the authors suggested a protective role of IL-27 in the inflammation that follows toxoplasmic infection (105). Moreover, the inflammatory roles of Th1 and Th17 cytokines and the regulatory roles of IL-10, TGF-β, and IL-27 in the immunologic responses following toxoplasmic infection have been reinforced in a later study concerning cytokine regulation in *T. gondii* infection (106).

Table XIV. Increased IL-17 expression in several autoimmune uveitic disorders.

Systemic or intraocular autoimmune disorder associated with uveitis	Main conclusions	Authors, year of publication
VKH	Elevated IL-23 serum expression and upregulated IL-17 expression in peripheral blood mononuclear cells (PBMCs) and CD41+ T-cells from VKH patients with active uveitis.	Chi W. *et al.*, 2007 (107)
VKH	Decreased IL-27 expression is associated with an elevated IL-17 response in active VKH patients. Increased Th17 and IL-17 expression in PBMCs from active VKH patients.	Wang C. *et al.*, 2012 (108)
VKH	Treatment with cyclosporine A and corticosteroids in VKH disease suppresses IFN-ɣ and IL-17 expression, which, in turn, correlates with disease activity.	Liu X. *et al.*, 2009 (109)
VKH	Increased IL-21 and IL-17 expression in VKH patients with chronic or recurrent disease. Recombinant IL-21 stimulated IL-17 production via PBMCs in VKH patients.	Li F. *et al.*, 2010 (110)
Birdshot retinochoroidopathy (BSRC)	IL-21, IL-23, and TGF-β may promote differentiation and expansion of a chronic Th17 cell response in BSRC patients.	Yang P. *et al.*, 2013 (111)
BSRC	Elevated IL-17 intraocular expression in BSRC.	Kuiper J.J. *et al.*, 2011 (112)
Behçet's disease (BD)	Elevated levels of IL-23, IL-17, and IFN-ɣ by PBMCs and increased frequencies of IL-17- and IFN-ɣ-producing T-cells in BD patients with active uveitis.	Chi W. *et al.*, 2008 (113)
BD	Levels of IL-17- and IFN-ɣ-producing CD4+ T-cells are elevated in the peripheral blood of BD patients.	Shimizu J. *et al.*, 2011 (114)
BD	IL-17 expression is elevated in BD patients with active uveitis. Anti-TNF-α therapy inhibits Th17 cell differentiation and may ameliorate intraocular inflammation in BD.	Sugita S. *et al.*, 2012 (115)
HLA-B27-associated uveitis	Elevated IL-17 and IFN-ɣ expression in the peripheral blood of HLA-B27-associated uveitis patients. The increase in Th17 cells and IL-17 may correlate with disease activity.	Zou W. *et al.*, 2014 (116)

One study analyzed the inflammatory cytokine and chemokine levels in the aqueous humour of patients with toxoplasmic and viral uveitis. The authors found that IL-17 was upregulated in most screened patients with toxoplasmic uveitis when compared to cataract patients without uveitis or with those with non-infectious intermediate uveitis. The authors suggested that the presence of elevated levels of IL-17 in the intraocular fluids of these patients may be a clue to a possible autoimmune mechanism that contributes to ocular inflammation following infection (117). These findings were reinforced in a study addressing various cytokine levels in the aqueous humour of patients with uveitis from various etiologies – infectious and non-infectious. Patients with toxoplasmic infection and subsequent uveitis had significantly increased intraocular levels of IL-17 (118).

Another recent study highlighted the upregulation of IL-17 expression in patients with acute ocular toxoplasmosis and demonstrated a pathogenic role for this cytokine in developing intraocular inflammation after *T. gondii* infection. The authors proposed a possible *in vivo* therapeutic approach for toxoplasmic retinochoroiditis based on the use of local anti-IL-17 antibodies (119).

3.1.4. IL-17 Induction

Several studies have shown that TGF-β and IL-6 are essential cytokines in Th17 differentiation and that IL-23 and IL-21 may also be crucial in Th17 cell expansion and activation (Table XV).

3.1.5. IL-17 Suppression

IFN-ɣ

Antigen-specific IL-17 production is exacerbated in the absence of IFN-ɣ, increasing the severity of uveitis in a murine model of spondyloarthritis. In this model, IL-17 blocking ameliorated intraocular inflammation in IFN-ɣ KO mice, attesting to the regulatory function that this cytokine plays in IL-17 production (120). Moreover, IL-17 expression was increased in the peripheral blood of patients with uveitis and scleritis, and also in an EAU model. In this same study, IFN-ɣ upregulated IL-27 expression by retinal cells, which, in turn, inhibited Th17 cell proliferation, reducing uveitis severity, and altering its clinical course (87). Another study involving an EAU animal model also showed that IFN-ɣ ameliorated uveitis through Th1 and Th17 cell inhibition and IL-10 upregulation (121).

Table XV. Cytokines involved in IL-17 induction.

Cytokines involved in IL-17 upregulation	Main conclusions	Authors, year of publication
TGF-β, IL-6, IL-23	IL-23 may expand the Th17 cell population, but it cannot induce this subset's differentiation from T-cell precursors. TGF-β induces Treg differentiation. The association between TGF-β and IL-6 induces Th17 cell differentiation.	Bettelli E. *et al.*, 2006 (96)
IL-6	IL-6 signaling blockade inhibits Th17 differentiation and induces antigen-specific Treg formation in EAU.	Haruta H. *et al.*, 2011 (122)
IL-6	Early treatment with an anti-IL-6 receptor monoclonal antibody ameliorated mice EAU and inhibited Th17 cell differentiation.	Hohki S. *et al.*, 2010 (123)
IL-6, IL-23	EAU induction is impaired in IL-6 and IL-23 knockout (KO) mice. Blocking of IL-6 and IL-23 ameliorates the clinical course of EAU through interference with Th17 cell differentiation and expansion. The anti-IL-6-receptor antibody ameliorates EAU by suppressing Th17 responses.	Yoshimura T. *et al.*, 2009 (124)
IL-21	EAU induction is impaired in IL-21 KO mice. There is decreased IL-17 expression in IL-21 KO mice. IL-21 signaling blockade inhibits IL-17 upregulation in EAU.	Wang L. *et al.*, 2011 (125)
IL-21	IL-21 and its receptor are upregulated in mouse EAU with increased IL-17 expression.	Liu L., *et al.*, 2009 (126)
IL-23	IL-23 is upregulated in the peripheral blood of VKH patients before and after cataract surgery; it is also correlated with intraocular inflammation.	Jiang S. *et al.*, 2010 (127)
IL-23	IL-23 and IL-1β induce Th17 expansion and cytokine response.	Wilson N. *et al.*, 2007 (128)
IL-23	IL-23 serum levels are elevated before and after cataract surgery in BD patients. IFN-ɣ and IL-27 serum levels are elevated after cataract surgery in BD patients.	Jiang S. *et al.*, 2011 (129)
IL-23	There is increased IL-17 and IL-23 expression in patients with active BD.	Na S. *et al.*, 2013 (130)

IL-27

IL-27 is a regulatory cytokine capable of inhibiting the differentiation of precursor cells into their Th17 phenotype. By blocking the production of Th17 cells, this cytokine has been implicated in the suppression of experimental autoimmune encephalomyelitis (EAE) and EAU (87, 131). A previous investigation addressing the regulatory cytokines, IL-27 and IL-10, in the development of uveitis found that mice retinal microglia and ganglion cells constitutively expressed IL-27 and that IL-27 production was elevated during uveitis (132).

IL-27 expression was found to be decreased in BD patients with active uveitis (133), and another study demonstrated elevated levels of IL-27 after cataract surgery in VKH patients, indicating that the upregulation of this cytokine during the first month following surgery might serve a protective function in postoperative inflammation (127). Similar results were found in BD patients after cataract surgery, as increased IL-27 serum levels were also evident during the postoperative period. These IL-27 levels correlated both with uveitis severity and IFN-ɣ levels (129).

3.1.6. Therapeutic Targets

Anti-IL-17

An anti-IL-17 monoclonal antibody was used to treat chronic non-infectious uveitis in patients with posterior and anterior segment disease. The treatment featured effects comparable to those of historical control patients with chronic non-infectious uveitis treated with infliximab. Specifically, this treatment was associated with improvements in visual acuity and intraocular inflammation reduction (134).

Another study conducted of an animal model of spondyloarthritis demonstrated that IL-17 blockade reduced intraocular inflammation and peripheral arthritis, although there was suspicion of retinal toxicity (120). Treatment of EAU with the anti-IL-17 antibody in rats also showed a reduction in intraocular inflammation and of T-cell proliferation during disease onset (135).

Secukinumab

Secukinumab, a human monoclonal antibody against IL-17, was used in a Phase III trial to treat chronic non-infectious uveitis associated with Behçet's disease; however, the study's primary outcome was not met (SHIELD study). Some authors have since claimed that the use of secukinumab in chronic uveitis has not been correctly assessed to date, since the other two trials enrolling patients with active and inactive non-infectious uveitis (not associated with Behçet's disease) (INSURE and ENDURE) were interrupted following the termination of SHIELD. In the SHIELD study, although there was no significant difference between the treated patients and controls, there was a reduction in concomitant immunosuppressant drugs and a trend toward a reduction in recurrence (136). Since then, other studies have shown results that highlighted the importance of IL-17 in the pathological mechanisms that lead to uveitis, suggesting that there is still an opportunity for another clinical trial using this or other monoclonal antibodies against IL-17 (or the IL-17 receptor) in its treatment.

3.2 IL-6

IL-6 belongs to a family of cytokines including IL-11, IL-31, ciliary neurotrophic factor (CNTF), Cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), osteopenia (OPN), and oncostatin M (OSM). It functions by binding either a cell surface (IL-6R) or a soluble IL-6 receptor (sIL-6R) (84). The binding of IL-6 to its receptor results in the activation of the JAK-STAT (JAK1, JAK2, STAT3) and mitogen-activated protein kinase (MAPK) pathways, ultimately leading to the expression of inflammatory cytokines, vascular endothelial growth factor (VEGF), and differentiation of naive $CD4^{+}$T cells into Th17 cells (137).

In EAU, treatment with an anti-IL-6 receptor antibody or, alternatively, EAU induction in IL-6-deficient mice results in dramatically reduced uveitic inflammation (124). Elevated levels of IL-6 have been detected in the aqueous humour (AqH) of patients with BD, VKH, sarcoid, idiopathic uveitis, acute retinal necrosis (ARN), and HLA-B27-associated uveitis when compared with controls (138-140). The uveitic disorders associated with dysregulated IL-6 or IL-6 receptor are listed in Table XVI.

Table XVI. Uveitic disorders associated with dysregulated IL-6 or IL-6 receptor (141-143).

Vogt–Koyanagi–Harada Syndrome

Toxoplasmosis
Fuchs Heterochromic Uveitis
Behçet Disease
Sarcoidosis
Idiopathic uveitis

Tocilizumab (Genentech, South San Francisco, CA, USA) is a monoclonal antibody against soluble and membrane-bound IL-6 receptor, and it has been used successfully in case reports in JIA uveitis refractory to prior anti-TNF-α therapy (144).

A previous study involving eleven eyes from seven patients with uveitic CME due to Birdshot choroidopathy, JIA, and idiopathic panuveitis showed improved visual acuity and central foveal thickness after treatment with tocilizumab (145). The STOP-Uveitis is a randomized, open-label safety, efficacy, and bioactivity clinical trial which evaluated the role of tocilizumab in patients with NIU. This study included 37 uveitis patients, and results at 6 months showed that tocilizumab (both 4 and 8 mg/kg) effectively improved visual acuity and reduced vitreous haze and central macular thickness in eyes with posterior NIU (146).

3.3 IL-23

IL-23 is a member of the IL-12 superfamily of cytokines. This family includes IL-12, IL-23, IL-27, and IL-35. The IL-23 receptor is composed of the IL-12Rb1 and IL-23R subunits that signal through tyrosine kinase 2(Tyk2), JAK2, STAT3, and STAT4 upon binding IL-23 (147).

In the context of inflammation, it is primarily produced by antigen-presenting cells such as dendritic cells and macrophages, and multiple lines of evidence point to IL-23 as the critical checkpoint used by naïve T cells to decide whether to become a homeostatic Th17 or pathogenic Th17 effector cell (88, 148), which means that in the absence of IL-23, Th17 cells can still develop, but do not become pathogenic.

As for its role in NIU, increased IL-23 levels have been found in the serum and the supernatants of peripheral blood mononuclear cells from patients with active VKH and Behçet's uveitis compared with patients with inactive uveitis and normal control subjects (107, 113).

Table XVII lists uveitic disorders associated with increased IL-23 levels or with single nucleotide polymorphisms (SNPs) in the IL-23R gene.

Table XVII. Uveitic disorders associated with increased IL-23 levels or single nucleotide polymorphisms (SNPs) in the IL-23R gene.

Przepiera-Bedzak *et al* (149)	Increased serum levels of IL-23 are associated with an increased risk of AAU in patients with spondyloarthropathy.
Velez *et al.* (150)	Increased IL-23 levels in the vitreous obtained from patients with posterior uveitis compared with non-uveitic control subjects.
Yang and Foster (111)	Elevated serum levels of IL-23 in patients with treatment-naïve Birdshot Choroidopathy.
SNPs in the IL-23R gene associated with an increased risk of inflammatory disease	In Ankylosing Spondylitis -associated uveitis (151).
	In Sarcoid uveitis (152).

AAU: acute anterior uveitis. SNP: single nucleotide polymorphisms.

Ustekinumab, which targets the p40 subunit shared by both IL-23 and IL-12, has been reported to effectively control uveitis in patients treated for coexisting severe psoriatic arthritis and plaque psoriasis (153) or BD (154). It is currently being studied in a phase II clinical trial to treat patients with uveitis - STELARA (clinicaltrials.gov, NCT02911116). In the STELABEC trial, ustekinumab will be tested in patients with BD and active posterior or panuveitis (clinicaltrials.gov, NCT02648581).

3.4 TNF-α

TNF-α is a member of the tumour necrosis factor family, which includes TNF-α, lymphotoxin, B-cell activating factor (BAFF), and a proliferation-inducing ligand (APRIL). TNF Receptor 1 (TNFR-1) is the primary receptor for either form, and it is ubiquitously expressed on all cells. TNFR-2 is only expressed on immune cells and only responds to membrane-bound TNF-α (84). It has both a membrane-bound form and a soluble form. These two forms have distinct actions in chronic infection, and the selective targeting of different pathways shows some promise in selectively ameliorating inflammation in autoimmunity while preserving antibacterial potency.

This cytokine is well known as a key mediator of inflammation and plays a crucial role during the early phase of a host's defence against bacterial, viral and parasitic infections, although high systemic levels of TNF-α can lead to vascular decompensation and death (155). It is produced by many cell types, including macrophages, monocytes, lymphocytes, mast cells, polymorphonuclear leukocytes, keratinocytes, astrocytes, microglial cells, intestinal paneth cells, and tumour cells (156). During inflammation, TNF-α is produced by Th1 cells in copious amounts as well as by inflammatory macrophages, and it acts synergistically with other cytokines, such as IFN-ɣ and IL-17, propagating the pro-inflammatory state (157, 158).

In the EAU model, neutralization of TNF-α suppresses disease (157), and mice deficient in TNFR1 are resistant to the development of uveitis (159). Moreover, one previous study analyzed the AqH levels of various pro-inflammatory cytokines in patients with NIU, including patients with VKH, BD, and HLA-B27-associated uveitis, and found an elevated TNF-α expression that correlated significantly with disease activity (103). When analyzing patients with idiopathic and HLA-B27-associated anterior uveitis, another study also found increased TNF-α levels in the AqH. However, in patients with intermediate uveitis from various causes, only serum TNF-α levels were significantly higher in patients with no difference from controls in AqH samples (160).

TNF-α off-label treatment of NIU patients led to expert-panel recommendations for their use in ocular inflammatory disorders (161). In this study, a panel of uveitis experts found good-quality evidence for recommending treatment with anti-TNF-α agents (infliximab and adalimumab) for ocular manifestations of BD, Juvenile Idiopathic Arthritis, and Spondyloarthropathy-associated or HLA-B27-associated Uveitis.

Since then, the VISUAL I and II trials subsequently led to adalimumab approval for treatment of patients with NIU (162, 163). The VISUAL I trial showed that in patients with active non-infectious intermediate uveitis, posterior uveitis, or panuveitis treated with adalimumab, the median time to treatment failure was 24 weeks versus 13 weeks in the placebo group, with a lower risk of uveitic flare or visual impairment than placebo.

Golimumab, a more recent anti-TNF-α agent, also showed success in treating NIU in several retrospective case studies (164, 165).

Etanercept is another well-known anti-TNF-α agent that has been successfully used in several autoimmune diseases. However, experts do not recommend its use to treat ocular inflammatory disease since there have been reports of the development of sarcoid-like uveitis in patients treated with etanercept (161).

3.5 IFN-ɣ

Interferon-gamma (IFN-ɣ) was one of the first recognized cytokines and is known to play well-characterized roles in immune responses to microbial infection (166). However, it also plays a role in the pathogenesis and development of autoimmune disease, and it is now clear that it is more accurately described as a modulator of inflammation since it seems to be capable of both pro- and anti-inflammatory activities, depending on the biological context. IFN-ɣ is the only characterized Type II interferon, as defined by its interaction with the IFN-ɣ receptor (IFNɣR). This receptor consists of a complex of two receptor chains, designated IFNɣR1 and IFNɣR2 (167). One of the important transcription factors expressed due to IFNɣR signaling is T-bet, the transcriptional regulator necessary for inducing and maintaining an interferon-gamma producing, Th1, differentiation state in CD4+ helper T cells (166). IFN-ɣ signaling acts to modulate the expression of several cytokines, including IL-12, which is a vital component of IFN-ɣ production in both innate and adaptive immune compartments and also drives helper cell differentiation towards a Th1 phenotype. Although it has been shown that IFN-ɣ decreases T cell differentiation to Th2 and Th17 lineages (168, 169), it is now clear that IFN-ɣ production can occur concurrently with IL-17 expression with pathogenic consequences (170, 171).

In a study measuring pro-inflammatory cytokine levels in the AqH of patients with active uveitis associated with BD, VKH, and HLA-B27 (103), patients with BD showed higher intraocular IFN-ɣ levels than patients with VKH or HLA-B27-associated disease. These results were consistent with another study, which also showed that levels of IFN-ɣ were significantly higher in the AqH of BD patients than those found in NIU from other causes (172). The authors concluded that, in BD, there may be a more extensive Th1 polarization in the intraocular cytokine environment. Table XVIII lists autoimmune diseases with an association with an IFN-ɣ dysregulation.

Table XVIII. Autoimmune Diseases associated with an increased IFN-ɣ expression.

Multiple Sclerosis (MS)	Examination of lymphocytes from MS patients' blood demonstrated increased IFN-ɣ production (173).
Systemic lupus erythematosus (SLE)	Deficiency of IFN-ɣ signaling In SLE models ameliorates disease (174).
Sjögren's syndrome (SjS)	IFN-ɣ deficiency ameliorates disease in SjS mouse models (175).

3.6 IL-10

IL-10 is the founding member of the IL-10 cytokine superfamily, which also includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29 (176). It was initially described as a secreted cytokine synthesis inhibitory factor (CSIF) produced by Th2 T cell clones, which inhibits the production of several cytokines from Th1 cells (177). There is a wide variety of cells of the innate and adaptive arms of the immune system capable of IL-10 expression, including macrophages, monocytes, dendritic cells (DCs), mast cells, eosinophils, neutrophils, natural killer (NK) cells, CD4 + and CD8 + T cells, and B cells (178). The IL-10 receptor (IL-10R) is a tetramer consisting of two α and two β subunits, where α are responsible for binding IL-10, and β is part of signal transduction. Like many other cytokines, receptor signaling is mediated by the JAK-STAT pathway, in this case, through JAK1, STAT1, and STAT3 (179).

Consequently, it can inhibit the production of proinflammatory cytokines, antigen presentation, and cell proliferation (180, 181). IL-10 performs these regulatory functions by binding to a specific cell surface receptor (IL-10R) that is made of two chains, IL-10R1 and IL-10R2.

The binding of IL-10 to IL-10R1 leads to conformational changes in IL-10 that allows its association with the IL-10R2 and the generation of IL-10/IL-10R complexes.

These complexes can suppress immune responses by multiple mechanisms, but inhibiting nuclear translocation of the NF-κb and its DNA-binding activity is considered the main one (182). IL-10 also inhibits major histocompatibility complex class II expression, limiting co-stimulation and reducing proinflammatory cytokine production by antigen-presenting cells (APC), particularly dendritic cells and macrophages (183).

Different studies have reported different IL-10 levels in the AqH and sera of patients with uveitic disorders, including non-infectious and infectious uveitis. Animal studies demonstrate the protective role of local IL-10 in uveitis, both in EAU and endotoxin-induced uveitis (EIU) (184-186). In patients with uveitis from various causes, elevated intraocular IL-10 levels have been identified (160, 187) and are attributed to the presence of regulatory mechanisms activated along with inflammation. The presence of consistently elevated levels of IL-10 in the AqH of IU patients, particularly in viral uveitis (118, 188-190), may be related to its ability to potently suppress the cellular immune response and increase the host susceptibility to infection. Increased IL-10 is thought to play an essential part in persistent infections caused by viruses, bacteria, and parasites, and it is known that Cytomegalovirus (CMV) and Epstein Barr virus (EBV) can induce IL-10 synthesis and encode their own IL-10 homologs (191-193).

The evaluation of intraocular IL-10 levels can be particularly helpful when there is a suspicion of Primary Intraocular Lymphoma. In these cases, an elevated IL-10/IL-6 ratio strongly suggests this diagnosis (194). Table XIX lists uveitic diseases that have been associated with an IL-10 dysregulation.

3.7 TGF-β

TGF-β1 is part of the TGF-β superfamily members, collectively coded by 33 different genes in mammals (195). The family comprises the three TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3; activins (Act); bone morphogenetic proteins (BMPs) and/or growth differentiation factors (GDFs) (196), which are all dimers. TGF-β1 is a homeostatic factor that keeps the immune system in balance and orchestrates the complex tissue repair processes following injury or infection in all organs (197).

Table XIX. Infectious and Non-Infectious Uveitic disorders associated with altered IL-10 levels.

Non-Infectious Uveitis (NIU)	Idiopathic Uveitis (198)	Low IL-10 levels in the AqH of patients.
	Fuchs' Uveitis Syndrome (199)	High IL-10 levels in the AqH of patients.
	VKH, BD, HLA-B27-associated (103)	Increased IL-10 levels in the AqH of patients and a positive correlation with disease activity.
Infectious Uveitis (IU)	ARN and Toxoplasma Chorioretinitis (188)	High IL-10 levels in patients with uveitis.
	Laboratory confirmed intraocular infection (not specified)(190)	IU Patients had higher levels of IL-10 than NIU patients.
	Herpetic Uveitis (189)	Significantly increased IL-10 levels in the AqH of IU patients compared with those with NIU.
	Toxoplasmosis and viral uveitis (HSV, HZV, and CMV) (118)	Increased IL-10 levels in viral uveitis, whereas IL-17A levels were augmented in toxoplasmic uveitis.

ARN: Acute Retinal Necrosis; AqH: Aqueous humour; HSV: Herpes Simplex Virus; HZV: Herpes Zoster Virus; CMV: Cytomegalovirus.

It is involved in about every aspect of the immune system and in regulating inflammation, either by inducing direct actions or by setting baseline states in immune cells that determine the reaction to other signaling events (200). It has a role in the development and survival of the T cell population in the thymus, such as CD8+ T cells, CD4+/CD25+/FoxP3+ T regulatory cells (Treg), intraepithelial lymphocytes, and invariant natural killer T cells. It becomes critical in suppressing autoreactive T cells that escape the thymus's negative selection process and manage to circulate to the periphery (201).

TGF-β is also vital for maintaining an inhibitory ocular microenvironment. Regarding its isoforms, there is very little to no detection of the other isoforms, other than TGF-β2, in healthy AqH (202). Macrophages treated with TGF-β2 express the characteristics of the aqueous humor-treated macrophages that mediate ACAID (Figure 2), (203). These TGF-β2 treated macrophages increase their F4/80 surface expression while reducing the expression of co-receptors for T cell activation (204, 205) and expressing TGF-β1 (206).

Moreover, TGF-β2 triggers the expression of FOXP3 in CD4+T cells and subsequently converts them to FOXP3+T regulatory cells (Tregs) (207, 208). A recent study showed an inhibitory role of TGF- β2 in EAU, given that treatment with TGF-β2 during the early stages of intraocular inflammation completely suppressed the immune response by inhibiting CD4+T cell proliferation and generating Tregs (209). The authors suggest that recombinant TGF-β2 could be a successful therapeutic agent for local treatment of human idiopathic anterior uveitis.

4. Regulatory T cells

Regulatory T cells (Tregs) are a cluster of CD4+T cells, which can be classified into two main types: thymus-derived or “natural” Treg cells and peripheral or “adaptive” Treg cells (210). So far, no distinct functional differences have been conclusively demonstrated between thymus-derived and peripheral Tregs, suggesting that the mechanistic repertoire of Treg function is specified by lineage and not mode of induction (211). Nonetheless, typically natural Tregs act mainly by contact with target cells through their surface markers, and peripheral Tregs act mostly by cytokine production. They can be mobilized in a variety of immune responses - including allergy, autoimmunity, inflammation, and tumoral immunity (212).

Natural Tregs are characterized as expressing both the CD4 T-cell co-receptor and CD25, a component of the IL-2 receptor. IL-2 and its CD25 receptor are vital for developing and maintaining a Treg pool (213). Tregs are, thus, phenotypically characterized as CD4+ CD25+. Moreover, they also express intracellularly a specific marker called FOXP3.

Peripheral Tregs are characterized by TGF-β and IL-10 production and play an essential role in maintaining T cell homeostasis and self-tolerance (214, 215)**,** particularly in peripheral immune tolerance (216).

FOXP3, also known as scurfin, is a protein involved in immune system responses, and it serves as a critical factor for the thymic development of CD4+CD25+ regulatory natural T-cell (217). It is critically important for the development and function of thymic Tregs (218), and it keeps the cells on the right developmental track toward a suppressive phenotype, being considered a prerequisite for stabilizing the Treg lineage (219). Furthermore, loss of FOXP3 expression impairs the suppressive activity of Tregs (217). It has been extensively used as a marker for CD4+CD25+ Tregs, distinguishing them from activated CD4+CD25–T cells.

However, FOXP3 detection requires intracellular staining, which limited the applicability of this phenotype to cell research. Later, a negative correlation between the IL-7 receptor (CD127) expression and FOXP3 expression was found, further characterizing Tregs as low CD127-expressing cells (220).

FOXP3+CD4+ non-Tregs are characterized by the absence of cell surface expression of CD39 and IFN-γ, IL-2, and IL-17 expression (221). CD39 is an ectonucleotidase that cleaves ATP to form AMP, which is then be cleaved by CD73 to form adenosine. Since extracellular ATP has a known proinflammatory effect, its cleavage to AMP and subsequent adenosine formation will induce a suppressive immune response (222).

CD39 has been reported on the surface of naive human Tregs, and it has been confirmed that it is predominantly expressed on CD4+ FOXP3+ T cells with an expression level that is proportional to FOXP3 (223). There is a growing body of evidence implicating CD39 expression as vital for natural Tregs' suppressive function (223, 224). A study analyzing Treg CD39 expression in patients with inflammatory bowel disease (IBD) found that although the total numbers of circulating FOXP3+ Treg cells did not differ between controls and patients with active IBD, there was significantly lower CD39 expression by Treg cells in active IBD (225). In patients with relapsing-remitting multiple sclerosis (MS), CD39+ Tregs isolated from patients have been shown to have impaired suppressive activity over the Th17 response (221), and RA patients with higher frequencies of circulating CD39+ Tregs were associated with a positive clinical response to MTX (226).

Moreover, some studies suggest that the expression of CD39 by human Tregs (222) is restricted to a subset of T regulatory effector memory cells (221) and that these cells are particularly capable of suppressing IL-17 production.

CD39 expression is intimately associated with CD4+CD25+FOXP3+ signatures and the acquisition of a memory (CD45RO+/RA-) phenotype. Furthermore, FOXP3 appears to drive CD39 expression as evidenced by retroviral transduction of CD4+CD25− T cells with FOXP3 (222). Other studies have also demonstrated amplification of the CD39 gene by FOXP3 (219). These findings suggested that the CD39 surface marker can be successfully used for routine isolation of functionally active human Tregs.

Recent studies have succeeded in dividing human Tregs into more homogenous subsets based on cell-surface marker expression. Dividing human Tregs into distinct subsets has been fruitful for improving our understanding of basic human Treg biology and will likely be critical to future studies of the role of human Tregs in autoimmune disease.

The most common approach to defining human Treg subsets is based on combining CD25 and CD127 expression with the expression of the classic markers for memory (CD45RO) and naïve (CD45RA) conventional T cells (227, 228).

This further isolated two suppressive FOXP3+Treg populations that are distinct in phenotype and function: CD25+CD127lowCD45RA+FOXP3+ (naïve Tregs) and CD25highCD127lowCD45RO+ FOXP3+ (memory Tregs).

Memory Tregs express the highest levels of CD25, FOXP3, and other markers of activated T cells, exhibit the most substantial suppressive capacities, exhibit hyporesponsiveness and undergo apoptosis in response to TCR stimulation (227, 229, 230).

Naïve Tregs express CD25 and FOXP3 at lower levels, exhibit weaker suppression, substantial proliferation, and resistance to apoptosis in response to TCR stimulation (227, 228, 230). Compared to naïve Tregs, human memory Tregs are more activated and more fully differentiated in their ability to perform strong suppression (230). A prospective study of Treg phenotype from human infants has provided considerable evidence to support this model by demonstrating naïve Treg conversion into memory Tregs starting around 18 months (231).

Memory Tregs cells seam to maintain long-lived tolerance to self-antigens and have been identified on mouse models in which self-antigens' expression could be turned on and off, showing the persistence of antigen-specific Treg cells even after antigen elimination (232). Human T-cells express the RO isoform of CD45 in the thymus and convert to CD45RA upon migration to peripheral tissues, switching back to CD45RO after antigen recognition and TCR stimulation (233). Therefore, circulating memory T-cells are phenotypically characterized as CD45RA-/CD45RO+.

Miyara *et al.* demonstrated that human FOXP3+Tregs could be further divided into three phenotypically distinct subpopulations: CD45RA+FOXP3(lo) resting Treg cells, CD45RA-FOXP3(hi) activated Treg cells, both demonstrating in vitro suppressive capacity, and CD45RA-FOXP3(lo) non-suppressive T cells (227). However, the role and characterization of memory Tregs in the physiopathology of autoimmune disease and the importance of CD39 expression on memory Tregs need to be further elucidated.

In Type 1 diabetes (T1D) patients, a study by Jin *et al*. found that patients had higher memory Tregs percentages than controls but showed impaired suppression with low FOXP3 and CD39 expression (234). These study results are in accordance with a previous study, also involving children with T1D, in which a lower expression of CD39 within Tregs was also found in patients (235).

The authors concluded that an impaired suppressive function of Tregs in T1D patients was due to a reduced CD39 expression on their memory subpopulation.

4.1 Tregs in Autoimmune Diseases

Several studies have analyzed circulating regulatory T cell frequencies in autoimmune diseases like Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Inflammatory Bowel Disease (IBD), Autoimmune Thyroid Disease (ATD), and T1D. However, there has been some discrepancy in results since there are reports documenting decreased, normal, and increased circulating Treg frequencies relative to health.

The reasons behind the reported inconsistencies are not fully understood. One potential explanation for the observed discrepancies is a lack of standardization between studies due to the absence of a uniformly recognized marker, which reliably defines a homogenous human Treg population (236). Table XX lists some of the autoimmune disorders associated with an altered circulating Treg frequency.

4.2 Tregs in Non-Infectious Uveitis

In NIU, a decrease in Treg frequency has been previously demonstrated (91) and has been associated with various causes of NIU, which include BD (92), VKH (93), and HLA-B27-associated anterior uveitis (237). Table XXI lists some of the uveitic disorders associated with a decrease in circulating Treg frequency or dysregulation. However, other studies failed to show this disease-associated reduction (238, 239), with one study reporting significant reductions in FOXP3+Treg levels in both controls and uveitis patients after *in vitro* dexamethasone treatment (238).

The Th17/Treg ratio has also been studied in NIU patients showing an elevation during active intraocular inflammation in HLA-B27-associated uveitis (237) and a positive correlation with disease activity score. Such observations suggest that both the elevation of Th17 cells and the reduction of the Treg subset may contribute to disease activation and progression. However, this may not be the case for all the uveitic conditions causing NIU since a recent study involving Birdshot Chorioretinopathy (BSCR) patients showed that the proportion of $CD4^{+}CD25^{hi}CD127^{low}$cells was higher in treated and untreated BSCR patients than in controls. Additionally, Th17 and $CD4^{+}CD25^{hi}CD127^{low}$cells levels were not significantly different between treated and untreated patients (240).

Table XX. Autoimmune diseases associated with an altered circulating Treg frequency.

SLE	Decreased CD4+CD25+ T cell frequency. (241)
	Normal frequency of the $CD4+CD25^{hi}$ subset in active disease. (242)
	Levels of circulating Tregs cells are reduced during flares. (243)
MS	Decrease in the relative frequency and suppressive function of $CD4^{+}CD25^{+}CD127^{low}FoxP3^{+}CD39^{+}$ Treg cells. (221)
	Decreased FOXP3 levels in Tregs may contribute to MS. (244)
	The frequency of Tregs in the peripheral blood did not differ between MS patients and healthy donors. (245)
RA	The frequency of peripheral blood Treg cells in patients with RA was significantly low compared with that of healthy controls. (246)
	Decrease in the $CD4+CD25^{high}$ regulatory T-cell population in the peripheral blood of individuals with early active RA prior to disease-modifying treatment. (247)

ATD	No differences were found in the frequency of Tregs as a percentage of $CD4^+$ cells between ATD patients and controls but patient´s Tregs were less capable of inhibiting the proliferation of T effector cells. (248)
	The proportion of Treg cells and the transcription factor forkhead box P3 (*FOXP3*) mRNA expression is decreased in Graves' Disease patients when compared with healthy subjects. (249)
IBD	Decrease of $CD4^+FOXP3^+$ Treg cells in peripheral blood of patients and an accumulation of Treg cells in inflamed mucosa. (250)
	A decrease in Tregs and an increase in Th17 cells were observed in the peripheral blood of IBD patients. (251)
	$CD4+CD25^{high}$ and FOXP3+ Treg cells are increased during remission but decreased during active disease. (252)
T1D	In contrast to previous studies of T1D patients, similar frequencies of $CD4^+CD25^+$ T-cells were observed in healthy control subjects and patients of similar age. There was no difference between T1D subjects of recent-onset versus those with established disease in terms of their $CD4^+CD25^+$ or T-cell frequency. (253)
	The percentages of CD4+CD25+ and CD4+CD25+Foxp3+ cells both in total lymphocytes and in CD4+ lymphocytes were significantly decreased in patients when compared to controls. (254)

SLE- Systemic Lupus Erythematosus; MS- Multiple Sclerosis; RA- Rheumatoid Arthritis; ATD- Autoimmune Thyroid Disease; IBD- Inflammatory Bowel Disease; T1D- Type 1 Diabetes.

Table XXI. Uveitic disorders associated with a decrease in circulating Treg frequency or dysregulation

NIU	20 patients with intermediate uveitis, posterior uveitis, and panuveitis.	T-regulatory cells are reduced in patients with active compared with inactive disease. (91)
BD	19 BD patients with ocular complications.	Decreased percentages of Treg cells may be a predictive marker of ocular attack in BD patients. (255)
	16 BD patients with refractory uveitis.	The percentage of Foxp3+ cells among CD4+ T cells was significantly decreased in patients with active uveitis compared with patients with inactive uveitis in the remission stage. (92)
VKH	30 patients with active VKH.	A significantly decreased frequency and diminished function of $CD4+CD25^{high}$ Treg cells are associated with active uveitis in patients with VKH syndrome. (93)
BSCR	12 patients, HLA-A29+ and with a history of untreated disease.	The percentage of CD4+CD25+Foxp3+ Tregs was significantly lower in BSCR patients compared with controls. (256)
HLA-B27-associated	20 patients with HLA-B27-associated AAU.	Increase in circulating Th17 cells and a significant decrease of Treg cells in patients compared to controls. (237)

NIU- Non-Infectious Uveitis; BD- Behçet Disease; VKH- Vogt-Koyanagi-Harada; BSCR- Birdshot Chorioretinopathy; AAU- acute anterior uveitis.

The heterogeneity of the different conditions causing NIU and the different strategies used to characterize Tregs may contribute to conflicting results and delay Tregs' possible use as biomarkers or even as therapeutic targets in NIU. Treg therapy in preclinical and clinical studies involves two main approaches: the systemic autologous antigen-specific and the local polyclonal strategies (257).

4.3 Therapeutic strategies

The use of T cells for treating diseases has emerged as an attractive target for new therapeutic strategies of translational medicine. In the last few years, there have been considerable efforts to understand the generation, activation, and mechanisms of action of Tregs, leading to the development of tools and treatment modalities.

Systemic autologous antigen-specific strategy

Only a few clinical studies in humans have been completed. They have been performed mostly in the transplantation setting and in several autoimmune diseases, such as Crohn's disease (CD) (258). In 20 patients with refractory CD, administration of antigen-specific Tregs was well tolerated and had dose-related efficacy based on a reduction in Crohn's Disease Activity Index in 40% of patients. In this study, ovalbumin-specific Treg cells (ova-Tregs) were isolated from patients' peripheral blood mononuclear cells, exposed to ovalbumin, and administrated intravenously. After intravenous administration, ovalbumin-specific type 1 Treg cells were specifically activated by ovalbumin in the gut of patients with Crohn's disease and displayed a bystander regulatory activity witnessed by the decrease of the disease symptoms in responder patients.

A previous study using Col-Treg cells, produced from collagen II-specific T cell receptor transgenic mice, demonstrated that these cells were able to display several modes of actions allowing the dampening of inflammation *in vitro*, such as anti-inflammatory cytokine production (IL-10 and IL-13), myeloid cell cytolysis (through Granzyme B production), and ATP hydrolysis (through CD39 expression) (259). *In vivo* data obtained in a mice model of EAU demonstrated the reduction of several uveitis features, such as vasculitis and vitritis, after intravenous administration of Col-Treg cells.

Local administration of ex vivo-activated polyclonal regulatory T cells

A different treatment strategy involves injecting pre-activated polyclonal Treg cells directly into the site of inflammation. In uveitis, this approach would be dependent on intravitreal injections of activated Tregs. In the eye, a therapeutic effect of Treg cells might be obtained with small cell numbers, without the requirement for *in vitro* Treg expansion. One study showed uveitis suppression after preactivated Polyclonal Treg intravitreal injection (260).

Disease control by these Treg cells was associated with an increase of IL-10 production and decreased reactive oxygen species produced by immune cell infiltrates. Based on these preclinical data, the UVEREG phase I/II clinical trial is currently assessing the safety of *ex vivo*-activated polyclonal Treg intravitreal injection in patients with refractory and end-stage NIU (ClinicalTrials.gov; NCT02494492).

II. AIMS

1. General Aims

1) To characterize Regulatory T cell frequency, subsets as well as their respective CD39 expression in patients with Non-Infectious Uveitis;

2) To characterize cytokine expression in patients with Non-Infectious and Infectious Uveitis.

2. Specific Aims

1) To analyze the peripheral Regulatory T cell subpopulations and respective CD39 expression, as well as inflammatory and anti-inflammatory cytokines in a group of patients with active NIU and compare them with normal controls.

2) To compare the peripheral Regulatory T cell subpopulations and respective CD39 expression, as well as inflammatory and anti-inflammatory cytokines, before and after treatment of NIU.

3) To analyze the local and peripheral inflammatory and anti-inflammatory cytokines in a group of patients with active Infectious Uveitis and compare them with normal controls.

III. MATERIAL AND METHODS

1. Patients

This study was performed in accordance with the Declaration of Helsinki. All patients and controls were recruited from the Ophthalmology Department of Egas Moniz Hospital, West Lisbon Hospital Center, between October 2014 and December 2016. The study protocol was approved by the Ethics Committee of Egas Moniz Hospital, West Lisbon Hospital Center, and by the Ethics Committee of NOVA Medical School. Informed consent was obtained from each patient.

All patients were over 18 years old and were diagnosed with active NIU at the time of inclusion. The diagnosis of active NIU followed the clinical criteria based on inflammatory cell reaction in the anterior chamber or vitreous as per standardization of uveitis nomenclature (SUN) and National Eye Institute (NEI) grading systems (16, 261). Active chorioretinal lesions and vasculitis were evaluated by indirect ophthalmoscopy, fundus autofluorescence, and fluorescein angiography. Any mentioned inflammatory sign (i.e., anterior chamber cell ≥0.5+, vitreous cells ≥0.5+, active retinal vasculitis, or active chorioretinal lesions) was enough to be eligible.

VKH disease was diagnosed according to the criteria established at the International Workshop on VKH disease (55). HLA-B27-associated AAU was diagnosed by HLA typing of peripheral blood cells and typical ocular manifestations (262). BD was diagnosed according to the criteria of the International Team Group for the Revision of the International Criteria for BD (263). Tubulointerstitial nephritis and uveitis syndrome (TINU) was diagnosed based on the reports of Mandeville *et al.* (264) and Mackensen and Billing (265). Patients presenting with active uveitis from a presumed viral/herpetic etiology were included in the infectious uveitis group.

The patient´s blood samples were collected at the time of diagnosis and at least three months after treatment. For patients to be included, uveitis resolution had to be obtained after treatment. For the uveitis to be considered inactive, no inflammatory signs could be present (i.e., no anterior chamber cell reaction, no vitreous cells, and no active retinal vasculitis or chorioretinal lesions).

Data collected from patients included demographic information, diagnosis, anatomical location according to the SUN criteria (16), and disease activity. The control group included sex- and age-matched healthy subjects without any history of autoimmune disease or intraocular inflammation.

Patients presenting with active uveitis from a presumed viral/herpetic etiology were included in the Infectious Uveitis group. At the time of sampling, all patients had active disease, and both blood and AqH samples were collected at presentation. Intraocular samples were also examined for the presence of cytomegalovirus (CMV), herpes simplex virus (HSV)-1 and 2, and varicella-zoster virus (VZV) by real-time polymerase chain reaction (PCR) analysis as previously described (266).

Controls were selected among healthy subjects undergoing cataract or refractive surgery, with no known history of intraocular inflammation.

2. Detecting T lymphocyte subpopulations by flow cytometry

Peripheral blood (10 mL) was collected from patients and controls using Ethylene Diamine Tetra-acetic Acid (EDTA) tubes. Multiparametric flow cytometry was performed. 100 microliters (μL) of all samples were labeled with the antibody combinations in one tube as follows: CD197-BV241 (Clone 150503, BDBiosciences™), CD45-V500 (Clone 2D1, BDBiosciences™), CD45RA-FITC (Clone HI100, Biolegend™), CD25-PE (Clone M-A251, Biolegend™), CD4-PerCP-Cy5.5 (Clone SK3, BDBiosciences™), CD39-PE-Cy7 (Clone A1, Biolegend™), CD127-APC (Clone A019D5, Biolegend™), CD3-APC-H7 (Clone SK7, BDBiosciences™).

All incubations were performed at room temperature in the dark. First, cells were incubated with appropriately titrated, fluorescently labeled antibodies for 15 minutes. Cell suspensions were then lysed and fixed with 2mL of BD FacsLyse (BD Biosciences) for ten minutes and centrifuged for 300 seconds at 1200 x g. The supernatant was aspirated and discarded. The cells were then washed with 2 mL of CellWash (BD Biosciences) and resuspended with 500 μL of BD FacsFlow (BD Biosciences) before the acquisition. More than 400,000 events were typically acquired on a 3-laser BD FACSCanto II flow cytometer (BD Biosciences) with BD FacsDiva software for each measurement. Set up and validation of the method included tube replicates in some samples.

The flow cytometry data were analyzed using Infinicyt 2.0 software (Cytognos, Spain). A unique template and strategy of analysis were created and applied to all the files to improve reproducibility.

The analysis of the CD4+T-cells included the following subpopulations: Tregs (CD3+CD4+CD25hiCD127lowcells), naïve CD4+ T-cells (CD3+CD4+CD45RA+CD197+), memory CD4+ T-cells (CD3+CD4+CD45RA-CD197+/-) and effector CD4+ T-cells (CD3+CD4+CD45RA+CD197-). Tregs were defined as CD3+CD4+CD25hiCD127lowcells. Naïve and memory Treg subsets were also identified as well as their respective CD39 expression according to the above described phenotypes (Fig. 4).

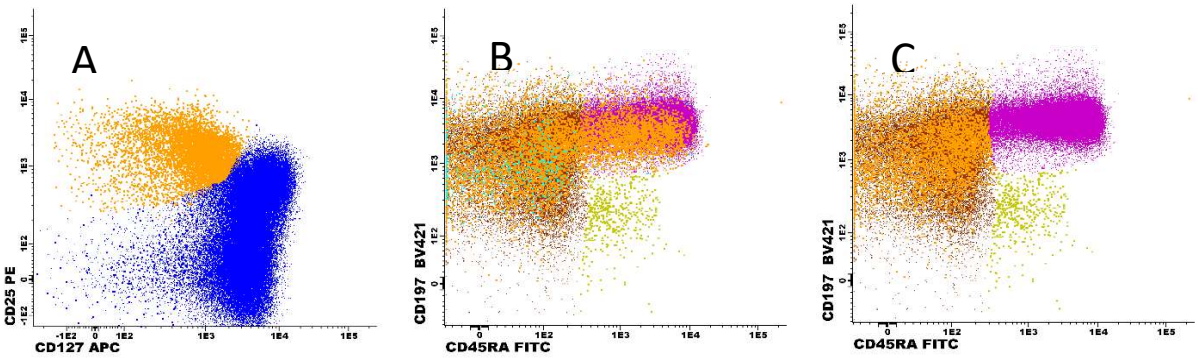

Fig. 4 - A. CD4+CD3+ auxiliary T-cells in red, CD4+CD3+CD25hiCD127low Treg population in violet. B. Naïve (CD45RA+CD197+) CD4+ T-cells in purple, memory (CD45RA-CD197+/-) CD4+ T-cells in brown, and effector (CD45RA+CD197-) CD4+ T-cells in green. The Treg population in gold is mostly concentrated in the memory subset. C. The CD39+ Treg cell subset in turquoise overlaying the previously displayed subsets.

Cells were assessed in absolute counts and percentage values (CD4+-T cells as a percentage of total CD3+-T cells; naïve, memory, effector, and Treg subsets as a percentage of total CD4+-T cells; and naïve and memory Tregs, as a percentage of total Tregs).

For each sample, the absolute cell counts were calculated by multiplying each population's fraction by the total white blood cell count derived from the complete blood count.

3. Quantification of serum cytokine levels by multiplexed flow cytometry

A multiplex bead-based immunoassay (BD CBA Flex Set, BD Biosciences) was used to determine the serum and AqH levels of TNF-α, IFN-ɣ, IL17A, and IL10. A similar single-plex bead-based immunoassay was used for TGF-β.

The protocol was performed following the instructions of the manufacturer. In brief, standards and serum samples were incubated with specific capture beads for one hour at room temperature. After adding the detection reagent, the mixtures were incubated for two hours at room temperature in the dark. After a final wash, beads were acquired in a BD FACS Canto II (BD Biosciences), previously set up for the BD CBA Flex Set. For each cytokine, a minimum of 300 beads was acquired per sample. The FCAP Array Software (BD Biosciences) was used for data analysis. Standard curves covered a 0–2,500 pg/mL concentration range and the minimum detection levels were: 0.13 pg/mL for IL10; 0.3 pg/mL for IL17; 1.8 pg/mL for IFN-γ and 0.7 pg/mL for TNF-α.

For TGF-β, analyzed separately, samples were previously activated with the Sample Activation Kit 1 (R&D, Minneapolis, MN, USA) according to the recommended procedure. After activation, samples were incubated with capture beads for two hours, washed, and incubated with a detection reagent. Acquisition and analysis were performed as described above. For TGF-β, standard curves covered a 0–10,000 pg/mL concentration range, and the minimum detection level was 14.9 pg/mL.

4. Statistical analysis

Categorical variables were expressed as absolute frequencies and percentages and analyzed using Fisher's exact test. The normality of distributions was assessed using the D'Agostino and Pearson test. Normally distributed data are presented as mean (SD) and non-normally distributed data as median (IQR). Two independent groups were compared using the Mann-Whitney U test, and paired data were compared using the Wilcoxon signed-rank test. A P-value of <0,05 indicated the presence of a statistically significant difference. The Spearman´s rank correlation test was used to analyze correlations between cytokine levels and T-lymphocyte subsets. Data were analyzed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla, California).

IV. RESULTS

1. Regulatory T cells and IL-17A levels in Non-infectious Uveitis

1.1. Clinical characterization

A total of 29 patients with a clinical diagnosis of active non-infectious uveitis were recruited. Regarding the different diagnoses, 3 had an idiopathic disease, 20 had HLA-B27-associated uveitis, 3 had BD, 2 had VKH disease, and 1 had TINU. Patients with HLA-B27-associated disease were also analyzed separately.

Patient demographics are listed in Table XXII. NIU patients included 13 males and 16 females, with an average age of 47 (range, 24-80 years old). At the time of sampling, all patients had active disease, and blood samples were collected at presentation. This was the first episode of symptomatic intraocular inflammation for all the patients included, and none had received previous topical or systemic treatment for ocular and/or extraocular symptoms.

Table XXII. Patient demographic characterization

	NIU patients n=29	**Healthy controls** n=15	***P* value**
Age (years) (Mean±SD)	24–80 (47,2 ±14,4)	20-86 (44,7 ±18,6)	0.683[a]
Male sex (%)	13 (44,8%)	8 (53,3%)	0.752[b]

[a]Unpaired *t* test; [b]Fisher's exact test.

1.2. Characterization of lymphocyte subsets in NIU patients and controls

In this study, we characterized several circulating T cell subsets, but no significant differences were found between patients and controls for any of the studied CD4 subpopulations, including the regulatory subsets and respective CD39 expression (Table XXIII).

Considering the distinct etiologies present in the NIU group, we further isolated HLA-B27+ patients and compared it both to healthy controls and other causes of NIU. However, again, no differences were found in both comparisons for any of the studied T-cell subsets.

Table XXIII. Characterization of lymphocyte subsets and serum cytokine levels in NIU patients and controls

					Group's comparisons			
					Total NIU vs. Control	NIU low vs. high severity	NIU Low Severity vs. Control	NIU High Severity vs. Control
T cell subsets	**Total NIU (n=29)**	**NIU Low Severity (n=13)**	**NIU High Severity (n=16)**	**Control (n=15)**	***P*-values**			
Cells/µL, median (IQR)								
Total Tregs	69 (35)	70 (36)	67 (32)	57 (40)	0,107	0,738	0,121	0,220
Naive Tregs	16 (14)	16 (22)	18 (11)	14 (16)	0,271	0,416	0,593	0,198
CD39+ naive Tregs	2 (2)	2 (1)	2 (2)	2 (1)	0,908	0,902	0,819	1,000
Memory Tregs	50 (18)	52 (24)	46 (15)	33 (33)	0,112	0,210	**0,053**	0,384
CD39+ memory Tregs	29 (14)	32 (13)	27 (21)	26 (20)	0,380	0,089	0,127	0,930
Percentages, median (IQR)								
(Within CD4+ T cells)								
Total Tregs (within CD4+ Tcells)	7,97 (2,25)	7,97 (1,81)	7,91 (2,69)	7,43 (1,22)	0,329	0,650	0,474	0,358
Naive Treg	1,89 (1,14)	1,84 (1,38)	2,03 (1,33)	1,81 (0,98)	0,691	0,101	0,503	0,220
CD39+ naive Tregs (within CD4+ T cells)	0,16 (0,17)	0,18 (0,17)	0,16 (0,18)	0,21 (0,18)	0,429	0,905	0,547	0,464
Memory Treg	5,5 (1,78)	5,8 (1,87)	5,34 (2,15)	5,27 (1,18)	0,664	0,812	0,578	0,838
CD39+ memory Tregs (within CD4+ T cells)	3,36 (2,07)	3,79 (2,20)	3,05 (2,76)	3,23 (1,40)	0,646	0,288	0,274	0,838
(Within Total Tregs)								
Total CD39+ Tregs	48,18 (23,19)	53,02 (20,07)	40,08 (26,99)	44,65 (15,89)	0,807	**0,083**	0,235	0,545
Naive Treg	27,86 (18,08)	22,59 (15,92)	28,08 (20,01)	28,02 (7,22)	0,695	**0,068**	0,185	0,626
Memory Tregs	72,14 (18,09)	77,41 (15,93)	71,93 (20,01)	71,98 (7,22)	0,695	**0,068**	0,185	0,626
CD39+ Memory Tregs	47,17 (22,98)	52,00 (21,48)	38,89 (26,12)	42,97 (15,91)	0,788	**0,062**	0,217	0,545
Serum Cytokine Levels	**(n=28)**	**(n=12)**						
pg/mL, median (IQR)								
IL-10	1,40 (1,23)	1,44 (1,21)	1,37 (1,83)	1,22 (1,27)	0,380	0,673	0,548	0,395
IL-17ª	2,295 (5,79)	2,73 (5,76)	1,91 (5,71)	1,20 (3,73)	**0,080**	0,766	**0,060**	0,241
TNF-α	0,28 (1,80)	0,30 (1,54)	0,28 (2,14)	0,00 (0,82)	0,129	0,976	0,231	0,159
INF-ɣ	2,835 (3,26)	2,91 (5,40)	2,74 (2,76)	2,81 (3,25)	0,743	1,000	0,792	0,800
TGF-β	2042 (640)	2007 (550)	2139 (718)	1962 (671)	0,650	0,397	0,943	0,446

NIU, Non-infectious Uveitis; IQR, interquartile range. Results are presented as medians and interquartile range, median (IQR). * Mann-Whitney U test

1.3. Serum cytokine levels in NIU patients and controls

As for cytokine evaluation (Fig. 5), a tendency for increased levels of IL-17A was found in the NIU group (p=0.08) compared to healthy controls (Fig. 6). No other differences were observed for the other cytokines evaluated. Again, we performed similar comparisons for the HLA-B27-associated subgroup. However, no differences were encountered in these comparisons for any of the cytokines evaluated.

We then assessed how cytokine levels correlated to the cellular subsets studied. As for the control group, only a positive correlation was observed between TGF-β levels and the percentages of total CD4 Tregs (r=0.604; p=0.017). Interestingly, the NIU group presented a distinct profile of correlations. We observed a negative correlation between IL-10 levels and the percentages of memory Tregs in NIU patients' peripheral blood (p=0.030; R=-0.411). This correlation was also present in the HLA-B27-associated uveitis subset (r=-0.411; p=0.007). Similarly, we observed a tendency for a negative correlation between TGF-β and the percentages of total CD39+ Tregs in the NIU group (r=-0.373; p=0.051).

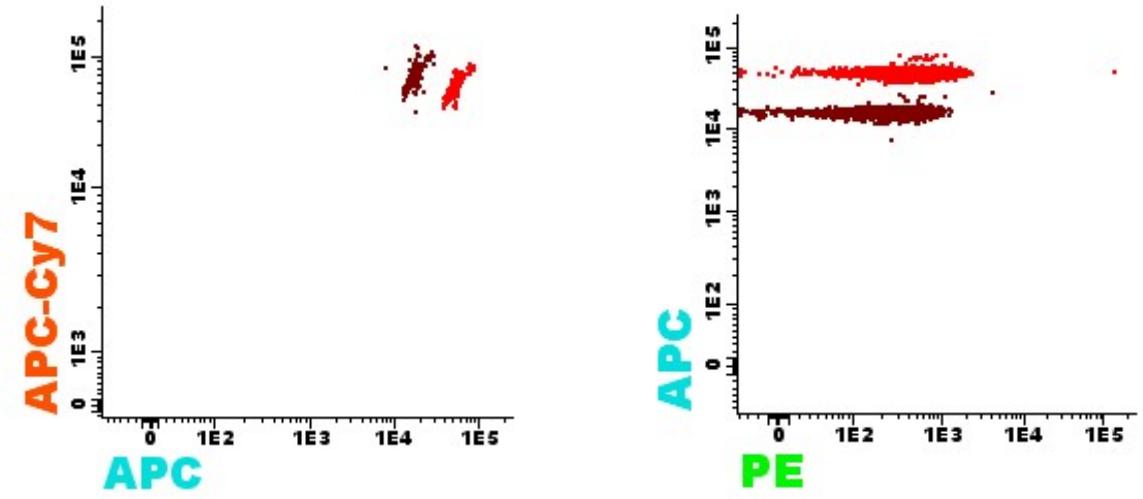

Fig. 5 -. Representation of serum cytokine levels measurement by multiplexed flow cytometry. (A) Identification of IL-17A (dark red) and IL-10 (red) CBA beads. (B) Determination of IL-17A and IL-10 serum sample concentration through PE fluorescence intensity.

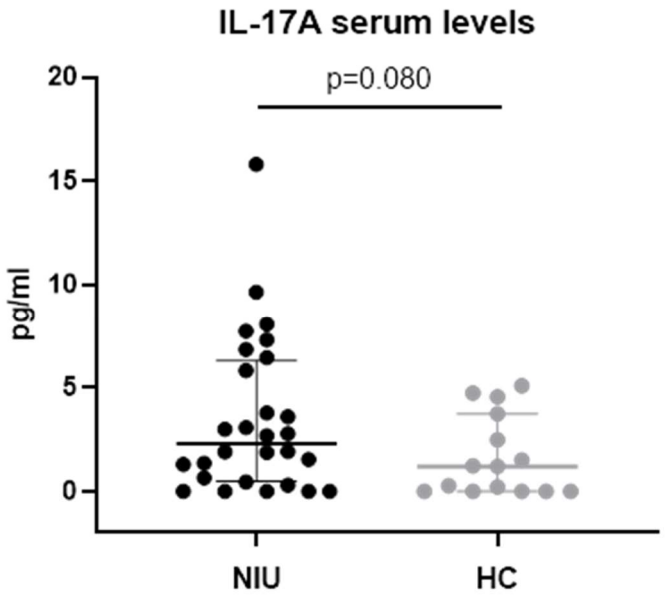

Fig. 6 - Scatter dot plot (median with IQR) of serum IL-17A levels in NIU and HC groups. NIU, Non-infectious uveitis; HC, Healthy Controls.

Regarding the evaluated pro-inflammatory cytokines, a positive correlation between IL-17A levels and the absolute counts of memory Tregs was found (r=0.465; p=0.013) in the NIU group (Fig. 7-A). We also observed a positive correlation between TNF-α and the absolute counts of both total memory Tregs and CD39+ memory Tregs (r= 0.418; p=0.027) in the NIU group (Fig. 7-B).

The inflammatory (TNF-α + IFN-ɣ + IL-17A) /anti-inflammatory (IL-10 + TGF-β) cytokine ratio showed a positively correlation with the absolute counts of both memory Tregs and CD39+ memory Tregs in the NIU group (r=0.406; p=0.032). In line with this, the IL-17A/IL-10 ratio was also positively correlated with the absolute counts of memory Tregs (r=0.417; p=0.030).

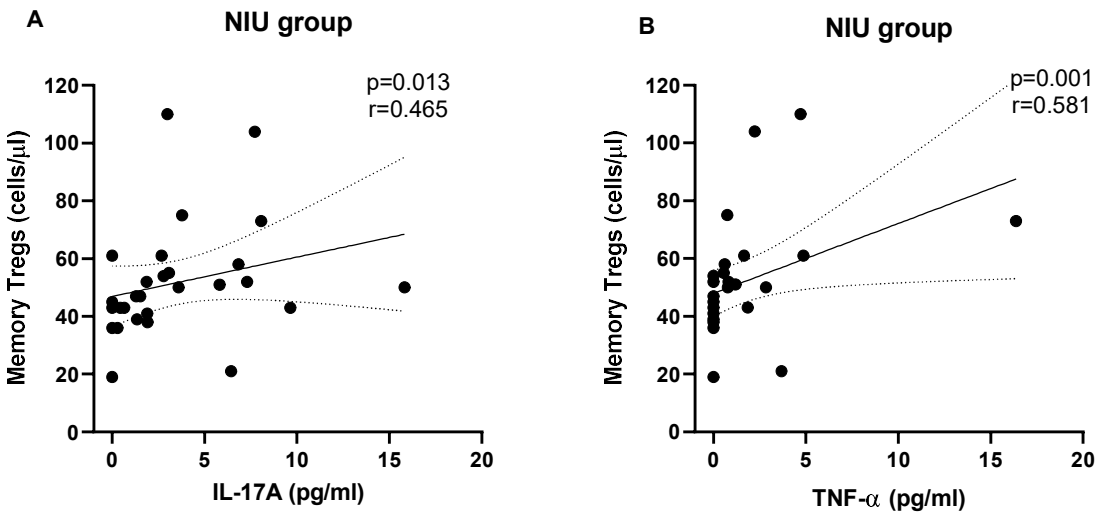

Fig. 7 - Memory Tregs counts correlations with IL-17 (A) and TNF-α (B) serum levels in NIU group. Spearman's correlation coefficients and p values are indicated. NIU, noninfectious uveitis.

1.4. Age and T-lymphocyte subsets and Cytokine levels

Since the mean age in both the patient and control group was similar (47.2 for patients and 44.7 for controls) but with a significant interval between minimum and maximal ages included (24-80 for patients and 20-86 for controls), we further separated both the patient and the control groups in two subsets -bellow (patients n=15, controls n=10) and over (patients n=14, controls n=5) 45 years old and compared cytokine levels as well as T lymphocyte subpopulations. We found a significant increase in total memory CD4+ T-cells in NIU patients younger than 45 years-old that was not present in controls (p=0.009), as well as an increase in IFN-ɣ levels (p=0.004).

1.5. IL-17A levels and T-lymphocyte subsets, and other Cytokine levels

As to evaluate IL-17A levels in NIU-patients, we separated both patients and controls into two groups-bellow (patients n=14, controls n=10) and over 2.17 pg/mL (mean + S.E.M. for IL-17A levels in the control group) (patients n=15, controls n=5). We analyzed possible correlations with T lymphocyte subsets and other cytokine levels in peripheral blood and found that patients with higher IL-17A levels also showed higher serum concentrations of memory (p=0.001) and naïve (p=0.003) Tregs as well as higher TNF-α (p<0.0001) and IFN-ɣ (p=0.016) levels.

1.6. Anterior Chamber cellular reaction or Vitreous haze severity and T-Lymphocyte subsets and Cytokine levels

Patients were further divided according to inflammatory cell reaction in the anterior chamber or vitreous as per standardization of uveitis nomenclature (SUN) and National Eye Institute (NEI) grading systems [25, 26] in two groups: (1) High severity (flare grade >2+ cell reaction or vitreous haze> 2+); (2) Low severity: flare grade <= 2+ cell reaction or vitreous haze<= 2+).

Sixteen patients were included in the NIU-high severity group (HLA-B27-associated uveitis, n=12; BD, n=1; TINU, n=1; idiopathic disease, n=2) and thirteen in the NIU-low severity group (HLA-associated uveitis, n=8; BD, n=2; VKH, n=2; idiopathic disease, n=1). (Table XXIII)

Comparing to controls, there was a tendency for increased memory Treg counts in the NIU-low severity group (p=0.053), but with no difference in CD39+ memory, Treg counts. There were also no differences in both these subsets between NIU-high severity patients and healthy subjects. Moreover, when analyzing percentages of Treg subsets within total Tregs, a tendency for higher levels of naïve Tregs (p=0.068), lower memory Tregs (p=0.068), and CD39+ memory Tregs (p=0,062) were also present in the NIU-low severity group compared to High severity patients.

Regarding serum cytokine levels, a tendency for higher IL-17A concentrations was also found in the NIU-low severity group when comparing to controls (p=0,06).

Finally, in the serum of NIU-low severity patients, positive correlations were found between IL-17A and TNF-α levels and memory Treg (respectively, r=0.767; p=0.005 and r=0.826; p=0.002) and CD39+ memory Treg counts (respectively, r=0.586; p=0.049 and r=0.714; p=0.012). As for the NIU-high severity group, a negative correlation was found between serum IL-10 levels and both percentages of total Tregs and memory Tregs (respectively, r=0.518; p=0.042 and r=0.553; p=0.029). (Fig. 8)

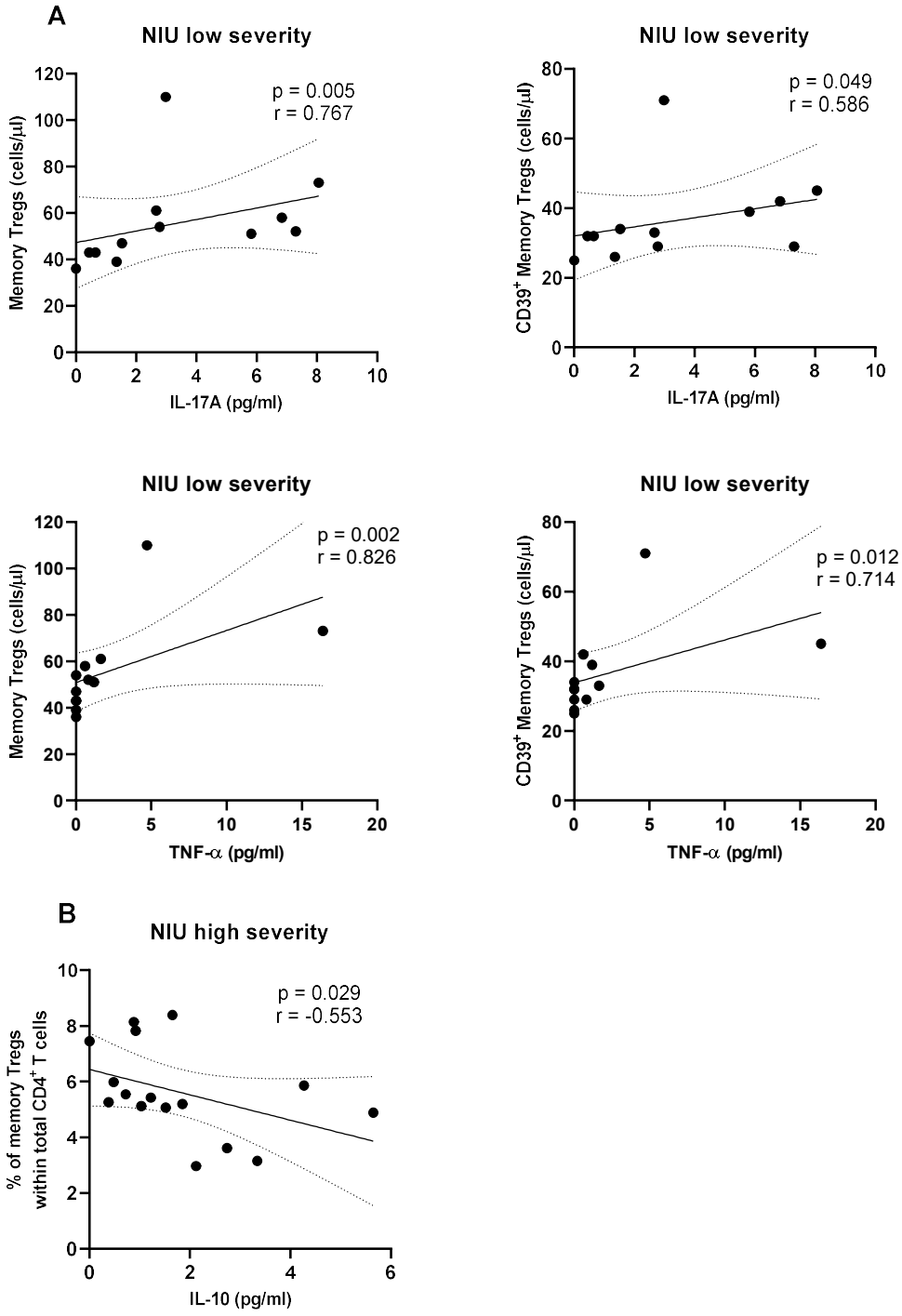

Fig. 8 - Correlation between serum cytokine levels and the distribution of Tregs subsets in NIU patients with low severity (A) and NIU patients with high severity (B). Spearman correlation coefficients and p-values are indicated.

2. T-Lymphocyte Regulatory Subsets and Inflammatory Cytokine Levels after Treatment of Non-Infectious Uveitis

2.1. Patients Demographics and Clinical Characteristics

A total of 15 patients with a clinical diagnosis of active NIU were recruited. The diagnosis included idiopathic disease, HLA-B27-associated uveitis, BD, and VKH disease. Fifteen healthy sex and age-matched healthy subjects were selected as controls. Table XXIV summarizes the features of the study population.

Table XXIV. Characteristics of the study population.

Patient	Age	Sex	Diagnosis	Systemic Treatment
1	62	M	HLA-B27-associated	No
2	35	F	HLA-B27-associated	No
3	29	F	HLA-B27-associated	No
4	31	M	HLA-B27-associated	No
5	62	F	HLA-B27-associated	No
6	49	F	HLA-B27-associated	No
7	49	M	Vogt-Koyanagi-Harada	Prednisolone, Cyclosporine
8	37	F	Vogt-Koyanagi-Harada	Prednisolone, Cyclosporine
9	36	M	HLA-B27-associated	Prednisolone
10	72	F	HLA-B27-associated	Prednisolone
11	41	M	HLA-B27-associated	Prednisolone
12	42	M	Behçet Disease	Prednisolone, cyclosporine, adalimumab
13	53	F	Behçet Disease	Prednisolone, azathioprine
14	58	F	Idiopathic	No
15	50	M	Idiopathic	No

All patients were treated with topical corticosteroids and cycloplegics. In three cases of HLA-B27-associated uveitis and the two cases of BD and VKH, patients also received therapy with systemic corticosteroids (prednisolone). Immunosuppressive therapy with cyclosporine A or azathioprine was necessary for the BD and VKH patients. In one case of BD, adalimumab was also initiated.

Patient demographics were seven males and eight females, with an average age of 47 years (range, 29-72 years). Control demographics were as follows: 8 males and 7 females, with an average age of 44.7 years (range, 20-86 years old).

2.2. Characterization of Regulatory T-Lymphocyte Subsets in NIU Patients and Controls

Fifteen NIU patients were evaluated before and after treatment and compared to controls at both time points. Only thirteen NIU patients had flow cytometry evaluations (Table XXV and XXVI).

Table XXV. Comparison of T cell subsets absolute counts in NIU AT, NIU BT, and control group

				Group's comparisons		
				NIU BT vs. Control	NIU AT vs. Control	NIU BT vs. NIU AT
T cell subsets	**NIU BT (n=13)**	**NIU AT (n=13)**	**Control (n=15)**	***P-value**		**#P-value**
Cells/µL, median (IQR)						
T cells	1203 (755)	1273 (779)	1326 (671)	0,865	0,409	0,542
CD4 T cells	723 (557)	935 (483)	671 (496)	0,435	0,183	0,636
Naïve	208 (258)	285 (185)	228 (197)	0,937	0,917	1,000
Memory	479 (232)	567 (253)	382 (324)	0,369	0,259	0,542
Effector	10 (15)	9 (22)	9 (10)	0,741	0,991	0,451
Tregs	69 (56)	70 (50)	57 (39)	0,166	0,146	0,852
Naive Tregs	14 (14)	17 (14)	14 (16)	0,883	0,674	0,830
$CD39^+$ naive Tregs	1 (1)	1 (1)	2 (1)	0,424	0,469	1,000
Memory Tregs	47 (33)	52 (24)	33 (33)	0,110	0,230	0,877
$CD39^+$ memory Tregs	28 (25)	26 (21)	26 (20)	0,759	0,865	0,989
CD8 ($CD4^-$) T cells	442 (265)	443 (303)	510 (380)	0,808	0,843	0,497
Naïve	96 (118)	124 (134)	109 (133)	0,883	0,643	0,625
Memory	179 (134)	187 (114)	183 (157)	0,580	0,643	0,735
Effector	122 (223)	152 (134)	165 (118)	0,474	0,381	0,735
CD4/CD8 ratio	1,85 (0,88)	1,86 (0,90)	1,73 (0,88)	0,609	0,519	0,831

NIU: Non-infectious Uveitis; NIU BT: NIU before treatment; NIU AT: NIU after treatment; IQR: interquartile range. Results are presented as medians and interquartile range, median (IQR). * Mann-Whitney U test. # Wilcoxon signed-rank test.

Table XXVI. Comparison of percentual values of T cell subsets in NIU AT, NIU BT, and control group

				Group's comparisons		
				NIU BT vs. Control	NIU AT vs. Control	NIU BT vs. NIU AT
T cell subsets	**NIU BT (n=13)**	**NIU AT (n=13)**	**Control (n=15)**	***P-value**		**#P-value**
Percentages, median (IQR)						

				Group's comparisons		
				NIU BT vs. Control	NIU AT vs. Control	NIU BT vs. NIU AT
T cell subsets	**NIU BT (n=13)**	**NIU AT (n=13)**	**Control (n=15)**	***P*-value**		**#*P*-value**
Within CD3 T cells						
$CD4^+$ T cells	64,64 (12,78)	64,99 (12,07)	63,33 (14,24)	0,611	0,519	0,748
$CD8^+$ (CD4-) T cells	35,02 (12,76)	35 (12,00)	36,67 (13,93)	0,608	0,517	0,787
Within CD4 T cells						
Naïve	32,68 (22,01)	31,34 (12,20)	33,62 (14,04)	0,577	0,357	0,497
Memory	65,57 (19,06)	66,31 (13,13)	65,16 (11,97)	0,409	0,359	0,455
Effector	1,12 (2,36)	0,94 (2,03)	1,14 (2,95)	0,914	0,865	1,000
Tregs	9,12 (2,71)	8,56 (3,54)	7,43 (1,22)	**0,048**	0,336	**0,011**
Naive Tregs	1,84 (1,43)	1,72 (0,96)	1,81 (0,98)	0,973	0,717	0,376
$CD39^+$ naive Tregs	0,11 (0,12)	0,13 80,14)	0,21 (0,18)	0,091	0,221	0,625
Memory Tregs	7,05 (2,67)	6,1 (3,12)	5,27 (1,18)	0,057	0,249	0,094
$CD39^+$ memory Tregs	3,87 (4,19)	3,3 (3,11)	3,23 (1,40)	0,300	0,795	0,244
Within Tregs						
Naive Treg	19,49 (19,35)	23,14 (13,07)	28,02 (7,22)	0,154	0,107	0,735
Memory Tregs	80,51 (19,35)	76,86 (13,08)	71,98 (7,22)	0,155	0,107	0,735
$CD39^+$ Tregs	48,18 (36,33)	46,66 (26,78)	44,65 (15,89)	0,777	0,917	0,685
Within CD8 (CD4-) T cells						
Naïve	22,08 (23,15)	22,82 (15,62)	21,02 (23,57)	0,743	0,777	0,376
Memory	39,79 (18,68)	38,3 (19,54)	35,54 (12,44)	0,313	0,359	1,000
Effector	30,54 (21,55)	38,59 (21,22)	39,11 (13,95)	0,129	0,608	0,376

NIU: Non-infectious Uveitis; NIU BT: NIU before treatment; NIU AT: NIU after treatment; IQR: interquartile range. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold. *Mann-Whitney U test. #Wilcoxon signed-rank test.

Thus, considering the flow cytometric analysis before treatment, patients showed increased percentages of total Tregs (p=0.048) compared to controls. Also, within total CD4+ T cells, there was a tendency for increased percentages of memory Tregs in NIU patients (p=0.057). No further statistically significant differences were observed when comparing the two groups.

However, the introduction of therapy changed this scenario, with patients showing a reduction in Treg cell levels (p=0.011) after treatment. Moreover, when comparing the after-treatment patient group with the control subjects, no significant differences were observed in percentages or absolute counts.

2.3. Serum Cytokine Levels

Despite the alterations reported for the cellular subsets, when regarding inflammatory cytokine levels, we observed no differences towards controls in patients with a clinical diagnosis of active NIU.

Nevertheless, lower levels of IL17A (p=0.030), as well as a tendency for lower levels of TNF-α (p=0.055), were present in patients after treatment (Fig. 8). Furthermore, the inflammatory (TNF-α + IFN-ɣ + IL17A)/ anti-inflammatory (IL10 + TGF-β) ratio also showed a significant reduction between both evaluations in the NIU group.

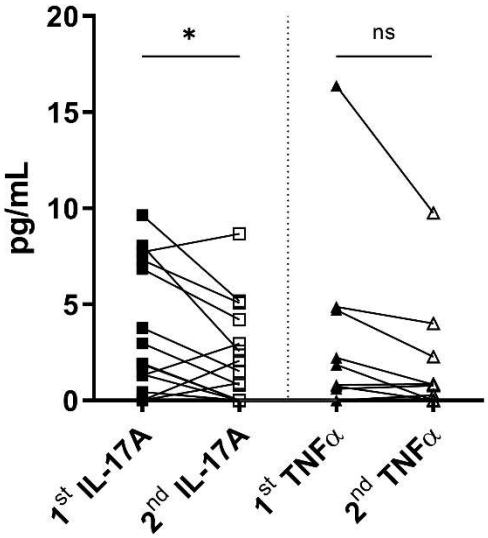

Fig. 9 - Scatter dot plot (median with IQR) of IL17A and TNF-α serum levels before (1st) and after (2nd) treatment of NIU group. Differences were tested using the Wilcoxon signed-rank test. *P-value <0.05; ns: non-significative.

3. Cytokine Profiles in the Peripheral Blood and Aqueous Humor of Patients with Herpetic Uveitis

Four patients with presumed HU and 8 controls were included. Table XXVII summarizes the demographic and clinical features of the HU group. One patient with a panuveitis associated with acute retinal necrosis was also included. All HU patients underwent anterior chamber puncture and AqH sampling, as previously described. Results of AqH by real-time PCR confirmed a VZV infection in all the cases tested.

Regarding serum cytokines, there were no significant differences observed between patients and controls. In AqH samples however, patients showed increased concentrations of IL10 (p=0.018), TNF-α (p=0.018) and IFN-ɣ (p=0.024).

Interestingly, the levels of serum and AqH cytokines differ between the two groups. While controls presented higher levels of IL10, IFN-ɣ and TGF-β in serum samples compared to those found in AqH (respectively, p=0.001; p=0.002 and p=0.001), in the patients' group, only TGF-β showed higher serum concentrations when compared to AqH (p=0.029), with comparable values for the other cytokines tested.

Table XXVIII shows the results and comparison of cytokine levels in the serum and AqH of both groups.

Table XXVII. Demographic and clinical features for the Herpetic Uveitis group.

Patient	Gender	Age (years)	Clinical Features at presentation	Systemic Features	PCR results
1	F	76	Unilateral anterior uveitis; sectorial iris atrophy; diffuse KPs; elevated IOP	None	+ VZV
2	M	45	Panuveitis- acute retinal necrosis	None	+ VZV
3	F	84	Unilateral keratouveitis; sectorial iris atrophy; diffuse KPs	None	+VZV
4	M	74	Unilateral anterior uveitis; elevated IOP; cataract; diffuse KPs; diffuse iris atrophy	None	+VZV

F- female; M- male; KPs- keratic precipitates; IOP- intraocular pressure; VZV- varicella-zoster virus; PCR- polymerase chain reaction.

Table XXVIII. Comparison of cytokine levels in Herpetic Uveitis patients and controls.

SERUM & AQUEOUS HUMOR (AqH) CYTOKINES	Controls, serum samples (n=8)	Controls, AqH samples (n=8)	HU, serum samples (n=4)	HU, AqH samples (n=4)	Controls vs. HU, serum samples	Controls vs. HU, AqH samples	Controls, Serum vs. AqH samples	HU, serum vs. AqH samples
pg/ml, median (IQR)								
IL-10	1,22 (0,42-1,69)	0,00 (0,00-0,00)	1,88	3,44	-	**0.018**	**0.001**	**-**
IL-17ª	1,20 (0,00-3,73)	1,20 (0,00-3,73)	0,20	0,99	**-**	**-**	**-**	**-**
TNF-α	0,00 (0,00-0,82)	0,00 (0,00-0,00)	0,18	0,91	-	**0.018**	-	**-**

INF-ɣ	2,81 (1,58-4,83)	0,00 (0,00-0,05)	2,08	0,87	-	**0.024**	**0.002**	-
TGF-β	1962 (1685-2356)	368 (0,00-565)	1861	374	-	-	**0.001**	**0.029**

HU, Herpetic Uveitis; IQR, interquartile range; AqH- Aqueous humour Mann-Whitney nonparametric U test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.

V. DISCUSSION

NIU can be associated with systemic autoimmune or inflammatory diseases like BD, ocular sarcoidosis, and VKH disease. HLA-B27-associated uveitis is the most common cause of acute anterior NIU, and patients with this condition may present with isolated uveitis or with an axial spondyloarthropathy.

Treg cells are essential for regulating immune responses and have a potential suppressive role in disease activity. They can be subdivided into two different populations: thymic and peripherally-derived and are responsible for the production of anti-inflammatory cytokines, which include transforming growth TGF-β and IL-10.

FOXP3 is a characteristic marker of Treg cells and has been used in various studies analyzing Treg activation (218), but since it is an intracellular molecule, its detection requires fixation and permeabilization of cells. Several authors have proposed using the co-expression of CD4 and CD25 associated with the absence, or the low expression of CD127 to identify the Treg subpopulation since a good correlation between these two phenotypes was previously reported (220, 267). Besides CD25, CD39 is also predominantly expressed on CD4+ FOXP3+ T-cells with an expression level proportional to FOXP3 (223, 268).

In this study, we defined Tregs as CD3+CD4+CD25+CD127- cells while also analyzing its memory and naive subsets and CD39 expression in patients' peripheral blood with active NIU, including a subset of 20 patients with HLA-B27-associated uveitis. Several studies have found decreased levels of Treg cells in patients with active uveitis from various causes (91-93), which led to a growing interest in establishing these cell populations as a biomarker in uveitis and possibly as a future therapeutic target (257). Moreover, a recent study further analyzing the frequency of CD4+CD25+FOXP3+ cells in patients with active uveitis and immunosuppressed patients in sustained clinical remission also found that TIGIT+ Tregs, T-bet+ Tregs, and the ratio of Tregs to Th1 cells were all significantly higher in remission NIU patients compared with patients with active uveitic disease (269).

TIGIT is a co-inhibitory molecule expressed by Tregs associated with the selective inhibition of Th1 and Th17 responses (270), while the expression by Tregs of the effector CD4 T-cell transcription factor T-bet has also been associated with Th1 suppression (271).

Nevertheless, results have been conflicting, as some authors have found no differences (238, 239) or even increased (272) Treg levels in patients with active disease.

In BSCR, an autoimmune disorder causing chronic inflammation of the posterior segment of the eye, a case-control study assessing Treg cell levels in the peripheral blood of patients with active disease found that the percentage of CD4+CD25+FOXP3+ Tregs was significantly lower when compared to controls but with no difference between CD4+CD25+ Tregs in both groups (256). However, another study with BSCR patients found that the proportion of CD4+CD25hiCD127low T cells was higher in patients than in controls (240). The authors suggested that Treg function rather than frequency needed to be studied.

In the present work, when studying CD4+CD25+CD127- cell levels in NIU patients, we did not find significant differences from controls, although this may be related to a different strategy used to characterize Tregs.

A recent study has also assessed different Treg subsets in autoimmune disease and found that the Treg compartment of patients with T1D Tregs contained a higher percentage of memory Tregs than healthy controls (234). When analyzing the CD39 expression of these Treg subpopulations, the same authors observed a decreased expression of this surface marker in memory Tregs, concluding that this may impair their suppressive function. A lower expression of CD39 in Tregs was also found in patients with active IBD (273).

To our knowledge, the memory Treg subpopulation and respective CD39 expression have never been studied in NIU. In our patients, there were no significant differences in memory Treg levels (percentage and absolute counts) between patients and controls. However, when further analyzing patients with a lower grade of anterior chamber or vitreous inflammatory cellular reaction, we found a tendency for higher memory Treg counts in these patients than controls with no respective increase in CD39+ expression.

Moreover, when analyzing Treg levels after uveitis resolution and treatment, one would expect that Treg levels would increase. However, our results have shown a higher percentage of both total and memory Tregs in patients with active inflammation when compared to controls and no significant differences from controls after treatment. It is interesting that although there were increased percentages of total and memory Tregs in patients with active disease, there was no significant difference between groups regarding CD39 expression, which may lead us to speculate whether these cells maintain their normal suppressive function.

Our results do not support the role of total Treg frequency alone as a uveitis biomarker, and since, to our knowledge, these are the first studies addressing Treg naïve and memory subsets and respective CD39 expression in the peripheral blood of NIU patients, further studies are needed to elucidate this matter.

Because of the heterogeneity and rarity of the uveitis entities included, activity of the disease and the different strategies used to characterize Tregs, we think that a larger multicenter study evaluating total Treg frequency as well as their naïve and memory subsets, CD39 and FOXP3 expression could be useful in establishing (or not) Tregs as biomarkers for NIU or even as future therapeutic targets. This may be of particular interest for CD39 expression, especially in the Treg memory subset, since this surface marker expression seems to be, as well as FOXP3, closely related to normal Treg immunosuppressive function through the CD39-CD73-adenosine pathway. This specific and activated Treg subset could be useful as a therapeutic target in auto-immune diseases, and recent work has shown, for example, that oral treatment with Polysaccharide A derived from the commensal *Bacteroides fragilis*, significantly increased the expression of CD39 on the surface of FOXP3+Tregs in addition to *in vitro* enhancement of CD39+FOXP3+Treg frequency (274). This expansion of a CD39+FOXP3+Treg subset could help establish a new paradigm in the treatment of several auto-immune diseases, including NIU.

We also observed a negative correlation between IL-10 levels and the percentages of memory natural Tregs in NIU patients' peripheral blood, including in the HLA-B27-associated subgroup. A tendency for a negative correlation was also found for TGF-β and the percentages of total CD39+ Tregs in the NIU group. Although IL-10 and TGF-β are suppressive cytokines and produced by Tregs, this may be explained by the fact that all these patients were in a very early stage of disease and Treg induction or that there is, in fact, an impaired regulatory cytokine production by Tregs in NIU patients.

Regarding inflammatory cytokine expression, a tendency for IL-17A elevation was found in the NIU group ($p=0.08$), and it would have been interesting to study CD4+IL-17+ T cells frequency in these patients. The Th17/Treg ratio may be more specific for disease activity than isolated Treg levels, and further studies can warrant its usage as an active disease biomarker.

Although we did not have data on Th17 cell levels, when analyzing the inflammatory (TNF-α + IFN-ɣ + IL-17A)/anti-inflammatory (IL-10 + TGF-β) cytokine ratio and the IL-17/IL-10 ratio, we did find a positive correlation between these ratios and the absolute counts of memory Tregs in the NIU group. We also found that higher IL-17A levels were associated with higher levels of memory and naïve Tregs, as well as higher serum concentrations of TNF-α and IFN-ɣ.

This scenario may suggest induction of Treg cells in an effort to suppress cytokine production in early stages of intraocular inflammation, an impaired memory Treg function, or even activation of a specific IL-17-producing peripheral Treg subset, as observed in other conditions, including inflammatory and infectious diseases (275). Although adaptative Tregs exert their suppressive effect via the production of anti-inflammatory cytokines such as IL-10 and TGF-β, a distinct population of peripheral Treg cells that are FoxP3+ and produce IL-17 has already been identified in patients with Crohn's disease (276) although, to our knowledge, this has never been described in uveitis.

Nevertheless, evaluation of Th17 levels could have given further insight on this correlation between IL-17 and memory Tregs. Finally, we have observed a positive correlation between TNF-α and the absolute counts of both total memory Tregs and CD39+ memory Tregs in the NIU group. Together with IL-17A, TNF-α is a key cytokine in uveitis pathogenesis (157, 238, 277), and, understandably, it may show an elevated concentration when there are active disease and memory Treg upregulation.

Accordingly, our results have thus shown a reduction in IL17A and TNF-α levels after uveitis resolution, as well as a reduction in the inflammatory/anti-inflammatory cytokine ratio. Although previous works have studied the total Treg cell frequency in active disease, there are few data evaluating individual Treg subsets as well as cytokine levels after treatment. Since we have evaluated patients before and after treatment, our results reinforce the notion that inflammatory cytokines like IL17A are fundamental in the disease pathogenesis and may represent a more tailored approach or treatment option in patients not responding to classical immunosuppression.

So far, IL-17 increased expression and dysregulation seem to be particularly important in the physiopathology of auto-immune conditions like psoriasis (278, 279) and ankylosing spondylitis (280, 281) and novel therapeutic strategies based on neutralization of IL-17 have had encouraging results (282-286).

However, results of clinical trials with the anti-IL-17 antibody Secukinumab in CD (287), RA (288) and refractory posterior NIU (136) have shown this drug to be ineffective or only partially effective, even though there is a strong body of evidence suggesting a pivotal role of IL-17 in the disease pathogenesis of CD (289), RA (290) and NIU (107, 112, 113, 116).

In the clinical trials on the efficacy and safety of secukinumab in the treatment of NIU, patients with Behçet's uveitis (SHIELD study); patients with active, noninfectious, non-Behçet's uveitis (INSURE study) and patients with quiescent, noninfectious, non-Behçet's uveitis (ENDURE study) were enrolled, but the primary endpoint for each study was not met, which led to early trial termination (136). Nevertheless, the secondary efficacy data from these studies suggest a beneficial effect of secukinumab in reducing concomitant immunosuppressants' use.

Our study results support and reinforce the role of IL-17 in NIU, and although secukinumab did not show efficacy in uveitis treatment, other anti-IL-17 antibodies may be better alternatives for IL-17 blockade and NIU treatment.

Ixekizumab is a humanized monoclonal antibody against IL-17A currently approved for psoriasis, arthritic psoriasis, and ankylosing spondylitis. Brodalumab is a recombinant, fully human monoclonal antibody that binds with high affinity to the interleukin 17 receptor (IL17R) and is approved for treating moderate-to-severe chronic plaque psoriasis. As it is well known that different anti-TNF-α antibodies have different efficacies and safety profiles in NIU treatment (291), it seems reasonable to assume that, given the amount of evidence demonstrating the importance of IL-17 in NIU pathogenesis, these different antibodies targeting the IL-17 pathway could also be studied for uveitis treatment (292).

There are NIU patients that do not respond to classical immunosuppressants or to anti-TNFα therapy, and therefore in these patients, it is vital to find drugs that target alternative pathways, further exploring the possible role of antibodies that target the IL-17 pathway (IL-17A or the IL-17 receptor). Additionally, monitoring IL-17 serum levels can also be an interesting option for patients with refractory NIU in whom there are doubts regarding current treatment efficacy.

Finally, our results in NIU patients have also shown a reduction in TNF-α levels after uveitis resolution. Along with IL-17A, TNF-α is a crucial cytokine in uveitis pathogenesis (156, 293), and there is a growing experience in managing refractory NIU with anti-TNF-α immunomodulatory treatment (291). In EAU, it has been shown that TNF-α inhibition improves intraocular inflammation through Th1 effector response suppression (157) and its neutralization in the afferent phase of the disease, reduces EAU disease scores, IRBP-specific cell proliferation, and expression of Th1, Th2, and Th17 cytokines (294).

These pre-clinical observations supported the completion of three clinical trials analyzing the efficacy and safety of adalimumab, the first fully human anti-TNFα monoclonal antibody, in patients with noninfectious intermediate, posterior, or panuveitis. These trials results found that adalimumab was associated with a lower risk of uveitic flare or visual impairment (163) and led to adalimumab's approval in posterior NIU.

Overall, our study has several strengths, such as the pioneer characterization of Treg subsets and respective CD39 expression in NIU, the prospective analysis after uveitis treatment and resolution, and the analysis of the major inflammatory and anti-inflammatory cytokines profiles involved in uveitis. These features provide new insight into the role of Tregs and cytokine profiles in NIU. We think it can be beneficial for NIU patients' approach and future treatment since some do not respond to classical immunosuppressants and need novel therapeutic targets.

Our study's main limitation is the small sample size, which can be attributed to the rarity of the uveitic disorders included and to our study design. We only recruited patients presenting with their first intraocular inflammation episode who had not received either topical or systemic treatment. Our initial estimates included about 20 patients for each group (patients before and after treatment and controls). After recruitment, it was possible to increase the first group of patients to 29, even though we could only include 15 patients with inactive and controlled uveitis. Some patients completed our first evaluation but were then lost to follow-up and did not return to our clinic.

Another limitation is the heterogeneity of uveitic conditions included in the present study. Among the active NIU patients included, three had an idiopathic disease, twenty had HLA-B27-associated uveitis, three had BD, two had VKH disease, and one had TINU. Although all these patients had uveitis as their primary complaint and clinical feature, ideally, each of these entities should be studied separately because they all have different immunological mechanisms culminating in intraocular inflammation. Taking this into consideration, we further isolated HLA-B27+ patients and compared it both to healthy controls and to other causes of NIU. However, no differences were found in both comparisons for any of the studied T-cell subsets or cytokine profiles.

Moreover, since most patients had an untreated concomitant systemic disease at the time of inclusion, it is reasonable to assume that inflammatory cytokine serum levels (IL-17, TNF-α, and IFN-ɣ) were also affected by active systemic inflammation, and this too must be taken into consideration when interpreting our results.

Regarding infectious uveitis, our initial aim was to analyze the peripheral lymphocyte subpopulations as well as local and peripheral inflammatory and anti-inflammatory cytokines in a group of patients with active infectious uveitis and compare them with normal controls. However, patient recruitment and inclusion for this group were particularly difficult because we could only include patients who, for clinical reasons, needed to confirm the etiology of their intraocular infection through anterior chamber puncture and AqH PCR analysis. Since we were only able to include four patients with a clinical suspicion of herpetic uveitis, we chose to focus on the local (AqH samples) and peripheral (serum) cytokines profiles, comparing them with eight normal controls undergoing cataract or refractive surgery, without a history of intraocular inflammation.

In this preliminary work, we included three patients with anterior herpetic uveitis and one patient with acute retinal necrosis, all with a confirmed VZV-associated ocular infection, as well as eight healthy controls. Our results showed that while were no significant differences between patients and controls regarding serum cytokine profiles, there were increased concentrations of IL-10, TNF-α, and IFN-ɣ in the AqH samples of patients.

Two previous studies have found similar results in viral uveitis, one including 14 patients (HSV- 8; VZV-5 and CMV- 1) (118) and other 5 patients (HSV- 2 and VZV-3) (189) who also underwent AqH cytokine analysis. This IL-10 elevation in intraocular fluids suggests a possible role of IL-10 as a biomarker for intraocular viral infection and may be responsible for the persistent and recurrent character of herpetic uveitis.

Although IL-10 is a well-known immunoregulatory cytokine, viruses have developed mechanisms by which they use IL-10´s anti-inflammatory mechanisms to evade effective T-cell responses, promoting viral tolerance and survival (295). As a result, viruses can persist for life in infected hosts possessing otherwise competent immune responses, and this may be the reason why herpetic viruses can be associated with persistent and recurrent uveitis.

It is known that sustained IL-10 expression during immune priming or secondary responses can favor persistence or chronic infections, and it is this delicate balance between the inflammatory response crucial to virus clearance and the IL-10-mediated immune regulation necessary for T cell homeostasis and host tissue protection that may be subverted by viruses to allow replication and spreading (295). IL-10 may actually be a therapeutic target for the treatment of persistent viral infection since it has been shown that, in mice, its genetic removal or administration of an anti-IL-10 receptor antibody successfully restores the effector T-cell response and results in viral elimination (296).

In AqH samples, patients also showed increased concentrations of TNF-α and IFN-ɣ, once again with no significant differences from controls in serum cytokine levels. This intraocular elevation in IL-10 and inflammatory cytokine levels reflects the local immune response secondary to VZV infection and shows how a further in-depth analysis of AqH´s cytokine concentrations may help clarify the mechanisms of uveitis-related ocular damages and enable a better understanding of the disease pathogenesis, according to the pathogens involved.

VI. CONCLUSIONS

In this study, we aimed to characterize regulatory T cell frequency and subpopulations (with respective CD39 expression) in patients with NIU, as well as cytokine expression in patients with non-infectious and infectious uveitis. We performed flow cytometric analysis of the peripheral blood of patients with active intraocular inflammation and compared them with healthy controls. Patients with non-infectious disease were then revaluated after treatment. In infectious uveitis cases, cytokine profiles present in the aqueous humour were also evaluated.

There were no significant differences between patients with active NIU and healthy controls regarding total Treg frequency, as well as their naïve and memory subsets and respective CD39 expression. These results do not support the use of total Treg frequency as a biomarker for active disease.

When analyzing Treg levels before and after uveitis resolution and treatment, our results have shown a higher percentage of both total and memory Tregs in patients with active inflammation and no significant difference from controls after treatment. However, although total and memory Tregs were increased in NIU patients with active disease, there was no significant difference between groups regarding CD39 expression in Tregs, suggesting a possible Treg dysfunction rather than reduction.

IL-17 levels in the serum of NIU patients have shown an elevation of this inflammatory cytokine levels in active intraocular inflammation and a reduction with disease remission. These results are in accordance with our extensive literature review on the roles of IL-17 in the physiopathology of uveitis and reinforce the need for more clinical trials addressing the efficacy of anti-IL-17 antibodies in the treatment of NIU.

The results in NIU patients have also shown a reduction in TNF-α levels after uveitis resolution, highlighting the already known role of this cytokine in active intraocular inflammation and providing additional evidence on the rationale behind anti-TNF-α antibody treatment of NIU.

In patients with a confirmed intraocular VZV infection, there were increased concentrations of IL-10, TNF-α, and IFN-ɣ in the AqH samples compared to healthy controls. While local TNF-α and IFN-ɣ expression may be related to the intraocular inflammation, IL-10 seems to be a candidate for viral/herpetic uveitis biomarker and, possibly, a target for local treatment of chronic or recurrent infection.

VII. FUTURE PERSPECTIVES

Uveitis is a potentially blinding disease affecting active young individuals. Its diagnosis can be particularly challenging since it can manifest itself by isolated intraocular inflammation or be associated with other systemic manifestations. It can also be infectious or non-infectious; chronic, acute, or recurrent; granulomatous or non-granulomatous and affect only the anterior segment of the eye, only the posterior segment, or the whole uveal tract. These unique features pose many difficulties when trying to establish the correct diagnosis, and there have been made extensive efforts to find novel biomarkers that can help in the differential diagnosis of non-infectious and infectious uveitis.

There is also much to learn about the immune mechanisms behind uveitis, and this search for biomarkers can lead the way to find new treatment targets for patients with disease refractory to conventional drugs.

Tregs have been a subject of extensive research in recent years, and there have been numerous studies addressing Treg frequency and function on various auto-immune diseases. That was also the case for non-infectious uveitis, leading to the possible therapeutic potential of specific regulatory T-cell immunotherapy for the treatment of non-infectious uveitis. However, results addressing Treg frequency or function in several auto-immune diseases and in NIU have been conflicting.

Our study results also do not support total Treg frequency alone as a biomarker for uveitis, but we think that additional work with a larger multicenter study in which patients can be further separated and analyzed according to different etiologies would be more elucidative. After analyzing our results, it also seems clear that future studies on this subject must address Treg subsets along with total Treg frequency and CD39 expression, as well as FOXP3 expression, because natural Tregs have different subpopulations performing unique functions, and there may be different mechanisms of disease for each separate entity.

We also would like to take a further insight into the relationship between Th17 cells and adaptative Tregs in uveitis because it seems to us that there is a sequenced interplay between these cells in both the active and resolution phase of uveitis.

Rather than thinking of uveitis as an imbalance in one cellular subset or one cytokine leading to an isolated treatment strategy, it is possible to think of it as a dynamic sequence of events in which we could act differently in different stages of disease.

Finally, considering our preliminary results in herpetic uveitis patients, we think IL-10 may be a viable intraocular biomarker for patients with persistent and recurrent uveitis associated with VZV infection. Since there is little published evidence concerning this matter, we intend to further expand the number of infectious uveitis patients included to either confirm these results and compare them with intraocular infection from other etiologies, including other herpes viruses (HSV- 1 and 2, CMV) and other causative agents like, for example, *Toxoplasma gondii*. Again, these are rare diseases, and since it is vital to analyze intraocular fluid samples in this setting, a multicenter study or a more extended recruitment period would be mandatory.

In conclusion, this study in non-infectious and infectious uveitis aimed to characterize lymphocyte subpopulations and cytokine expression in uveitis in order to better understand the underlying immune mechanisms and contribute to finding new biomarkers for disease activity as well as novel treatment targets. It is only with medical investigation that we can move forward and become better clinicians, when we are finally able to understand what lies beneath.

VIII.REFERENCES

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IX. APPENDIX I

Full articles

1- Guedes MC, Borrego LM, Proença RD. Roles of Interleukin-17 in Uveitis. Indian J Ophtalmol. 2016 Sep; 64(9): 628-634.

2- Guedes MCE, Arroz MJ, Martins C, Angelo-Dias M, Proença RD, Borrego LM. Regulatory T cells and IL-17A levels in noninfectious uveitis. Graefes Arch Clin Exp Ophthalmol. 2020 Jun; 258(6):1269-1278.

3- Guedes MC, Arroz MJ, Martins C, Angelo-Dias M, Borrego LM, Proença RD. T-Lymphocyte Regulatory Subsets and Inflammatory Cytokine Levels after Treatment of Non-Infectious Uveitis. (Submitted)

4- Guedes MC, Martins C, Arroz MJ, Angelo-Dias M, Borrego LM, Proença RD. Cytokine Profiles in the Peripheral Blood and Aqueous Humor of Patients with Herpetic Uveitis. (Accepted for publication in Journal of Ophthalmic Inflammation and Infection.)

Review Article

Roles of interleukin-17 in uveitis

Marta Catarina Esteves Guedes, Luis Miguel Borrego[1], Rui Daniel Proença[2]

Th17 cells, a CD4+ T-cell subset, produce interleukin (IL)-17, a pro-inflammatory cytokine that has been shown to be involved in several forms of infectious and noninfectious uveitis. Here, we explore the roles of this IL in uveitic disorders as well as in experimental autoimmune uveitis, the possible pathogenic implications of several cytokines associated with IL-17 and analyze the current outcomes and goals for drugs aiming for the IL-17 pathway.

Key words: Interferon-γ, interleukin-6, interleukin-17, interleukin-23, interleukin-27, secukinumab, transforming growth factor-β, uveitis

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Th17 cells are a subset of CD4+ T-cells responsible for the production of interleukin (IL)-17. This T-cell subset is responsible for the production of IL-17A, a pro-inflammatory cytokine involved in several autoimmune diseases.

Both IL-6 and transforming growth factor (TGF)-β are necessary for Th17 differentiation and IL-17 expression; they feature complementary roles since the sole presence of TGF-β induces T-regulatory (Treg) cell production from CD4+ T-cells.[1-3] IL-6 needs to be present to promote the differentiation of a Th17 cell subset in addition to concurrent Treg cell inhibition.[4] Although IL-6 and TGF-β have synergistic roles in Th17 cell differentiation, IL-23 is also important for Th17 cell expansion and activation.[1] By contrast, interferon (IFN)-γ and IL-27 show a regulatory role in uveitis induction through the suppression of Th17 differentiation [Fig. 1].[5,6]

IL-17 induces the production of other inflammatory cytokines such as IL-6, granulocyte colony-stimulating factor (CSF), granulocyte-macrophage-CSF, IL-1, TGF-β, and tumor necrosis factor (TNF)-α; chemokines such as monocyte chemoattractant protein-1, cytokine-induced neutrophil chemoattractant, and macrophage inflammatory protein-2; prostaglandin E2; intercellular adhesion molecule-1; and matrix metalloproteinases. It is also involved in the recruitment of neutrophils, monocytes, and Th1 cells acting with other inflammatory cytokines to induce inflammation in target tissues.[7]

Th1 cells have been previously implicated in the induction of uveitis in experimental autoimmune uveitis (EAU), but since then, several authors have reported the crucial role of IL-17-producing Th17 cells in the genesis of experimental uveitis in mice. They have pointed that treatment with anti-IL-17 antibodies reduces the induction and severity of uveitis in EAU. Moreover, the degree of intraocular inflammation was reduced in IL-17 knockout mice,[8] and although both Th1 and Th17 cells are activated during EAU,[6,8] it has been proposed that Th17 cells are responsible for retinal inflammation in the early stages of uveitis whereas Th1 expression is increased during the late phases and resolution of the disease.[6] It has thus been shown that Th1 and Th17 cells are implicated in the genesis of EAU and may be synergistic with each other, having definitive functions at different stages of the disease.

In addition to its obvious pro-inflammatory role in the induction of EAU, IL-17 expression is also increased in the human peripheral blood of patients suffering from autoimmune and infectious uveitis.

This review aims to explore the roles of this IL in uveitic disorders as well in EAU. The possible pathogenic implications of several cytokines associated with IL-17 induction and suppression will also be discussed, and the current outcomes and goals for drugs aiming for the IL-17 pathway are analyzed. A systematic literature search was carried out using the PubMed and EMBASE databases with the search terms, "uveitis," "interleukin-17," and IL-17," until July 2015. Bibliographies of the retrieved literature were manually searched.

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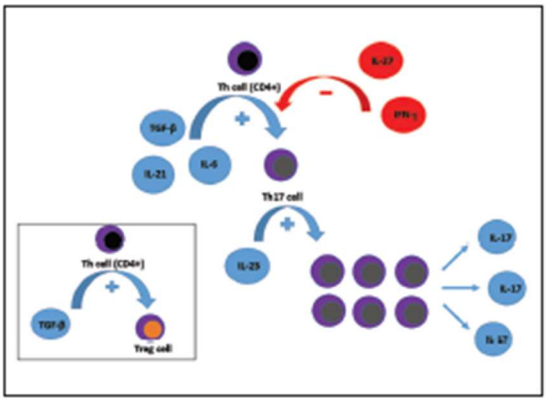

Figure 1: Both interleukin-6 and interleukin-21 can cooperate with transforming growth factor-β to promote Th17 differentiation from CD4+ Th-cells while interleukin-23 is important for Th17 cell expansion and activation. Interferon-γ and interleukin-27 have regulatory roles through the suppression of Th17 differentiation. Note that the sole presence of transforming growth factor-β induces Treg cell production from CD4+ Th-cells

Experimental Autoimmune Uveitis

IL-17 is crucial in the development of EAU in mice, as well as in other models of experimental autoimmune disorders.[6,8] In EAU, the intraocular expression of IL-17 is elevated in mice with uveitis and it promotes the release of inflammatory mediators from ARPE-19 cells, disrupting the retinal pigment epithelium barrier function.[9]

There is abundant information regarding the role of Th17 cells and IL-17, its main pro-inflammatory cytokine, in the induction and clinical severity of EAU, although some studies have inconsistently determined the specific timing of increased IL-17 expression in this condition.

The Th1 cytokine, IFN-γ, has a complex role in EAU and can be protective, suggesting that IL-17 has an important inflammatory effect given that IFN-γ can inhibit IL-17 expression through Th17 suppression. In a study addressing the different functions of Th1 and Th17 cells in EAU, IL-17$^{-/-}$ mice showed no difference in terms of uveitis severity concerning the early stages of the disease, and after anti-IFN-γ and anti-IL-4 antibody treatment and concomitant increase in Th17 expression, only the late stages of the disease were affected, showing the aforementioned differential response of Th1 and Th17 cell subsets during the clinical course of EAU.[8]

In another study concerning the monophasic and relapsing phases of EAU, the authors concluded that IFN-γ-producing cells may be responsible for initiating recurrence in the relapsing form of EAU. Conversely, IL-17-producing cells might be implicated in the primary mechanisms related to intraocular inflammation, thus exhibiting different roles in the monophasic and relapsing forms of the disease.[10]

Previous data have also shown that even though a Th1- or Th17-driven response can initiate uveitis in an animal model, IL-17 plays a critical role in the induction of EAU; moreover, anti-IL-17 treatment can reduce the severity of antigen-induced autoimmune uveitis in mice. The same authors suggested that IL-23 may be a key element in the early stages of intraocular inflammation, and that its function may even surpass the expansion and activation of Th17 cells. Furthermore, there seems to be an exacerbation of the Th17 response and increased disease severity with inhibition of the IL-12-IFN-γ pathway, reinforcing the previously described regulatory activities of these Th1 cytokines in Th17 differentiation.[11]

Noninfectious Uveitis

Increased IL-17 expression has been proven in several studies measuring its aqueous humor or peripheral blood concentration in noninfectious uveitic disorders [Table 1], as well as in other autoimmune diseases such as rheumatoid arthritis,[23] ankylosing spondylitis,[24] inflammatory bowel disease,[25] systemic lupus erythematosus,[26] and psoriasis,[27] in which circulating IL-17 was accessed.

In a study measuring IL-15, IL-17, IFN-γ, TNF-α, and IL-10 levels in the aqueous humor of patients with active autoimmune uveitis from different etiologies, including Behçet's disease (BD), Vogt–Koyanagi–Harada (VKH) disease, and human leukocyte antigen-B27-associated-uveitis, IL-17 levels were found to be higher in these patients than in control subjects, and these levels correlated with disease activity.[28] These results were confirmed by another study that measured IL-17 levels in the peripheral blood of a large group of patients with autoimmune uveitis with and without associated systemic disease. IL-17 levels were elevated in the serum of uveitis patients when compared to controls, and they also served as a marker for disease activity.[29] When comparing intraocular and serum IL-17 levels, one study used a multiplex immunoassay to determine IL-17 levels in paired aqueous humor and serum samples of birdshot retinochoroidopathy (BSRC) patients.[17] The authors not only found an increased intraocular IL-17 expression in BSRC patients when compared to age-related cataract controls but also found that IL-17 levels in aqueous humor were higher than their concurrent serum levels.

All of these studies suggest that IL-17 may be used as a possible biomarker in autoimmune uveitis. Moreover, a recent study revealed a novel association between IL-17A locus polymorphisms and panuveitis, suggesting that IL-17A may also be a possible genetic risk factor for panuveitis.[30]

Infectious Uveitis

IL-17 has been implicated in the development of toxoplasmic encephalitis in an animal model, and since then, there have been studies that have analyzed its role in the induction and maintenance of intraocular inflammation caused by an infectious agent. In a previous study, upregulation of IL-17 levels in mice chronically infected with *Toxoplasma gondii* that lacked the IL-27 receptor was observed, and the authors suggested a protective role of IL-27 in the inflammation that follows toxoplasmic infection.[31] Moreover, the inflammatory roles of Th1 and Th17 cytokines and the regulatory roles of IL-10, TGF-β, and IL-27 in the immunologic responses following toxoplasmic infection have been reinforced in a later study concerning cytokine regulation in *T. gondii* infection.[32]

One study analyzed the inflammatory cytokine and chemokine levels in the aqueous humor of patients with

Table 1: Studies demonstrating increased interleukin-17 expression in several autoimmune uveitic disorders that are not cited in the text

Systemic or intraocular autoimmune disorder associated with uveitis	Main conclusions	Authors, year of publication
VKH	Elevated IL-23 serum expression and upregulated IL-17 expression in PBMCs and CD41+ T-cells from VKH patients with active uveitis	Chi *et al.*, 2007[12]
	Decreased IL-27 expression is associated with an elevated IL-17 response in active VKH patients. Increased Th17 and IL-17 expression in PBMCs from active VKH patients	Wang *et al.*, 2012[13]
	Treatment with cyclosporine A and corticosteroids in VKH disease suppresses IFN-γ and IL-17 expression which, in turn, correlates with disease activity	Liu *et al.*, 2009[14]
	Increased IL-21 and IL-17 expression in VKH patients with the chronic or recurrent disease. Recombinant IL-21 stimulated IL-17 production via PBMCs of VKH patients	Li *et al.*, 2010[15]
BSRC	IL-21, IL-23, and TGF-β may promote differentiation and expansion of a chronic Th17 cell response in BSRC patients	Yang and Foster, 2013[16]
BSRC	Elevated IL-17 intraocular expression in BSRC	Kuiper *et al.*, 2011[17]
BD	Elevated levels of IL-23, IL-17, and IFN-γ by PBMCs and increased frequencies of IL-17- and IFN-γ-producing T-cells in BD patients with active uveitis	Chi *et al.*, 2008[18]
	Levels of IL-17 and IFN-γ-producing CD4+ T-cells are elevated in the peripheral blood of BD patients	Shimizu *et al.*, 2012[19]
	IL-17 expression is elevated in BD patients with active uveitis. Anti-TNF-α therapy inhibits Th17 cell differentiation and may ameliorate intraocular inflammation in BD	Sugita *et al.*, 2012[20]
HLA-B27-associated uveitis	Elevated IL-17 and IFN-γ expression in the peripheral blood of HLA-B27-associated uveitis patients. The increase in Th17 cells and IL-17 may correlate with disease activity	Zou *et al.*, 2014[21]
VKH, BD	Elevated IL-17F and IL-23A gene copy number variants	Hou *et al.*, 2015[22]

VKH: Vogt-Koyanagi-Harada, BSRC: Birdshot retinochoroidopathy, BD: Behçet's disease, PBMCs: Peripheral blood mononuclear cells, IFN-γ: Interferon gamma, IL: Interleukin, TGF: Transforming growth factor, HLA: Human leukocyte antigen

toxoplasmic and viral uveitis. The authors found that IL-17 was upregulated in the majority of screened patients with toxoplasmic uveitis when compared to cataract patients without uveitis or with those with noninfectious intermediate uveitis. The authors suggested that the presence of elevated levels of IL-17 in the intraocular fluids of these patients may be a clue to a possible autoimmune mechanism that contributes to ocular inflammation following infection.[33] These findings were reinforced in a study addressing various cytokine levels in the aqueous humor of patients with uveitis from various etiologies - infectious and noninfectious. Patients with toxoplasmic infection and subsequent uveitis had significantly increased intraocular levels of IL-17A, whereas patients with viral uveitis showed increased IL-1β and IL-10 expression.[34] The authors postulate that this different cytokine pattern in intraocular fluids may help understand disease pathogenesis and even be used as a specific diagnostic marker for each etiology.

Another recent study highlighted the upregulation of IL-17 expression in patients with acute ocular toxoplasmosis and demonstrated a pathogenic role for this cytokine in the development of intraocular inflammation after *T. gondii* infection. The authors proposed a possible *in vivo* therapeutic approach for toxoplasmic retinochoroiditis based on the use of local anti-IL-17 antibodies.[35]

Interleukin-17 Induction

Several studies have shown that TGF-β and IL-6 are important cytokines in Th17 differentiation, and that IL-23 and IL-21 may also be crucial in Th17 cell expansion and activation [Table 2 and Fig. 1].

Interleukin-17 Suppression

Interferon-γ

Antigen-specific IL-17 production is exacerbated in the absence of IFN-γ increasing the severity of uveitis in a murine model of spondyloarthritis. In this model, IL-17 blocking ameliorated intraocular inflammation in IFN-γ knockout mice, suggesting the regulatory function that this cytokine plays in IL-17 production.[45] Moreover, IL-17 expression was increased in the peripheral blood of patients with uveitis and scleritis and also in an EAU model. In the animal model used in this study, IFN-γ upregulated IL-27 expression by retinal

Table 2: Cytokines involved in interleukin-17 induction

Cytokines involved in IL-17 upregulation	Main conclusions	Authors, year of publication
TGF-β, IL-6, IL-23	IL-23 may expand the Th17 cell population, but it cannot induce this subset×s differentiation from T-cell precursors. TGF-β induces Treg differentiation. The association between TGF-β and IL-6 induces Th17 cell differentiation	Bettelli *et al.*, 2006[29]
IL-6	IL-6 signaling blockade inhibits Th17 differentiation and induces the antigen-specific Treg formation in EAU	Haruta *et al.*, 2011[36]
	Early treatment with an anti-IL-6-receptor monoclonal antibody ameliorated mice EAU and inhibited Th17 cell differentiation	Hohki *et al.*, 2010[37]
IL-6, IL-23	EAU induction is impaired in IL-6 and IL-23 KO mice. Blocking of IL-6 and IL-23 ameliorates the clinical course of EAU through interference with Th17 cell differentiation and expansion. The anti-IL-6-receptor antibody ameliorates EAU by suppressing Th17 responses	Yoshimura *et al.*, 2009[38]
IL-21	EAU induction is impaired in IL-21 KO mice. There is decreased IL-17 expression in IL-21 KO mice. IL-21 signaling blockade inhibits IL-17 upregulation in EAU	Wang *et al.*, 2011[39]
	IL-21 and its receptor are upregulated in mouse EAU with increased IL-17 expression	Liu *et al.*, 2009[40]
IL-23	IL-23 is upregulated in the peripheral blood of VKH patients before and after cataract surgery; it is also correlated with intraocular inflammation	Jiang *et al.*, 2010[41]
	IL-23 and IL-1β induce Th17 expansion and the cytokine response	Wilson *et al.*, 2007[42]
	IL-23 serum levels are elevated before and after cataract surgery in BD patients. IFN-γ and IL-27 serum levels are elevated after cataract surgery in BD patients	Jiang *et al.*, 2011[43]
	There is increased IL-17 and IL-23 expression in patients with active BD	Na *et al.*, 2013[44]

IFN-γ: Interferon gamma, TGF: Transforming growth factor, EAU: Experimental autoimmune uveitis, KO: Knockout, VKH: Vogt-Koyanagi-Harada, BD: Behçet's disease, IL: Interleukin

cells which, in turn, inhibited Th17 cell proliferation, reducing uveitis severity and altering its clinical course.[6] Another study involving an EAU animal model also showed that IFN-γ ameliorated uveitis through Th1 and Th17 cell inhibition and IL-10 upregulation.[46] IFN-γ may also play a key role in infectious disease pathogenesis since the presence of anti-IFN-γ autoantibodies seems to be associated with nontuberculous mycobacterial infections.[47]

Interleukin-27

IL-27 is known as a regulatory cytokine that is capable of inhibiting the differentiation of precursor cells into their Th17 phenotype. By blocking the production of Th17 cells, this cytokine has been implicated in the suppression of experimental autoimmune encephalomyelitis and EAU.[6,48] A previous investigation addressing the regulatory cytokines, IL-27 and IL-10, in the development of uveitis found that mice retinal microglia and ganglion cells constitutively expressed IL-27, and that IL-27 production was elevated during uveitis.[49]

IL-27 expression was found to be decreased in BD patients with active uveitis,[50] and another study demonstrated elevated levels of IL-27 after cataract surgery in VKH patients, indicating that the upregulation of this cytokine during the 1st month following surgery might serve a protective function in postoperative inflammation.[43] Similar results were found in BD patients after cataract surgery as increased IL-27 serum levels were also evident during the postoperative period. These IL-27 levels correlated both with uveitis severity and IFN-γ levels.[43]

Therapeutic Targets

Anti-interleukin-17

An anti-IL-17 monoclonal antibody was used in the treatment of chronic noninfectious uveitis in patients with posterior and anterior segment disease. The treatment featured effects comparable to those of historical control patients with chronic noninfectious uveitis that were treated with infliximab. Specifically, this treatment was associated with improvements in visual acuity and the reduction of intraocular inflammation.[51]

Another study conducted of an animal model of spondyloarthritis demonstrated that IL-17 blockade reduced intraocular inflammation and peripheral arthritis although there was suspicion of retinal toxicity.[45] Treatment of EAU with the anti-IL-17 antibody in rats also showed a reduction in intraocular inflammation and of T-cell proliferation during disease onset.[52]

Secukinumab

Secukinumab, a human monoclonal antibody against IL-17, was used in a Phase III trial for the treatment of chronic noninfectious uveitis associated with BD; however, the

study's primary outcome was not met (SHIELD study). Some authors have since claimed that the use of secukinumab in chronic uveitis has not been correctly assessed to date, since the other two trials enrolling patients with active and inactive noninfectious uveitis not associated with BD (INSURE and ENDURE) were interrupted following the termination of SHIELD. In the SHIELD study, although there was no significant difference between the treated patients and controls, there was a reduction in the use of concomitant immunosuppressant drugs and a trend toward a reduction in recurrence.[53]

Recently, another study demonstrated good results, in terms of both efficacy and safety, using intravenous secukinumab in the treatment of 37 patients with active noninfectious intermediate, posterior, or panuveitis who required corticosteroid-sparing immunosuppressive therapy.[48] This may suggest that there is still an opportunity for Phase III clinical trials using this or other monoclonal antibodies against IL-17 (or the IL-17 receptor) for noninfectious uveitis treatment.

Interleukin-17 Pathway

In addition to IL-17, there are other possible candidates for the treatment of noninfectious uveitis.

Ustekinumab, a monoclonal antibody directed at the IL-23 and IL-12 p40 subunit, was already approved for the treatment of psoriasis and it may be a future option for the treatment of uveitis patients, since a different study addressing the therapeutic effect of STA-5326 (another IL-12/IL-23 inhibitor) showed EAU clinical improvement and suppressed IL-17 production.[54]

Tocilizumab, a monoclonal antibody directed against the IL-6 receptor, has been successfully used in the treatment of refractory uveitis[55] and was approved for the treatment of rheumatoid and systemic juvenile idiopathic arthritis. It is currently being studied for use in noninfectious and juvenile idiopathic arthritis-associated uveitis (clinicaltrials.gov). Another monoclonal antibody against the IL-6 receptor, sarilumab, is also currently being studied in a Phase II trial accessing subcutaneous administration in patients with active noninfectious intermediate, posterior, or panuveitis (clinicaltrials.gov).

Conclusions

Various studies have highlighted the importance of the IL-17 pathway in the development of various forms of infectious and noninfectious uveitis. Although the main Phase III trial addressing the use of a human anti-IL-17 monoclonal antibody in Behçet's-associated uveitis has failed to meet its primary outcomes, it seems adequate to continue the investigation concerning neutralization of the primary IL-17 pathway cytokines in the treatment of chronic noninfectious uveitis. Furthermore, the IL-17 pathway may also serve as a therapeutic target for infectious uveitis like toxoplasmic retinochoroiditis although always in addition to infection targeted therapy.

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Conflicts of interest

There are no conflicts of interest.

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INFLAMMATORY DISORDERS

Regulatory T cells and IL-17A levels in noninfectious uveitis

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Abstract

Purpose Regulatory T cells (Tregs) have been intensively studied in a myriad of autoimmune diseases. As for noninfectious uveitis (NIU), results have been contradictory, and studies have failed to demonstrate a consistent reduction in Treg cell frequency in patients with active disease. The present study aims to characterize T lymphocyte subsets, including naïve and memory Tregs as well as their respective CD39 expression, in the peripheral blood of NIU patients. Inflammatory as well as suppressive cytokine profiles were also evaluated.

Methods T cell subpopulations were evaluated by multiparametric flow cytometry using anti-CD3, anti-CD4, anti-CD45, anti-CD45RA, anti-CD197, anti-CD25, anti-CD127, and anti-CD39. Treg cells were defined as CD3 + $CD4^{+}CD25^{hi}CD127^{low}$. A multiplex bead-based immunoassay was used to determine TNF-α, IFN-ɣ, IL-17A, IL-10, and TGF-β levels.

Results Twenty-nine patients with active NIU were included as well as 15 sex- and age-matched controls. There were no significant differences in T lymphocyte subsets, including Tregs, between patients and controls. However, patients with a lower grade of anterior chamber or vitreous inflammatory cellular reaction showed higher memory Treg counts than controls, with no respective increase in CD39+ expression, and a tendency for higher IL-17A levels ($p = 0.06$). This IL-17A elevation was present in the total NIU group ($p = 0.08$) as well as a positive correlation between IL-17A levels and the absolute counts of memory Tregs ($p = 0.013$; $R = 0.465$). Patients with higher IL-17A levels also showed higher serum concentrations of memory ($p = 0.001$) and naïve ($p = 0.003$) Tregs as well as elevated TNF-α ($p < 0.0001$) and IFN-ɣ ($p = 0.016$) levels. Negative correlations were observed between IL-10 and TGF-β levels and the percentages of memory ($p = 0.030$; $R = -0.411$) and total CD39+ Tregs ($p = 0.051$; $R = -0.373$) in the peripheral blood of NIU patients.

Conclusion Our results showed that total Treg levels were not reduced in patients with NIU. Further characterization of Treg subsets, including memory Tregs and respective CD39 expression, may provide additional insight on the role of Treg cells in NIU. Consistent high levels of circulating IL-17A in NIU patients are in accordance with previous studies and reinforce this cytokine's vital role in uveitis pathogenesis and its possible use as a therapeutic target.

Keywords Regulatory T lymphocyte · Memory Treg · Noninfectious uveitis · IL-17A · CD39

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Introduction

Noninfectious uveitis (NIU) is a potentially blinding disease affecting the anterior and/or posterior segments of the eye causing up to 10% of legal blindness in developed countries [1–3].

It can be either idiopathic, mostly local, or secondary and associated with a systemic disease. Human leucocyte antigen-B27 (HLA-B27)-associated uveitis is the most common cause of acute anterior NIU and may (or not) be a part of a systemic inflammatory condition, most frequently an axial spondyloarthritis. Other causes include a myriad of diseases like Behçet's disease (BD), ocular sarcoidosis (OS), and Vogt-Koyanagi-Harada disease (VKH).

The role of regulatory T cells (Tregs) in the pathogenesis of NIU has been accessed by several studies showing that these cells may be decreased in patients with active uveitis [4], BD [5], and VKH disease [6]. Tregs are responsible for the production of anti-inflammatory cytokines, which include transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) and have a potential suppressive role in disease activity. Unfortunately, these results have been difficult to interpret since other studies failed to show this reduction [3, 7].

CD4 + CD25 + Foxp3+ T cells are widely considered to be the classic Treg population. However, Foxp3 is an intracellular molecule, and its detection requires fixation and permeabilization of cells. Consequently, some authors took advantage of an alternatively described phenotype: the co-expression of CD4 and CD25 associated with the absence or the low expression of CD127 (α-chain of IL-7 receptor). A good correlation between these two phenotypes was reported in healthy subjects, autoimmune diseases, and HIV infected patients [8–11].

Besides CD4, CD25, and CD127, the expression of CD39 has been considered to be of interest in this population. To avoid ATP-induced pathological effects, ATP can be hydrolyzed into adenosine and phosphate by a cascade of enzymes, of which CD39 is the most important. CD39 has been reported to be found on the surface of human and murine naive Tregs. More importantly, CD39+ CD4 + T cells do express Foxp3, and it has been confirmed that CD39 is predominantly expressed on human CD4+ Foxp3+ T cells and that its expression level is proportional to the Foxp3 expression level [12, 13]. These findings suggested that the CD39 surface marker can be successfully used for routine isolation of functionally active human Tregs [13].

Further differentiation of Treg subsets has also been accessed in autoimmune disease. A previous study addressing resting, effector, and memory Tregs as well as CD39 expression in Tregs of type 1 diabetes patients (T1D) [14] found that in these patients, Tregs contained a higher percentage of memory Tregs than healthy controls and that CD39 expression on Tregs and memory Tregs was lower than controls. The authors concluded that a defective suppressive function of Tregs in T1D patients was due to a reduced CD39 expression on memory Tregs. Another study assessing CD39 expression by Treg lymphocytes in patients with inflammatory bowel disease (IBD) also found that CD39 expression was lower in active disease and increased significantly after treatment [15]. To our knowledge, Treg subsets and CD39 expression have not been previously studied in NIU.

Regarding Th17 cells and IL-17 expression, an increase on the Th17/Treg cells ratio has been recently demonstrated in patients with HLA-B27-associated uveitis [16]. These patients also showed a positive correlation between this ratio and disease activity score. When analyzing this Th17/Treg balance in BD, another study showed a disturbed ratio and increased IL-17 levels as well as decreased IL-10 levels when comparing to controls [17]. In fact, interleukin-17 (IL-17), as well as other pro-inflammatory cytokines in the IL-17 pathway, seems to play a key role in uveitis pathogenesis [18]. Previous studies have shown an elevated IL-17 expression in VKH [19], BD [20], birdshot retinochoroidopathy [21], and HLA-B27-associated uveitis [22].

Besides IL-17, tumor necrosis factor-α (TNF-α) expression may also account for disease progression and severity [3, 23, 24], and TNF-α antagonists are frequently used as rescue therapy in a clinical setting.

The purpose of the present study was to evaluate different T lymphocyte subpopulations, including CD4+ Tregs and their subsets as well as the respective CD39 expression in the peripheral blood of patients with active NIU. Although different etiologies were included, a subset of patients with HLA-B27-associated uveitis was analyzed separately. The cytokine expression profile of patients with active disease was also evaluated for inflammatory (IL-17A, TNF-α, interferon-ɣ (IFN-ɣ)) and anti-inflammatory (TGF-β and IL-10) cytokines.

Methods

Patients

Patients were recruited from the Ophthalmology Department of Egas Moniz Hospital, West Lisbon Hospital Center, between October 2014 and October 2016.

All patients presented with active NIU from multiple causes. The diagnosis of active NIU followed the clinical criteria based on inflammatory cell reaction in the anterior chamber or vitreous as per standardization of uveitis nomenclature (SUN) and National Eye Institute (NEI) grading systems [25, 26]. Active chorioretinal lesions and vasculitis were evaluated by indirect ophthalmoscopy, fundus autofluorescence, and fluorescein angiography. Any mentioned inflammatory sign (i.e., anterior chamber cell ≥ 0.5+, vitreous cells ≥ 0.5+, active retinal vasculitis, or active chorioretinal lesions) was enough to be eligible.

VKH disease was diagnosed according to the criteria established at the International Workshop on VKH disease [27]. HLA-B27-associated AAU was diagnosed by HLA typing of peripheral blood cells and typical ocular manifestations [28]. BD was diagnosed according to the criteria of the International Team Group for the Revision of the International Criteria for BD [29]. Tubulointerstitial nephritis and uveitis syndrome (TINU) was diagnosed based on the reports of Mandeville et al. [30] and Mackensen and Billing [31].

Data collected from patients included demographic information, diagnosis, anatomical location according to the SUN criteria [26], and disease activity.

The control group included sex- and age-matched healthy subjects without any history of autoimmune disease or intraocular inflammation.

Detecting T lymphocyte subpopulations by flow cytometry

Ten milliliter of fasting blood were collected from patients and controls using ethylene diamine tetraacetic acid (EDTA) tubes. Multiparametric flow cytometry was performed, 100 microliters (μL) of all samples were labeled with the antibody combinations in one tube as follows: CD197 BV241 (Clone 150,503), CD45 V500 (Clone 2D1), CD45RA FITC (Clone HI100), CD25 PE (Clone M-A251), CD4 PerCP-Cy5.5 (Clone SK3), CD39 PE-Cy7 (Clone A1), CD127 APC (Clone A019D5), and CD3 APC-H7 (Clone SK7).

Reagents were purchased from either Becton-Dickinson Biosciences or BioLegend. All incubations were performed at room temperature in the dark. Cells were incubated with appropriately titrated, fluorescently labeled antibodies for 15 min. Cell suspensions were then lysed and fixed with 2 milliliters (mL) of FACS Lyse (Becton Dickinson) and centrifuged for 240 s at 1200 *g*. The supernatant was aspirated and discarded. The cells were then washed with 2 mL of CellWash (Becton Dickinson) and resuspended with 500 μL of FACSFlow (Becton Dickinson) prior to analysis. For each measurement more than 400,000 events were typically acquired on a 3-laser FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA) with BD FACSDiva software. The flow cytometry data was analyzed using Infinicyt 2.0 software (Cytognos S.L., Spain).

The T lymphocyte subpopulations analyzed included CD4+ T cells, naïve CD4+ T cells (CD3 + CD4 + CD45RA + CD197+), memory CD4+ T cells (CD3 + CD4 + CD45RA-CD197±), and effector CD4+ T cells (CD3 + CD4 + CD45RA + CD197−). Tregs were defined as CD3 + $CD4^{+}CD25^{hi}CD127^{low}$cells as previously described [23]. Naïve and memory Treg subsets were also identified including their respective CD39 expression (Fig. 1).

For each sample, the absolute cell counts were calculated by multiplying the fraction of each population by the total white blood cell count derived from the complete blood count.

Quantification of serum cytokine levels by multiplexed flow cytometry

A multiplex bead-based immunoassay (BD CBA Flex Set, BD Biosciences, San Jose, CA, USA) was used to determine the serum levels of TNF-α, IFN-γ, IL-17A, and IL-10. A similar single-plex bead-based immunoassay was used for TGF-β.

The protocol was performed following the instructions of the manufacturer. In brief, standards and serum samples were incubated with specific capture beads for 1 h at room temperature. After adding the detection reagent, the mixtures were incubated for 2 h at room temperature in the dark. After a final wash, beads were acquired in a BD FACS Canto II, previously set up for the BD CBA Flex Set. For each cytokine, a minimum of 300 beads were acquired per sample. The FCAP Array Software (BD Biosciences) was used for data analysis. Standard curves covered a 0–2500 pg/mL concentration range and the minimum detection levels were 0.13 pg/mL for IL10; 0.3 pg/mL for IL17; 1.8 pg/mL for IFN-γ; and 0.7 pg/mL for TNF-α.

For TGF-β, analyzed separately, samples were previously activated with the Sample Activation Kit 1 (R&D, Minneapolis, MN, USA) according to the recommended procedure. After activation, samples were incubated with capture beads for 2 h, washed, and incubated with detection reagent. Acquisition and analysis were performed as described above. For TGF-β, standard curves covered a 0–10,000 pg/mL concentration range, and minimum detection level was 14.9 pg/mL.

Statistical analysis

Categorical variables were expressed as absolute frequencies and percentages and analyzed using the Fisher's exact test. Normality of distribution was assessed using the D'Agostino and Pearson test. Normally distributed data are presented as mean (SD) and nonnormally distributed data as median (IQR). The unpaired *t* test or Mann-Whitney test were used to compare each 2 independent groups. A p value of < 0.05 indicated the presence of a statistically significant difference. The Spearman's rank correlation test was used to analyze correlations between cytokine levels and T lymphocyte subsets. Data were analyzed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

A total of 29 patients with a clinical diagnosis of active non-infectious uveitis were recruited. Regarding the different diagnosis, 3 had idiopathic disease, 20 had HLA-B27-associated uveitis, 3 had BD, 2 had VKH disease, and 1 had TINU. Patients with HLA-B27-associated disease were also analyzed separately.

Patient demographics are listed in Table 1. NIU patients included 13 males and 16 females, with an average age of 47 (range, 24–80 years old). At the time of sampling, all patients had active disease, and blood samples were collected at

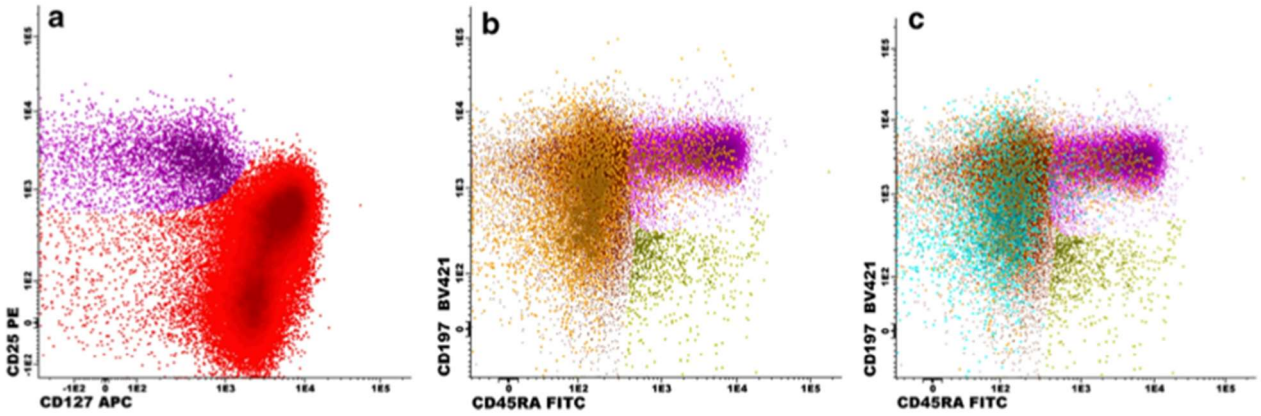

Fig. 1 **a** CD4 + CD3+ auxiliary T-cells in red, CD4 + CD3 + $CD25^{hi}CD127^{low}$ Treg population in violet. **b** Naïve (CD45RA + CD197+) CD4+ T cells in purple, memory (CD45RA-CD197±) CD4+ T cells in brown and effector (CD45RA + CD197-) CD4+ T cells in green. The Treg population in gold is mostly concentrated in the memory subset. **c** The CD39+ Treg cell subset in turquoise overlaying the previously displayed subsets

presentation. This was the first episode of symptomatic intraocular inflammation for all the patients included, and none had received previous topical or systemic treatment for ocular and/or extraocular symptoms.

Characterization of lymphocyte subsets in NIU patients and controls

In this study, we characterized several circulating T cell subsets, but no significant differences were found between patients and controls for any of the studied CD4 subpopulations, including the regulatory subsets and respective CD39 expression (Table 2).

Taking in consideration the distinct etiologies present in the NIU group, we further isolated HLA-B27+ patients and compared it both to healthy controls and to other causes of NIU. However, again, no differences were found in both comparisons for any of the studied T cell subsets.

Serum cytokine levels in NIU patients and controls

As for cytokine evaluation (Fig. 2), a tendency for increased levels of IL-17A was found in the NIU group ($p = 0.08$) compared to healthy controls (Fig. 3). No further differences were observed for the other cytokines evaluated. Again, we performed similar comparisons for the HLA-B27-associated subgroup. However, no differences were encountered in these comparisons for any of the cytokines evaluated.

We then assessed how cytokine levels correlated to the cellular subsets studied. As for the control group, only a positive correlation was observed between TGF-β levels and the percentages of total CD4 Tregs ($r = 0.604$; $p = 0.017$). Interestingly, the NIU group presented a distinct profile of correlations. We observed a negative correlation between IL-10 levels and the percentages of memory Tregs in the peripheral blood of NIU patients ($p = 0.030$; $R = -0.411$). This correlation was also present in the HLA-B27-associated uveitis subset ($r = -0.411$; $p = 0.007$). Similarly, we observed a tendency for a negative correlation between TGF-β and the percentages of total CD39+ Tregs in the NIU group ($r = -0.373$; $p = 0.051$).

Regarding the evaluated pro-inflammatory cytokines, a positive correlation between IL-17A levels and the absolute counts of memory Tregs was found ($r = 0.465$; $p = 0.013$) in the NIU group (Fig. 4a). We also observed a positive correlation between TNF-α and the absolute counts of both total memory Tregs and $CD39^+$ memory Tregs ($r = 0.418$; $p = 0.027$) in the NIU group (Fig. 4b).

The inflammatory (TNF-α + IFN-γ + IL-17A)/anti-inflammatory (IL-10 + TGF-β) cytokine ratio showed a positively correlation with the absolute counts of both memory Tregs and $CD39^+$ memory Tregs in the NIU group ($r = 0.406$; $p = 0.032$). In line with this, the IL-17A/IL-10 ratio was also positively correlated with the absolute counts of memory Tregs ($r = 0.417$; $p = 0.030$).

Table 1 Study participants demographic characteristics

	NIU patients $n = 29$	Healthy controls $n = 15$	p value
Age (years) (Mean ± SD)	24–80 (47.2 ± 14.4)	20–86 (44.7 ± 18.6)	0.683[a]
Male sex (%)	13 (44.8%)	8 (53.3%)	0.752[b]

[a] Unpaired t test

[b] Fisher's exact test

Age and T lymphocyte subsets and cytokine levels

Since the mean age in both the patient and healthy donors group was similar (47.2 for patients and 44.7 for controls)

Table 2 Comparison of Tregs subsets and cytokines between NIU patients and controls.

					Group's comparisons			
					Total NIU vs control	NIU low vs high severity	NIU low severity vs control	NIU high severity vs control
T cell subsets	Total NIU ($n = 29$)	NIU low severity ($n = 13$)	NIU High severity ($n = 16$)	Control ($n = 15$)	*p values			
Cells/μL, median (IQR)								
Total Tregs	69 (35)	70 (36)	67 (32)	57 (40)	0.107	0.738	0.121	0.220
Naive Tregs	16 (14)	16 (22)	18 (11)	14 (16)	0.271	0.416	0.593	0.198
CD39+ naive Tregs	2 (2)	2 (1)	2 (2)	2 (1)	0.908	0.902	0.819	1.000
Memory Tregs	50 (18)	52 (24)	46 (15)	33 (33)	0.112	0.210	0.053	0.384
CD39+ memory Tregs	29 (14)	32 (13)	27 (21)	26 (20)	0.380	0.089	0.127	0.930
Percentages, median (IQR)								
(Within CD4+ T cells)								
Total Tregs (within CD4+ T cells)	7.97 (2.25)	7.97 (1.81)	7.91 (2.69)	7.43 (1.22)	0.329	0.650	0.474	0.358
Naive Treg	1.89 (1.14)	1.84 (1.38)	2.03 (1.33)	1.81 (0.98)	0.691	0.101	0.503	0.220
CD39+ naive Tregs (within CD4+ T cells)	0.16 (0.17)	0.18 (0.17)	0.16 (0.18)	0.21 (0.18)	0.429	0.905	0.547	0.464
Memory Treg	5.5 (1.78)	5.8 (1.87)	5.34 (2.15)	5.27 (1.18)	0.664	0.812	0.578	0.838
CD39+ memory Tregs (within CD4+ T cells)	3.36 (2.07)	3.79 (2.20)	3.05 (2.76)	3.23 (1.40)	0.646	0.288	0.274	0.838
(Within total Tregs)								
Total CD39+ Tregs	48.18 (23.19)	53.02 (20.07)	40.08 (26.99)	44.65 (15.89)	0.807	0.083	0.235	0.545
Naive Treg	27.86 (18.08)	22.59 (15.92)	28.08 (20.01)	28.02 (7.22)	0.695	0.068	0.185	0.626
Memory Tregs	72.14 (18.09)	77.41 (15.93)	71.93 (20.01)	71.98 (7.22)	0.695	0.068	0.185	0.626
CD39+ memory Tregs	47.17 (22.98)	52.00 (21.48)	38.89 (26.12)	42.97 (15.91)	0.788	0.062	0.217	0.545
Serum cytokine Levels	($n = 28$)	($n = 12$)						
pg/mL, median (IQR)								
IL-10	1.40 (1.23)	1.44 (1.21)	1.37 (1.83)	1.22 (1.27)	0.380	0.673	0.548	0.395
IL-17A	2.295 (5.79)	2.73 (5.76)	1.91 (5.71)	1.20 (3.73)	0.080	0.766	0.060	0.241
TNF-α	0.28 (1.80)	0.30 (1.54)	0.28 (2.14)	0.00 (0.82)	0.129	0.976	0.231	0.159
INF-γ	2.835 (3.26)	2.91 (5.40)	2.74 (2.76)	2.81 (3.25)	0.743	1.000	0.792	0.800
TGF-β	2042 (640)	2007 (550)	2139 (718)	1962 (671)	0.650	0.397	0.943	0.446

Patients were further divided according to anterior chamber cellular reaction (high severity, tyndall grade > 2+ cell reaction; low severity, tyndall grade <= 2+ cell reaction)

NIU, noninfectious uveitis; IQR, interquartile range

Results are presented as medians and interquartile range, median (IQR)

*Mann-Whitney U test

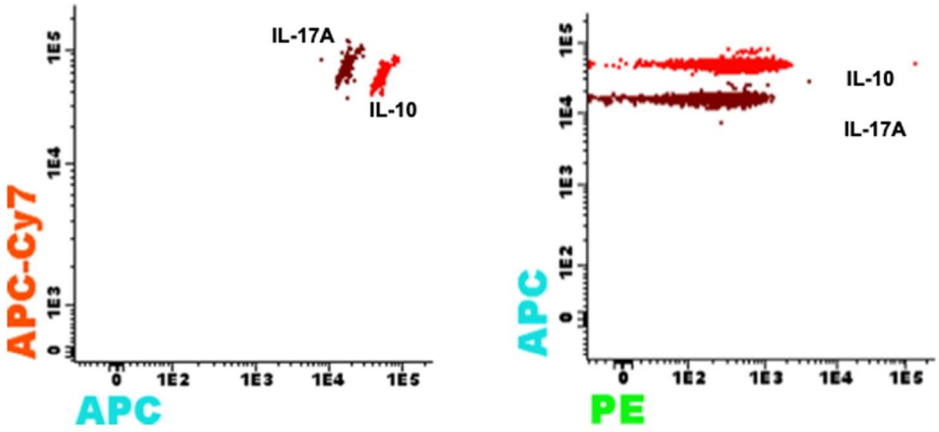

Fig. 2 Representation of serum cytokine levels measurement by multiplexed flow cytometry. **a** Identification of IL-17A (dark red) and IL-10 (red) CBA beads. **b** Determination of IL-17A and IL-10 serum sample concentration through PE fluorescence intensity

but with a big interval between minimum and maximal ages included (24–80 for patients and 20–86 for controls), we further separated both the patient and the control groups in two subsets–below (patients $n = 15$, controls $n = 10$) and over (patients $n = 14$, controls $n = 5$) 45 years old (y.o.) and compared cytokine levels as well as T lymphocyte subpopulations.

We found a significant increase in total memory CD4+ T-cells in NIU patients younger than 45 years old that was not present in controls ($p = 0.009$) as well as an increase in IFN-ɣ levels ($p = 0.004$).

IL-17A levels and T lymphocyte subsets and other cytokine levels

As to evaluate IL-17A levels in NIU-patients, we separated both patients and controls in two groups–below (patients $n = 14$, controls $n = 10$) and over (patients $n = 15$, controls $n = 5$) 2.17 pg/mL (mean + S.E.M. for IL-17A levels in the control group)–and analyzed possible correlations with T lymphocyte subsets and other cytokine levels in peripheral blood.

We found that patients with higher IL-17A levels also showed higher serum concentrations of memory ($p = 0.001$) and naïve ($p = 0.003$) Tregs as well as higher TNF-α ($p < 0.0001$) and IFN-ɣ ($p = 0.016$) levels.

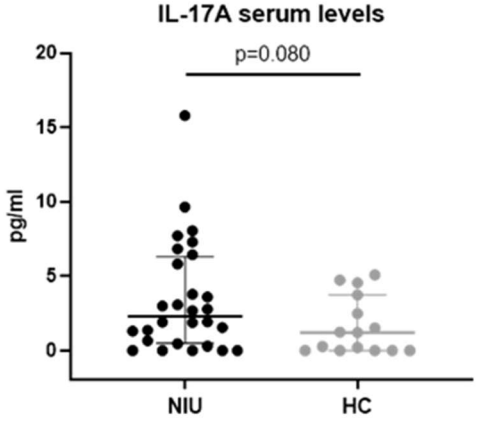

Fig. 3 Scatter dot plot (median with IQR) of serum IL-17A levels in NIU and HC groups. NIU, noninfectious uveitis; HC, healthy controls

Anterior chamber cellular reaction or vitreous haze severity and T lymphocyte subsets and cytokine levels

Patients were further divided according to inflammatory cell reaction in the anterior chamber or vitreous as per standardization of uveitis nomenclature (SUN) and National Eye Institute (NEI) grading systems [25, 26] in two groups:

- High severity (tyndall grade > 2+ cell reaction or Vitreous Haze > 2+)
- Low severity (tyndall grade <= 2+ cell reaction or Vitreous Haze <= 2+)

Sixteen patients were included in the NIU-high severity group (HLA-B27-associated uveitis, $n = 12$; BD, $n = 1$; TINU, $n = 1$; idiopathic disease, $n = 2$) and thirteen in the NIU-low severity group (HLA-associated uveitis, $n = 8$; BD, $n = 2$; VKH, $n = 2$; idiopathic disease, $n = 1$) (Table 2).

Comparing to controls, there was a tendency for increased memory Treg counts in the NIU-low severity group ($p = 0.053$) but with no difference in CD39+ memory Treg counts. There were also no differences in both these subsets between NIU-high severity patients and healthy subjects. Moreover, when analyzing percentages of Treg subsets within total Tregs, a tendency for higher levels of naïve Tregs ($p = 0.068$), lower memory Tregs ($p = 0.068$), and CD39+ memory Tregs ($p = 0.062$) were also present in the NIU-low severity group compared to high severity patients.

Regarding serum cytokine levels, a tendency for higher IL-17A concentrations was also found in the NIU-Low severity group when comparing to controls ($p = 0.06$).

Finally, in the serum of NIU-low severity patients, positive correlations were found between IL-17A and TNF-α levels and memory Treg (respectively, $r = 0.767$; $p = 0.005$ and $r = 0.826$; $p = 0.002$) and CD39+ memory Treg counts

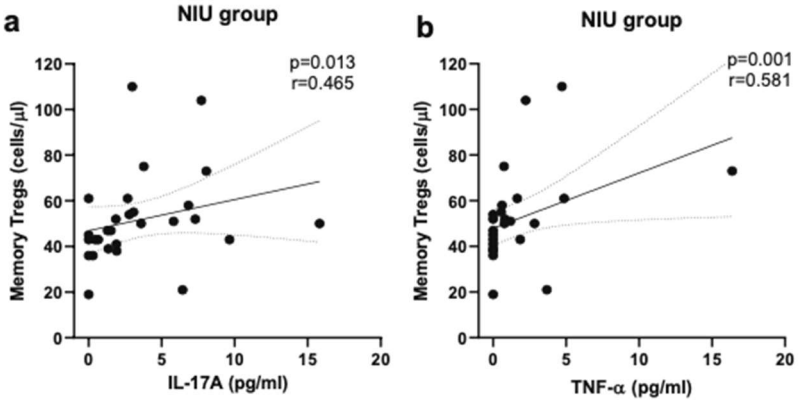

Fig. 4 Memory Tregs counts correlations with **a** IL-17A and **b** TNF-α serum levels in NIU group. Spearman's correlation coefficients and *p* values are indicated. NIU, noninfectious uveitis

(respectively, $r = 0.586$; $p = 0.049$ and $r = 0.714$; $p = 0.012$). As for the NIU-high severity group, a negative correlation was found between serum IL-10 levels and both percentages of total Tregs and memory Tregs (respectively, $r = 0.518$; $p = 0.042$ and $r = 0.553$; $p = 0.029$) (Fig. 5).

Discussion

Regulatory T (Treg) cells are important for the regulation of the immune response and are responsible for the production of anti-inflammatory cytokines, which include transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) and have a potential suppressive role in disease activity.

In the present work, we analyzed Treg cell levels and subsets as well as cytokine production in the peripheral blood of patients with active NIU, including a subset of 20 patients with HLA-B27-associated uveitis.

Several studies have found decreased levels of Treg cells in patients with active uveitis from various causes [4–6] which led to a growing interest in establishing Tregs as biomarkers in uveitis and possibly as a future therapeutic target [32]. However, results have been contradictory as other authors did not find this association [3, 7].

While looking at 20 patients with active HLA-B27-associated uveitis, a previous study found an increased frequency of $CD4^+IL\text{-}17^+$ T cells, a decreased percentage of $CD4^+CD25^+Foxp3^+$ Treg cells, and an increase in the Th17/Treg ratio when comparing patients to healthy subjects [16]. In the present work, when studying $CD4^+CD25^{hi}CD127^{low}$ cell levels in NIU patients, we did not find significant differences from controls, although this may be related to a different strategy used to characterize Tregs.

A recent study has also assessed different Treg subsets in autoimmune disease and found that the Treg compartment of patients with T1D Tregs contained a higher percentage of memory Tregs than healthy controls [14]. When analyzing the CD39 expression of these Treg subpopulations, the same authors observed that there was a decreased expression of this surface marker in memory Tregs, concluding that this may impair their suppressive function. A lower expression of CD39 in Tregs was also found in patients with active IBD [15]. To our knowledge, the memory Treg subpopulation and respective CD39 expression has never been studied in NIU. In our patients, there were no significant differences in memory Treg levels (percentage and absolute counts) between patients and controls. However, when further analyzing patients with a lower grade of anterior chamber or vitreous inflammatory cellular reaction, we found a tendency for higher memory Treg counts in these patients compared with controls with no respective increase in CD39+ expression. This may be related to an impaired memory Treg function since there was also a tendency for higher IL-17A levels in this group. The fact that these differences were not found in the NIU-high severity group may be related to the different distribution and heterogeneity of the uveitic conditions included in each group.

We also observed a negative correlation between IL-10 levels and the percentages of memory Tregs in the peripheral blood of NIU patients, including in the HLA-B27-associated subgroup. A tendency for a negative correlation was also found for TGF-β and the percentages of total CD39+ Tregs in the NIU group. Although IL-10 and TGF-β are suppressive cytokines and produced by Tregs, this may be explained by the fact that all these patients were in a very early stage of disease and Treg induction or that there is in fact an impaired regulatory cytokine production by Tregs in NIU patients.

It would therefore seem reasonable to conclude that, when studying autoimmune diseases, it is insufficient to only assess total Treg levels, although more studies are needed to verify the possible association between memory Treg (and respective CD39+ expression), regulatory cytokine production, and NIU.

Regarding inflammatory cytokine expression, a tendency for IL-17A elevation was found in the NIU group ($p = 0.08$), and it would have been interesting to study $CD4^+IL\text{-}17^+$ T cells frequency in these patients. The Th17/Treg ratio may be more specific for disease activity than isolated Treg levels,

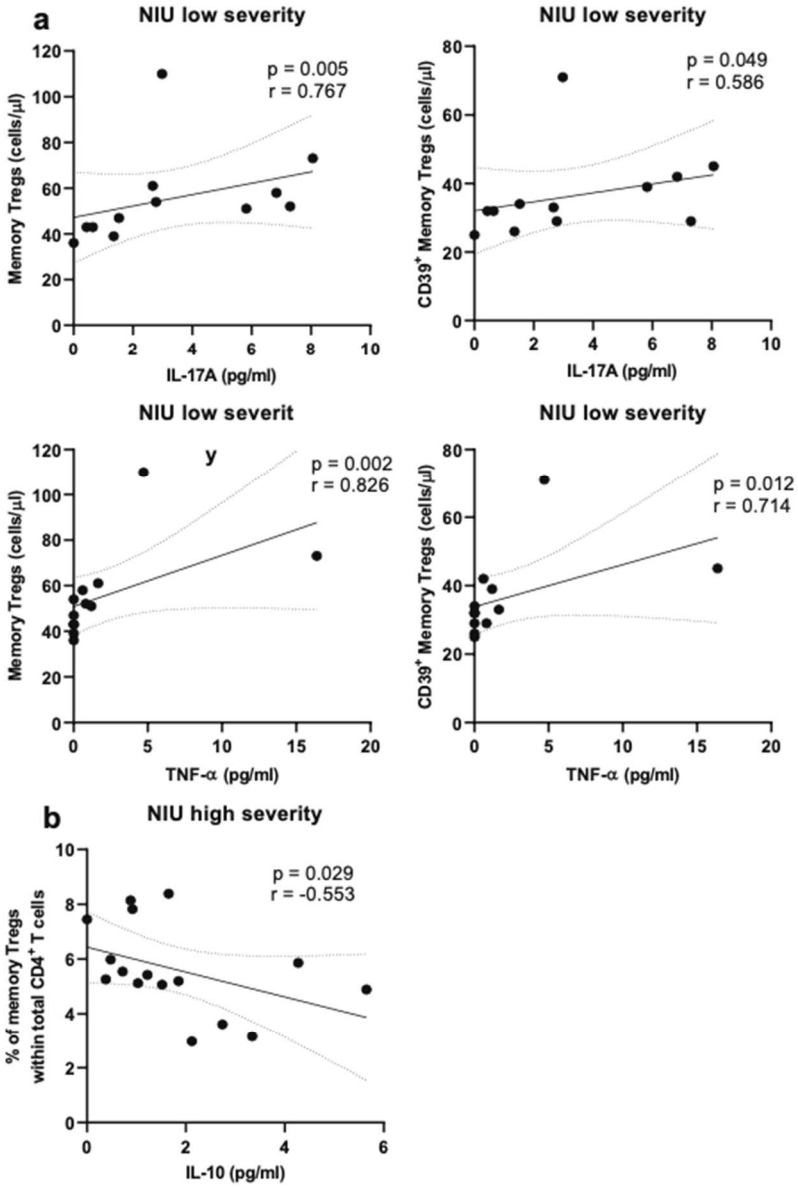

Fig. 5 Correlation between serum cytokine levels and the distribution of Tregs subsets in **a** NIU patients with low severity and **b** NIU patients with high severity. Spearman correlation coefficients and *p*-values are indicated

and further studies can possibly warrant its usage as an active disease biomarker. Although we did not have data on Th17 cell levels, when analyzing the inflammatory (TNF-α + IFN-γ + IL-17A)/anti-inflammatory (IL-10 + TGF-β) cytokine ratio and the IL-17/Il-10 ration, we did find a positive correlation with the absolute counts of memory Tregs in the NIU group. We also found that higher IL-17A levels were associated with higher serum concentrations of memory and naïve Tregs as well as higher TNF-α and IFN-γ levels. This may represent a Treg cell induction in an effort for suppressive cytokine production in early stages of intraocular inflammation or activation of a specific IL-17-producing Treg subset. In fact, although conventional Tregs exert their suppressive effect via production of anti-inflammatory cytokines such as IL-10 and TGF-β, there is growing evidence that these cells also secrete pro-inflammatory cytokines in inflammatory conditions [33] and a distinct population of Treg cells that are FoxP3+ and produce IL-17 that has already been identified in patients with Crohn's disease [34] although, to our knowledge, this has never been described in uveitis. Nevertheless, evaluation of Th17 levels could have given further insight on this correlation between IL-17 and memory Tregs.

Finally, we have observed a positive correlation between TNF-α and the absolute counts of both total memory Tregs and $CD39^+$ memory Tregs in the NIU group. In fact, together with IL-17A, TNF-α is a key cytokine in uveitis pathogenesis

[3, 23, 24], and it is understandable that it may show an elevated frequency when there is active disease and memory Treg upregulation.

The main limitation of our study is the small sample size. Moreover, since most patients had untreated concomitant systemic disease at the time of inclusion, it is reasonable to assume that IL-17A and TNF-α levels were also affected by active systemic inflammation. Despite these limitations, results presented here suggest that IL-17A is associated with active NIU and that Treg cell levels alone are insufficient for use as a biomarker for active disease. The further characterization of the interactions between Treg subsets, including memory Tregs and respective CD39 expression, and inflammatory and anti-inflammatory cytokines profiles is mandatory. Finally, the IL-17A elevation present in these patients underlines the importance of the IL-17 pathway in the pathogenesis of NIU and reinforces the possibility of this cytokine use as a future therapeutic target.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The study protocol was approved by the ethics committee of Egas Moniz Hospital, West Lisbon Hospital Center. Informed consent was obtained from each patient. This study was performed in accordance with the Declaration of Helsinki.

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T-Lymphocyte Regulatory Subsets and Inflammatory Cytokine Levels after Treatment of Non-Infectious Uveitis

(Tregs and Cytokines in Non-Infectious Uveitis)

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T-Lymphocyte Regulatory Subsets and Inflammatory Cytokine Levels after Treatment of Non-Infectious Uveitis

Abstract

Introduction: The reduction of Regulatory T-cells (Treg) and IL17A expression have been associated with non-infectious uveitis (NIU). We aimed to characterize Treg subsets and cytokine profiles in NIU patients, before and after treatment.

Methods: Treg subsets were analyzed by flow cytometry using anti-CD3, anti-CD4, anti-CD45, anti-CD45RA, anti-CD197, anti-CD25, anti-CD127 and anti-CD39. TNF-α, IFN-ɣ, IL17A, IL10 and TGF-β serum levels were quantified using a multiplex immunoassay.

Results: Patients with active NIU showed increased percentages of total (p=0,048) and memory (p=0,057) Tregs without significant differences from controls for CD39 expression in both subsets. After treatment, there was a reduction in Treg percentages (p=0,011) and in the serum levels of IL17A (p=0,030) and TNF-α (p= 0,055).

Conclusion: Memory Tregs and their CD39 expression may provide a further insight regarding Treg cell function in NIU, but more studies are needed to support the use of Tregs as biomarkers. IL17A and TNF-α seem to be associated with disease activity.

Keywords: Regulatory T-Lymphocytes; IL17; TNF-α; Non-Infectious Uveitis.

Tregs and Cytokines in Non-Infectious Uveitis

Introduction

Regulatory T-cells (Tregs) have a suppressive role in inflammation, mainly by the production of the inhibitory cytokines interleukin-10 (IL10) and transforming growth factor- β (TGF-β). The recognition of their suppressive abilities led to the enormous number of studies in distinct autoimmune diseases, with the premise that there must be a Treg reduction or dysfunction in the presence of active and uncontrolled inflammation.[1]

In active non-infectious uveitis (NIU), a Treg reduction has been previously demonstrated[2] and has been associated with several causes of active NIU including Behçet´s disease (BD)[3], Vogt-Koyanagi-Harada (VKH) syndrome[4] and HLA-B27-associated anterior uveitis.[5] Furthermore, an upregulation of peripheral Tregs has been associated with disease remission after immunosuppressive treatment.[6] However, this is not consensual, as some studies failed to show a Treg reduction in patients with active NIU.[7, 8] In fact, the study by Molins and collaborators even reported a significant reduction in FOXP3+Treg levels after *in vitro* dexamethasone treatment, in both healthy controls and uveitis patients,[8] when one would expect an increase in Treg levels after treatment.

The possible role of individual Treg subsets in autoimmune disease has also been addressed in recent studies. A study comparing Treg subsets between patients with Type 1 Diabetes (T1D) and controls, found higher percentages of memory Tegs in patients though with lower CD39 expression, denoting a possible impairment in Teg function. [9] Moreover, lower CD39 expression in Treg lymphocytes was observed in inflammatory bowel disease (IBD) patients with active disease, with a significant increase in CD39 expression after treatment. [10] CD39 is present on the surface of human naive Tregs and is predominantly expressed on human CD4+FOXP3+ T cells in a level that seems to be proportional to FOXP3 expression.[11] This has led to the use of CD39 in the isolation of

functionally active human Tregs.[12] These Treg subsets and CD39 expression have not been extensively studied in NIU.

The Th17/Treg ratio has also been addressed in NIU patients showing an elevation during active intraocular inflammation in HLA-B27-associated uveitis and a positive correlation with disease activity score.[5] These data highlight the importance of the inflammatory cytokine IL17A in uveitis[13] and suggest that both the elevation of Th17 cells and the reduction of Tregs may contribute to the activation of the disease. However, this may not be the case for all the uveitic conditions causing NIU. A recent study involving patients with Birdshot corioretinopathy (BSCR) showed that the proportion of $CD4^{+}CD25^{hi}CD127^{low}$ Tregs was higher in treated and untreated BSCR patients than in controls, and that the levels of $CD4^{+}CD25^{hi}CD127^{low}$ and Th17 cells were not significantly different between treated and untreated patients.[14] This heterogeneity in the different conditions causing NIU and the different strategies used to characterize Tregs may contribute to conflicting results and delay their possible use as biomarkers or even as therapeutic targets in NIU.

The present work aimed to characterize the Treg subsets in NIU patients, before and after treatment. Inflammatory (IL17A, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ)) and suppressive (TGF-β and IL10) cytokines were also evaluated using a multiplex bead-based immunoassay.

Materials and Methods

Patients

This study was performed in accordance with the Declaration of Helsinki. All patients were recruited between January 2015 and December 2016. The study protocol was approved by the ethics committee and informed consent was obtained from each patient.

All patients were over 18 years-old and were diagnosed with NIU at the time of inclusion. The diagnosis of active NIU followed the clinical criteria based on inflammatory cell reaction in the anterior chamber or vitreous as per Standardization of Uveitis Nomenclature and National Eye Institute grading systems.[15] Active chorioretinal lesions and vasculitis were evaluated by indirect ophthalmoscopy, fundus autofluorescence and fluorescein angiography. Any mentioned inflammatory sign (i.e. anterior chamber cell ≥0.5+, vitreous cells ≥0.5+, active retinal vasculitis or active chorioretinal lesions) was enough to be eligible.

VKH disease was diagnosed according to the criteria established at the International Workshop on VKH disease.[16] HLA-B27-associated AAU was diagnosed by HLA typing of peripheral blood cells and typical ocular manifestations.[17] BD was diagnosed according to the criteria of the International Team Group for the Revision of the International Criteria for BD.[18]

Patient´s blood samples were collected at the time of diagnosis and at least three months after treatment. For patients to be included, uveitis resolution had to be obtained after treatment. For the uveitis to be considered inactive, no inflammatory signs could be present (i.e. no anterior chamber cell reaction, no vitreous cells and no active retinal vasculitis or chorioretinal lesions).

Detecting Regulatory T-Lymphocyte Subsets

Peripheral blood was collected from patients and controls using Ethylene Diamine Tetra-acetic Acid (EDTA) tubes. Multiparametric flow cytometry was performed: 100 microliters (μL) of all samples were labeled with the antibody combinations in one tube as follows: CD197-BV241 (Clone 150503, BDBiosciences™), CD45-V500 (Clone 2D1, BDBiosciences™) , CD45RA-FITC (Clone HI100, Biolegend™), CD25-PE (Clone M-A251, Biolegend™), CD4-PerCP-Cy5.5 (Clone SK3, BDBiosciences™), CD39-PE-Cy7 (Clone A1, Biolegend™), CD127-APC (Clone A019D5, Biolegend™), CD3-APC-H7 (Clone SK7, BDBiosciences™).

All incubations were performed at room temperature in the dark. First, cells were incubated with appropriately titrated, fluorescently labeled antibodies for 15 minutes. Cell suspensions were then lysed and fixed with with 2mL of BD FacsLyse (BD Biosciences) for ten minutes, and centrifuged for 300 seconds at 1200 x g. The supernatant was aspirated and discarded. The cells were then washed with 2 mL of CellWash (BD Biosciences) and resuspended with 500 μL of BD FacsFlow (BD Biosciences) prior to acquisition. For each measurement more than 400,000 events were typically acquired on a 3-laser BD FACSCanto II flow cytometer (BD Biosciences) with BD FacsDiva software. Set up and validation of the method included tube replicates in some samples.

The flow cytometry data were analyzed using Infinicyt 2.0 software (Cytognos, Spain). To improve reproducibility a unique template and strategy of analysis was created and applied to all the files. In fact, all the files were analyzed by two different experts with similar results (data not shown).

The analysis of the $CD4^+$T-cells included the following subpopulations:, Tregs ($CD3^+CD4^+CD25^{hi}CD127^{low}$cells), naïve $CD4^+$ T-cells ($CD3^+CD4^+CD45RA^+CD197^+$), memory $CD4^+$ T-cells ($CD3^+CD4^+CD45RA^-CD197^{+/-}$) and effector CD4+ T-cells ($CD3^+CD4^+CD45RA^+$CD197-). Tregs were defined as $CD3^+CD4^+CD25^{hi}CD127^{low}$cells.

Naïve and memory Treg subsets were also identified as well as their respective CD39 expression according to the above described phenotypes [Fig. 1].

Cells were assessed in absolute counts and percentage values ($CD4^+$-T cells as a percentage of total $CD3^+$-T cells; naïve, memory, effector and Treg subsets as a percentage of total $CD4^+$-T cells; and naïve and memory Tregs, as a percentage of total Tregs).

For each sample, the absolute cell counts were calculated by multiplying the fraction of each population by the total white blood cell count derived from the complete blood count.

Quantification of Serum Cytokine Levels by Multiplexed Flow Cytometry

A multiplex bead-based immunoassay (BD CBA Flex Set, BD Biosciences) was used to determine the serum levels of TNF-α, IFN-γ, IL17A and IL10. A similar single-plex bead-based immunoassay was used for TGF-β.

The protocol was performed following the instructions of the manufacturer. In brief, standards and serum samples were incubated with specific capture beads for one hour at room temperature. After adding the detection reagent, the mixtures were incubated for two hours at room temperature in the dark. After a final wash, beads were acquired in a BD FACSCanto II (BD Biosciences), previously set up for the BD CBA Flex Set. For each cytokine, a minimum of 300 beads were acquired per sample. The FCAP Array Software (BD Biosciences) was used for data analysis. Standard curves covered a 0–2,500 pg/mL concentration range and the minimum detection levels were: 0.13 pg/mL for IL10; 0.3 pg/mL for IL17; 1.8 pg/mL for IFN-γ and 0.7 pg/mL for TNF-α.

For TGF-β, analyzed separately, samples were previously activated with the Sample Activation Kit 1 (R&D, Minneapolis, MN, USA) according to the recommended

procedure. After activation, samples were incubated with capture beads for two hours, washed and incubated with detection reagent. Acquisition and analysis were performed as described above. For TGF-β, standard curves covered a 0–10,000 pg/mL concentration range, and minimum detection level was 14.9 pg/mL.

Statistical Analysis

Categorical variables were expressed as absolute frequencies and percentages and analyzed using the Fisher's exact test. Normality of distributions was assessed using the D'Agostino and Pearson test. Normally distributed data are presented as mean (SD) and non-normally distributed data as median (IQR). Each two independent groups were compared using the Mann-Whitney *U* test, and paired data were compared using Wilconxon signed-rank test. A *P*-value of <0,05 indicated the presence of a statistically significant difference. Data were analyzed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla, California).

Results

Patients Demographics and Clinical Characteristics

A total of 15 patients with a clinical diagnosis of active NIU were recruited. The diagnosis included idiopathic disease, HLA-B27-associated uveitis, BD and VKH disease. Fifteen healthy sex and age-matched healthy subjects were selected as controls. Table 1 summarizes the features of the study population.

All patients were treated with topical corticosteroids and cycloplegics. In three cases of HLA-B27-associated uveitis and in the two cases of BD and VKH, patients also received therapy with systemic corticosteroids (prednisolone). Imunosuppressive therapy with cyclosporine A or azathioprine was necessary in the BD and VKH patients. In one case of BD, adalimumab was also initiated.

Patient demographics were as follows: seven males and eight females, with an average age of 47 years (range, 29–72 years). Control demographics were as follows: 8 males and 7 females, with an average age of 44,7 years (range, 20–86 years old).

Characterization of Regulatory T-Lymphocyte Subsets in NIU Patients and Controls

Fifteen NIU patients were evaluated before and after treatment and compared to controls at both time points. Only thirteen NIU patients had flow cytometry evaluations [Table 2a, 2b].

Thus, considering the flow cytometric analysis before treatment, patients showed increased percentages of total Tregs ($p=0.048$) when compared to controls. Also, within total $CD4^+$ T cells, there was a tendency for increased percentages of memory Tregs in NIU patients ($p=0.057$). No further statistically significant differences were observed when comparing the two groups.

However, the introduction of therapy changed this scenario, with patients showing a reduction in Treg cell levels ($p=0.011$), after treatment. Moreover, when comparing the after-treatment patient group with the control subjects, no significant difference differences were observed in percentages or absolute counts.

Serum Cytokine Levels

Despite the alterations reported for the cellular subsets, when regarding inflammatory cytokine levels, we observed no differences towards controls in patients with a clinical diagnosis of active NIU.

Nevertheless, lower levels of IL17A ($p=0.030$) as well as a tendency for lower levels of TNF-α ($p=0.055$) were present in patients after treatment [Fig. 2]. Furthermore, the

inflammatory (TNF-α + IFN-ɣ + IL17A)/ anti-inflammatory (IL10 + TGF-β) ratio also showed a significant reduction between both evaluations in the NIU group.

Discussion

NIU can be associated with systemic autoimmune or inflammatory diseases like BD, ocular sarcoidosis and VKH disease. HLA-B27-associated uveitis is the most common cause of acute anterior NIU and patients with this condition may present with only uveitis or with an axial spondyloarthropathy.

Treg cells play an indispensable role in establishing self-tolerance, maintaining immune homeostasis and preventing autoimmune diseases.[19, 20] FOXP3 is a characteristic marker of Treg cells and has been used in various studies analyzing Treg activation.[21] However, since it is an intracellular molecule, FOXP3 detection requires fixation and permeabilization of cells. Several authors have proposed using another well-known Treg surface marker: the co-expression of CD4 and CD25 associated with the absence or the low expression of CD127 (α-chain of IL7 receptor) since a good correlation between these two phenotypes was previously reported.[22, 23] Besides CD25, CD39 is also predominantly expressed on CD4+ FOXP3+ T-cells with an expression level proportional to FOXP3.[11, 12] In this study, we defined Tregs as CD3+CD4^{+}CD25^{hi}CD127^{low}cells while also analyzing its memory and näive subsets and CD39 expression.

Treg cell frequency has been previously shown to be decreased in uveitis.[2-4] Furthermore, it has been suggested that these cells could serve as biomarkers for active NIU since previous reports show a higher CD4+CD25+FOXP3+ Treg frequency in patients in clinical remission compared with patients with active disease.[6] However, results have been inconsistent with a few studies showing no significant differences in

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Treg levels between patients and controls in active NIU[8] and VKH.[7] In BSCR, an autoimmune disorder causing chronic inflammation of the posterior segment of the eye, a case-control study assessing Treg cell levels in the peripheral blood of patients with active disease found that the percentage of $CD4^+CD25^+$ $FOXP3^+$ Tregs was significantly lower when compared to controls but with no difference between $CD4^+CD25^+$ Tregs in both groups.[24] However, another study with BSCR patients found that the proportion of $CD4^+CD25^{hi}CD127^{low}$ T cells was higher in patients than in controls.[14] The authors suggested that Treg function rather than frequency needed to be studied.

In fact, one would expect that Treg levels would increase after uveitis resolution and treatment, but our results have shown an increased percentage of both total and memory Tregs in patients with active inflammation when comparing to controls and no significant difference from controls after treatment. It is interesting that although there were increased percentages of total and memory Tregs in patients, there was no significant difference between groups regarding CD39 expression, which may lead us to speculate whether these cells maintain their normal suppressive function.

Although one can argue that this inconsistency across studies may be a consequence of the different strategies used to quantify Tregs, it seems reasonable to conclude that there are insufficient data addressing Treg frequency and function in NIU and that more studies are needed before Treg levels can be considered as biomarkers for active disease.

Regarding inflammatory cytokines in NIU, both IL17A and TNF-α are considered to be key cytokines in uveitis pathogenesis [13, 25, 26] and while anti-IL17 therapy is not yet available, there is growing experience in managing refractory NIU with anti-TNF-α immunomodulatory treatment. Accordingly, our results have thus shown a reduction in IL17A and TNF-α levels after uveitis resolution, as well as a reduction in the inflammatory/anti-inflammatory cytokine ratio.

Several studies have been consistent in demonstrating the importance of the IL17 pathway in NIU and an increase in IL17A has already been found in VKH disease,[27] BSCR,[28] BD[29] and HLA-B27-associated uveitis.[30] These results suggest that IL17 may be used as a possible biomarker in NIU or even as a therapeutic target.

Our main study limitations are the heterogeneity of the uveitic conditions included and the small sample size. Nonetheless, NIU is a rare condition and previous studies addressing Treg cell frequency also included relatively small sample sizes.[5, 8]

It is important to highlight the importance of more studies looking for biomarkers in NIU. Although previous works study total Treg cell frequency in active disease, there are few data evaluating individual Treg subsets as well as cytokine levels after treatment. Since we have evaluated patients before and after treatment, our results reinforce the notion that inflammatory cytokines like IL17A are fundamental in the disease pathogenesis and may represent a more tailored approach or treatment option in patients not responding to classical imunosuppression. Finally, the role of Treg lymphocytes in NIU may be further elucidated if memory Tregs and respective CD39 expression are also addressed in studies analyzing Treg frequency and function.

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Table 1- Characteristics of the study population.

Patient	Age	Sex	Diagnosis	Systemic Treatment
1	62	M	HLA-B27-associated	No
2	35	F	HLA-B27-associated	No
3	29	F	HLA-B27-associated	No
4	31	M	HLA-B27-associated	No
5	62	F	HLA-B27-associated	No
6	49	F	HLA-B27-associated	No
7	49	M	Vogt-Koyanagi-Harada	Prednisolone, Cyclosporine
8	37	F	Vogt-Koyanagi-Harada	Prednisolone, Cyclosporine
9	36	M	HLA-B27-associated	Prednisolone
10	72	F	HLA-B27-associated	Prednisolone
11	41	M	HLA-B27-associated	Prednisolone
12	42	M	Behçet Disease	Prednisolone, cyclosporine, adalimumab

13	53	F	Behçet Disease	Prednisolone, azathioprine
14	58	F	Idiopathic	No
15	50	M	Idiopathic	No

Table 2a Comparison of T cell subsets absolute counts in NIU AT, NIU BT, and control group

				Group's comparisons		
				NIU BT vs Control	NIU AT vs Control	NIU BT vs NIU AT
T cell subsets	**NIU BT (n=13)**	**NIU AT (n=13)**	**Control (n=15)**	***P-value**		**#P-value**
Cells/µL, median (IQR)						
T cells	1203 (755)	1273 (779)	1326 (671)	0,865	0,409	0,542
CD4 T cells	723 (557)	935 (483)	671 (496)	0,435	0,183	0,636
Naïve	208 (258)	285 (185)	228 (197)	0,937	0,917	1,000
Memory	479 (232)	567 (253)	382 (324)	0,369	0,259	0,542
Effector	10 (15)	9 (22)	9 (10)	0,741	0,991	0,451
Tregs	69 (56)	70 (50)	57 (39)	0,166	0,146	0,852
Naive Tregs	14 (14)	17 (14)	14 (16)	0,883	0,674	0,830
$CD39^+$ naive Tregs	1 (1)	1 (1)	2 (1)	0,424	0,469	1,000
Memory Tregs	47 (33)	52 (24)	33 (33)	0,110	0,230	0,877
$CD39^+$ memory Tregs	28 (25)	26 (21)	26 (20)	0,759	0,865	0,989
CD8 ($CD4^-$) T cells	442 (265)	443 (303)	510 (380)	0,808	0,843	0,497
Naïve	96 (118)	124 (134)	109 (133)	0,883	0,643	0,625
Memory	179 (134)	187 (114)	183 (157)	0,580	0,643	0,735
Effector	122 (223)	152 (134)	165 (118)	0,474	0,381	0,735
CD4/CD8 ratio	1,85 (0,88)	1,86 (0,90)	1,73 (0,88)	0,609	0,519	0,831

NIU: Non-infectious Uveitis; NIU BT: NIU before treatment; NIU AT: NIU after treatment; IQR: interquartile range.
Results are presented as medians and interquartile range, median (IQR).
* Mann-Whitney U test.
# Wilcoxon signed-rank test.

Table 2b Comparison of T cell subsets percentages in NIU AT, NIU BT, and control group

				Group's comparisons		
				NIU BT vs Control	NIU AT vs Control	NIU BT vs NIU AT
T cell subsets	**NIU BT (n=13)**	**NIU AT (n=13)**	**Control (n=15)**	***P-value**		**#P-value**
Percentages, median (IQR)						
Within CD3 T cells						
$CD4^+$ T cells	64,64 (12,78)	64,99 (12,07)	63,33 (14,24)	0,611	0,519	0,748
$CD8^+$ (CD4-) T cells	35,02 (12,76)	35 (12,00)	36,67 (13,93)	0,608	0,517	0,787
Within CD4 T cells						
Naïve	32,68 (22,01)	31,34 (12,20)	33,62 (14,04)	0,577	0,357	0,497
Memory	65,57 (19,06)	66,31 (13,13)	65,16 (11,97)	0,409	0,359	0,455
Effector	1,12 (2,36)	0,94 (2,03)	1,14 (2,95)	0,914	0,865	1,000
Tregs	9,12 (2,71)	8,56 (3,54)	7,43 (1,22)	**0,048**	0,336	**0,011**
Naive Tregs	1,84 (1,43)	1,72 (0,96)	1,81 (0,98)	0,973	0,717	0,376
$CD39^+$ naive Tregs	0,11 (0,12)	0,13 80,14)	0,21 (0,18)	0,091	0,221	0,625

Memory Tregs	7,05 (2,67)	6,1 (3,12)	5,27 (1,18)	0,057	0,249	0,094
CD39⁺ memory Tregs	3,87 (4,19)	3,3 (3,11)	3,23 (1,40)	0,300	0,795	0,244
Within Tregs						
Naive Treg	19,49 (19,35)	23,14 (13,07)	28,02 (7,22)	0,154	0,107	0,735
Memory Tregs	80,51 (19,35)	76,86 (13,08)	71,98 (7,22)	0,155	0,107	0,735
CD39⁺ Tregs	48,18 (36,33)	46,66 (26,78)	44,65 (15,89)	0,777	0,917	0,685
Within CD8 (CD4-) T cells						
Naïve	22,08 (23,15)	22,82 (15,62)	21,02 (23,57)	0,743	0,777	0,376
Memory	39,79 (18,68)	38,3 (19,54)	35,54 (12,44)	0,313	0,359	1,000
Effector	30,54 (21,55)	38,59 (21,22)	39,11 (13,95)	0,129	0,608	0,376

NIU: Non-infectious Uveitis; NIU BT: NIU before treatment; NIU AT: NIU after treatment; IQR: interquartile range.
Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.
* Mann-Whitney U test.
# Wilcoxon signed-rank test.

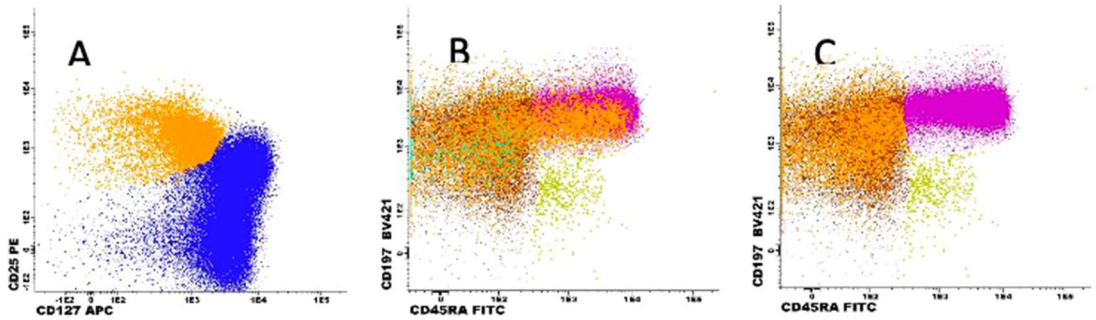

Fig. 1- A. CD4+CD3+ auxiliary T-cells in blue, CD4+CD3+CD25^{hi}CD127^{low} Treg population in orange. B. Naïve (CD45RA+CD197+) CD4+ T-cells in purple, memory (CD45RA-CD197+/-) CD4+ T-cells in brown and effector (CD45RA+CD197-) CD4+ T-cells in green. Tregs are displayed in orange and CD39+ Treg cells in turquoise, both overlaying the previously defined subsets. C. Memory Treg population in orange, overlaying the different CD4+ T-cell subsets.

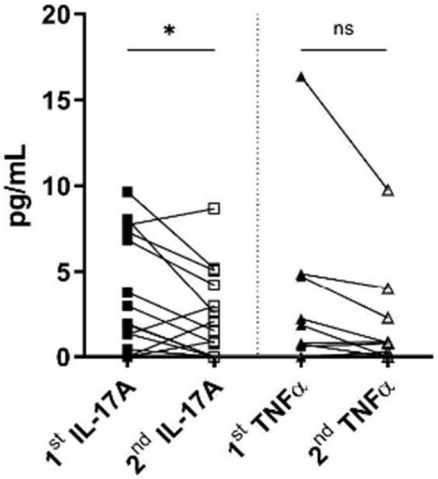

Fig. 2- Scatter dot plot (median with IQR) of IL17A and TNF-α serum levels before (1^{st}) and after (2^{nd}) treatment of NIU group. Differences were tested using Wilcoxon signed-rank test. *P-value <0.05; n.s, non-significative.

Cytokine Profiles in the Peripheral Blood and Aqueous Humor of Patients with Herpetic Uveitis

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To the Editor,

Introduction

Uveitis is an intraocular inflammation with several infectious or non-infectious etiologies. The characterization of local and systemic immune profiles in infectious viral uveitis could help recognize different clinical entities and contribute to a more targeted treatment approach.

One previous study addressing cytokine and chemokine profiles in aqueous humor (AqH) of infectious and non-infectious uveitis patients found that interleukin (IL)-1β and IL-10 levels were increased in viral uveitis whereas IL-17 was elevated in toxoplasmic uveitis(1). IL-10 is of particular interest when studying cytokine profiles in viral infections since it has been shown to suppress the host cellular immune response and favor viral replication, increasing the susceptibility to infection(2) and viral persistence(3).

In this study, we aimed to characterize cytokine profiles in the peripheral blood and AqH of patients with herpetic uveitis (HU) and compare them with healthy controls.

Methods

Patients

For this study, both patients and controls were recruited from the Ophthalmology Department of Egas Moniz Hospital, West Lisbon Hospital Center, between October 2014 and October 2016.

Patients presenting with active uveitis from a presumed viral/herpetic etiology were included in the uveitis group. The diagnosis of active uveitis followed the clinical criteria

based on inflammatory cell reaction in the anterior chamber or vitreous as per standardization of uveitis nomenclature (SUN) and National Eye Institute (NEI) grading systems(4).

At the time of sampling, all patients had active disease and both blood and AqH samples were collected at presentation. Intraocular samples were examined for the presence of cytomegalovirus (CMV), herpes simplex virus (HSV)-1 and 2, and varicella zoster virus (VZV) by real-time polymerase chain reaction (PCR) analysis as previously described(5).

Controls were selected among healthy subjects undergoing cataract or refractive surgery, with no known history of intraocular inflammation.

The study protocol was approved by the Ethics Committee of Egas Moniz Hospital, West Lisbon Hospital Center, and informed consent was obtained from each patient.

Sample collection

The AqH samples were collected with a 30-gauge needle under topical anesthesia and sterile conditions by slit lamp with the aid of one drop of povidone iodine before and after puncturing the anterior chamber. The AqH samples of control subjects were collected with a 30-gauge needle before starting surgery. Undiluted aqueous samples of at least 0.1mL were collected from each subject and immediately sent to the laboratory for analysis.

Peripheral blood samples were also collected in order to obtain serum.

Quantification of serum cytokine expression by multiplexed flow cytometry

A multiplex bead-based immunoassay (BD CBA Flex Set, BD Biosciences, San Jose, CA, USA) was used to determine serum and AqH levels of TNF-α, IFN-γ, IL-17A and IL-10. A similar single-plex bead-based immunoassay was used for TGF-β.

The protocol was performed following the instructions of the manufacturer. In brief, standards and serum samples were incubated with specific capture beads for 1 hour at room temperature. After adding the detection reagent, the mixtures were incubated for 2 hours at room temperature in the dark. After a final wash, beads were acquired in a BD FACS Canto II, previously set up for the BD CBA Flex Set. For each cytokine, at least 300 beads were acquired per sample. The FCAP Array Software (BD Biosciences) was used for data analysis. Standard curves covered a 0–2500 pg/mL concentration range and the minimum detection levels were: 0.13 pg/mL for IL10; 0.3 pg/mL for IL17A; 1.8 pg/mL for IFN-γ and 0.7 pg/mL for TNF-α.

For TGF-β, analyzed separately, samples were previously activated with the Sample Activation Kit 1 (R&D, Minneapolis, MN, USA) according to the recommended procedure. After activation, samples were incubated with capture beads for 2 hours, washed and incubated with detection reagent. Acquisition and analysis were performed as described above. For TGF-β, standard curves covered a 0-10000 pg/mL concentration range, and minimum detection level was 14.9 pg/mL.

Statistical analysis

The Mann-Whitney *U* test was used to compare each 2 independent groups. A *P* value of <0.05 was considered for statistical significance. Data were analyzed using GraphPad Prism, version 8 for Windows (GraphPad Software, La Jolla, California).

Results

Four patients with presumed HU and 8 controls were included. **Table 1** summarizes the demographic and clinical features for the HU group. One patient with a panuveitis associated with acute retinal necrosis was also included. All HU patients underwent anterior chamber puncture and AqH sampling as previously described. Results of AqH by real-time PCR confirmed a VZV infection in all the cases tested.

Regarding serum cytokines, there were no significant differences observed between patients and controls.

In AqH samples however, patients showed increased concentrations of IL10 (p=0.018), TNF-α (p=0.018) and IFN-ɣ (p=0.024).

Interestingly, the levels of serum and AqH cytokines differ within the two groups. While controls presented higher levels of IL10, IFN-ɣ and TGF-β in serum samples compared to those found in AqH (respectively, p=0.001; p=0.002 and p=0.001), in the patients' group, only TGF-β showed higher serum concentrations when compared to AqH (p=0.029), with comparable values for the other cytokines tested.

Table 2 shows the results and comparison of cytokine levels in the serum and AqH of both groups.

Discussion

Despite including just a few HU cases, our preliminary results show an elevation of intraocular TNF-α and IFN-ɣ levels which is likely associated with active disease and anterior chamber inflammation.

The increase in IL-10 levels also found in patients´ AqH samples is, as described in a previous study (1), probably related to the viral etiology, in this case herpetic. However, since all the patients included tested positive for VZV infection, it is possible that other herpetic etiologies, such as HSV-1 and 2 or CMV, show different serum and intraocular cytokine profiles.

The immunosuppressive ability of IL-10 to impair T-cell responses leading to persistent viral infection has already been demonstrated in mice (6, 7), reinforcing its possible use as a biomarker for viral infection or even as a therapeutic target since it has been shown that *in vivo* administration of an antibody against the murine IL-10 receptor completely prevented the viral persistence by restoring T-cell function (3).

In conclusion, although more studies are needed to confirm our findings, elevated intraocular IL-10, TNF-α and IFN-ɣ levels seem to be associated to VZV-associated uveitis.

List of Abbreviations

AqH- Aqueous humor

CMV- cytomegalovirus

HSV- herpes simplex virus

HU- herpetic uveitis

IFN-ɣ- Interferon-Ɣ

IL- Interleukin

PCR- polymerase chain reaction

TGF-β- Transforming Growth Factor β

TNF-α- Tumor Necrosis Factor α

VZV- varicella zoster virus

Declarations:

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Egas Moniz Hospital, West Lisbon Hospital Center, and informed consent was obtained from each patient.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

MG was involved in patients and controls recruitment, data collection, review of literature, and drafting of the manuscript. MJA, CGM and LMB were involved in the data collection and flow cytometric analysis as well as results interpretation and final editing of the manuscript. MAD was responsible for the statistical analysis. RDP was involved in supervision, fine tuning, and final editing of the manuscript. The author(s) read and approved the final manuscript.

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Table 1 - Demographic and clinical features for the HU group.

Patient	Gender	Age (years)	Clinical Features at presentation	Sytemic Features	PCR results
1	F	76	Unilateral anterior uveitis; sectorial iris atrophy; diffuse KPs; elevated IOP	None	+ VZV
2	M	45	Panuveitis- acute retinal necrosis	None	+ VZV
3	F	84	Unilateral queratouveitis; sectorial iris atrophy; diffuse KPs	None	+VZV
4	M	74	Unilateral anterior uveitis; elevated IOP; cataract; diffuse KPs; diffuse iris atrophy	None	+VZV

F- female; M- male; KPs- keratic precipitates; IOP- intraocular pressure; VZV- varicella zoster virus; PCR- polymerase chain reaction.

Table 2 - Comparison of cytokine levels in HU patients and controls.

SERUM & AQUEOUS HUMOR (AqH) CYTOKINES	Controls, serum samples (n=8)	Controls, AqH samples (n=8)	HU, serum samples (n=4)	HU, AqH samples (n=4)	Controls vs HU, serum samples	Controls vs HU, AqH samples	Controls, Serum vs AqH samples	HU, serum vs AqH samples
pg/ml, median (IQR)								
IL-10	1,22 (0,42-1,69)	0,00 (0,00-0,00)	1,88	3,44	-	**0.018**	**0.001**	-
IL-17A	1,20 (0,00-3,73)	1,20 (0,00-3,73)	0,20	0,99	-	-	-	-
TNF-α	0,00 (0,00-0,82)	0,00 (0,00-0,00)	0,18	0,91	-	**0.018**	-	-
INF-ɣ	2,81 (1,58-4,83)	0,00 (0,00-0,05)	2,08	0,87	-	**0.024**	**0.002**	-
TGF-β	1962 (1685-2356)	368 (0,00-565)	1861	374	-	-	**0.001**	**0.029**

HU, Herpetic Uveitis; IQR, interquartile range; AqH- Aqueous humour

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.