

Original Research

Increased local expression of P-glycoprotein on CD4⁺ T-cells in vitreous of patients with non-infectious uveitis: a pilot study

Running title: P-glycoprotein in vitreous CD4⁺ cells

Ravichandra Tagirasa, PhD^{1,3}; Hrishikesh Kaza, DNB²; Soumyava Basu, MS^{1,2}

Ocular Immunology¹ and Retina and Uveitis² services, Brien Holden Eye Research Centre, Mithu Tulsi Chanrai Campus, L V Prasad Eye Institute, Bhubaneswar, India

National Cancer Institute, NIH, Bethesda, MD, USA³(*current affiliation*)

Corresponding author:

Soumyava Basu, MS

Retina and Uveitis services, Kallam Anji Reddy Campus, L V Prasad Eye Institute, Hyderabad – 500034, India (*current affiliation*)

Email: basu@lvpei.org; Phone: +91-40-68102020; Fax: +91-23548339

No. of tables: 1

No. of figures: 2

Word count

Abstract: 181

Main text: 1476

Keywords: Uveitis; Multi-drug resistance; P-glycoprotein; Vitreous; Mitochondria

Abstract

Nearly a third of uveitis patients are unable to achieve adequate inflammation control with conventional anti-inflammatory therapy. Several factors are known to influence responsiveness to anti-inflammatory therapy. In this pilot study, we have investigated the local expression of P-glycoprotein, an efflux-transport protein with role in multi-drug resistance, in vitreous CD4⁺ T-cells of patients with non-infectious uveitis (NIU). CD4⁺ T-cells were isolated from vitreous and peripheral venous blood samples of NIU patients undergoing therapeutic vitrectomy. Rhodamine-123, a substrate of P-glycoprotein, whose retention inside cells is inversely proportional to P-glycoprotein function, was used to assay this transporter protein. In addition, cells were stained with IFN- γ , IL-17, GM-CSF and FoxP3, and analysed by flow cytometry. T-cell mitochondria were imaged by Mitotracker Red and confocal microscopy. Vitreous CD4⁺ T-cells expressed significantly higher P-gp and pro-inflammatory (IL-17⁺, IFN γ ⁺IL17⁺) cytokine expression than matching blood samples. Mitochondrial fission was noted in vitreous T-cells and fusion in blood. We concluded that NIU is associated with higher P-glycoprotein expression and pro-inflammatory state in vitreous than in blood. This supports P-glycoprotein inhibition and adjunctive local anti-inflammatory treatment in management of NIU.

Introduction

Corticosteroids and non-steroidal immunosuppressive therapy are the mainstay of treatment of non-infectious uveitis (NIU). However, up to a third of uveitis patients do not achieve control of inflammation with standard dose of corticosteroids [1]. Even non-steroidal immunosuppressives achieve corticosteroid sparing control of uveitis in only 36 – 61% patients (depending on the drug), after one year of treatment [2]. Hence, there is a need to identify putative features in inflamed tissues that can potentially be modified to improve the

efficacy of anti-inflammatory therapy. While several mechanisms are known to influence resistance to corticosteroids in inflammatory diseases [3], we investigated the expression of multi-drug resistance (MDR) protein, P-glycoprotein (P-gp), in CD4⁺ T-cells vitreous samples of NIU.

P-gp or MDR1, is a transmembrane efflux protein belonging to a superfamily of ATP binding cassette (ABC) transporter proteins. It is responsible for extrusion of xenobiotic or cytotoxic compounds from cells, and is found in normal tissues of kidney, liver, intestine, as well as the blood-brain and blood-retinal barriers. It is also expressed in different stages of lymphoid cell development. Among its substrates are endogenous compounds, and a wide range of therapeutic drugs, most notably anti-cancer drugs, anti-HIV drugs and corticosteroids [4]. While P-gp has been extensively investigated in non-ocular inflammatory diseases [5,6], its role in uveitis remains relatively unknown. We recently demonstrated higher MDR protein expression in blood CD4⁺ cells of patients with non-infectious uveitis, who were non-responders to immunomodulatory therapy [7]. In this study, we have compared P-gp expression in vitreous CD4⁺ T-cells to matched blood samples and demonstrated its association with pro-inflammatory cytokine secretion and mitochondrial fission within the T-cells.

Methods

We investigated four patients with non-infectious uveitis who reported to the uveitis clinic of our institute. The study was approved by the institutional review board of LV Prasad Eye Institute, Bhubaneswar, and adhered to tenets of Declaration of Helsinki. Written, informed consent was obtained from all patients. All four patients presented with pan uveitis with $\geq 2+$ vitritis, received tailored investigations for etiological diagnosis, and were under treatment with oral \pm local corticosteroids for varying durations. Each received tailored investigations for etiological diagnosis. Patients 1 and 4 were labelled idiopathic, while patients 2 and 3 were

diagnosed as probable ocular sarcoidosis. All patients received pars plana vitrectomy for collection of vitreous samples, followed by CD4⁺ T-cell isolation from vitreous and blood samples of each patient, as per previously described protocol [7,8].

P-gp functional assay: P-gp function in vitreous and blood T-cells was assessed by flow cytometry with the dye Rhodamine-123 (Rh-123), whose retention inside cells inversely correlates with P-gp function [7,9]. Briefly, the cells were washed with phosphate-buffered saline (PBS), and then incubated for 30 minutes in dark with Rh-123 (1 μ M/mL). These cells were then washed twice with RPMI-1640 and incubated for 2 hours at 37° C. These were then washed twice with FACS buffer (1% fetal bovine serum-PBS) and acquired by flow cytometry (CytoFLEX S, Beckman Coulter, Indianapolis, IN). The analysis was done by CytExpert Software (Beckman Coulter, Indianapolis, IN).

Intracellular cytokine assay: Intracellular cytokine staining for IFN γ , IL-17, TNF α , GM-CSF and FoxP3 in T-cells was done following activation with phorbol 12-myristate acetate (PMA) (50 ng/mL) and ionomycin (1 μ g /mL), as previously described [8].

Mitochondrial fusion assay: Patient 4's blood and vitreous T-cells were also assayed for mitochondrial morphology. The cells were incubated with 100 ng/mL of MitoTracker Red 580 (Invitrogen) for 30 min at 37° C, washed twice with PBS, and imaged by confocal microscopy.

Results

We included two patients each of sarcoidosis and idiopathic panuveitis, who received therapeutic vitrectomy for uncontrolled inflammation. The P-gp expression and cytokine data of individual patients is provided in Table 1. Overall, P-gp expression (Rh-123^{lo}) was significantly higher on vitreous CD4⁺ T-cells compared to matched blood samples of patients

with NIU (Figure 1A-B). Both IL-17 and IL-17⁺IFN γ ⁺ dual positive cells were also significantly higher in vitreous than blood samples (Figure 1C-D). GM-CSF was also significantly increased in vitreous samples while no such difference was noted in FoxP3 (data not shown). The vitreous T-cells showed predominantly mitochondrial fission while blood T-cell mitochondria showed fusion characteristics (Figure 2). Interestingly, the vitreous CD4⁺ cells appeared much larger in size than those from blood, possibly due to differences in stage of activation between the two samples.

Discussion

Sub-optimal control of inflammation by conventional anti-inflammatory therapy has been a long-standing challenge in the management of uveitis [10]. Prolonged or recurrent intraocular inflammation can potentially lead to photoreceptor damage and permanent visual loss [11]. Our study highlights a putative mechanism for resistance to anti-inflammatory therapy that is more pronounced in the intraocular compartment than in peripheral circulation. It is the first demonstration of enhanced local P-gp expression on CD4⁺ T cells in uveitis eyes.

Our results bear striking resemblance to a recent report on P-gp expression in clinically inflamed sections of the gut in Crohn's disease [12]. P-gp expression in the inflamed gut was significantly higher than in uninvolved gut or in the peripheral blood. Notably, higher P-gp expression was associated with pro-inflammatory Th17.1 cells (CCR6⁺CXCR3⁺CCR4⁻), producing both IL-17 and IFN γ , in clinically inflamed tissue. While we could not perform chemokine characterisation of vitreous T-cells in our patient samples, it is likely that the IL-17⁺IFN γ ⁺ dual positive cells in our samples also represent the Th17.1 subset. We have reported dual and triple positive (IL-17⁺IFN γ ⁺TNF α ⁺) autoreactive T-cells in the vitreous of patients with tuberculosis-associated uveitis – such cells can be expected in other forms of autoimmune uveitis as well [8]. In addition, our earlier study found the predominant CD4⁺ T-

cell populations in the uveitis eyes were effector (CD45RO⁺) and central (CD45RO⁺, CCR7⁺) memory T-cells [8]. These T-cell subsets are also known to be predominant among CD4⁺ T-cells expressing P-gp [13]. Taken together, the intraocular milieu of T-cell subsets appears well-matched for high expression of P-gp.

To further substantiate the functional difference between intraocular and peripheral blood T-cells, we observed their mitochondrial characteristics by live cell confocal microscopy. Mitochondrial dynamics control T cell fate by regulating their metabolic profile [14]. Fission or fragmented mitochondria has been linked to effector function in T-cells whereas fusion or elongated tubular mitochondria are required for generation of memory T-cells. Mitochondrial fission is associated with excessive ROS production and activation of NFκB and MAP Kinase, which leads to the generation of pro-inflammatory mediators [15]. We found that CD4⁺ T-cells from the vitreous had fragmented mitochondria while those from blood had elongated mitochondria. Though tested in only one sample, this provides additional evidence of the phenotypic difference between vitreous and blood CD4⁺ T-cells.

Our investigation was only a pilot study, limited by the number of samples and by lack of controls such as infectious uveitis. In addition, the functional impact of P-gp expression in CD4⁺ T-cells is not limited to extrusion of xenobiotic compounds [4]. P-gp has also been suggested to have a role in cytokine secretion. Indeed, P-gp expression in rheumatoid arthritis has been found to correlate with disease activity rather than refractoriness to anti-inflammatory therapy [16]. Conversely, pro-inflammatory cytokines such as IL-2 and TNFα have been found to up-regulate P-gp expression on lymphocytes [17]. Therefore, it is possible that high P-gp expression in the eye is an outcome of prolonged intraocular inflammation and not the reverse.

Nonetheless, our study highlights the potential of enhanced resistance to the therapeutic effects of anti-inflammatory drugs in the intraocular compartments, as compared to the peripheral circulation. It supplements our recent study on MDR proteins in blood CD4⁺ cells in non-responders to immunosuppressive therapy in non-infectious uveitis [7]. Together, both the studies underscore the need for P-gp inhibition strategies and high therapeutic concentrations of these drugs in the eye, to counter the cellular resistance mechanisms. Several P-gp inhibitors with different degrees of efficacy and specificity for the P-gp transporter protein, have been identified and many have completed Phase III clinical trials [19]. Incidentally, cyclosporine A is one of the earliest known P-gp inhibitors and its role as an adjunctive P-gp inhibiting agent in refractory NIU, was suggested in our recent study [7]. The other approach is to attain higher local concentrations of drugs in the eye. This can be achieved by using systemic therapies that have high penetration across the blood-retinal barrier, or by intraocular delivery of anti-inflammatory drugs. Local anti-inflammatory therapy has recently been in focus with the introduction of sustained-release corticosteroid implants [20], as well as non-steroidal drugs such as sirolimus [21]. Besides evading the systemic adverse effects of these drugs, the high concentration in the eye is likely to offset the drug efflux from increased P-gp expression as well as the higher pro-inflammatory state in the eye.

In summary, intraocular inflammation in NIU is characterised by higher P-gp expression and pro-inflammatory cytokine secretion than the peripheral circulation. P-gp expression is associated with mitochondrial fission in intraocular T-cells. Therapeutic strategies should be directed towards P-gp inhibition and higher intraocular concentrations of anti-inflammatory drugs to counter the P-gp induced resistance.

Statement of ethics: The study was approved by the institutional review board of LV Prasad Eye Institute, Bhubaneswar, and adhered to tenets of Declaration of Helsinki. Written, informed consent was obtained from all patients.

Conflicts of interest: None to declare.

Funding/ Support: SB is supported by DBT/Wellcome Trust India Alliance Intermediate Fellowship in Clinical and Public Health Research

Author contributions

RT: acquisition, analysis, and interpretation of data; revision of manuscript

HK: acquisition, interpretation of data; revision of manuscript

SB: analysis, and interpretation of data; initial draft and revision of manuscript

References

1. Schewitz LP, Lee RW, Dayan CM, Dick AD. Glucocorticoids and the emerging importance of T cell subsets in steroid refractory diseases. *Immunopharmacol Immunotoxicol.* 2009;31:1-22.
2. Jabs D. Immunosuppression for the uveitides. *Ophthalmology.* 2018;125:193-202.
3. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet.* 2009;373:1905-17.
4. Bossennec M, Di Roio A, Caux C, Ménétrier-Caux C. MDR1 in immunity: friend or foe? *Oncoimmunology.* 2018;7:e1499388.
5. Picchianti-Diamanti A, Rosado M, Scarsella M, Laganà B, D'Amelio R. P-glycoprotein and drug resistance in systemic autoimmune diseases. *Int J Mol Sci.* 2014;15:4965-76. doi: 10.3390/ijms15034965
6. Tsujimura S, Saito K, Nakayamada S, Nakano K, Tanaka Y. Clinical relevance of the expression of P-glycoprotein on peripheral blood

- lymphocytes to steroid resistance in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2005;52:1676-83. doi:10.1002/art.21032
7. Tagirasa R, Rana K, Kaza H, Parai D, Damera S, Basu S; Role of multidrug resistance proteins in non-responders to immunomodulatory therapy for non-infectious uveitis. *Trans. Vis. Sci. Tech.*2020;9:12.
 8. Tagirasa R, Parmar S, Barik MR, Devadas S, Basu S. Autoreactive T cells in immunopathogenesis of TB-associated uveitis. *Invest Ophthalmol Vis Sci.* 2017;58:5682-91.
 9. Petriz J, Garcia-Lopez J. Flow cytometric analysis of P-glycoprotein function using rhodamine 123. *Leukemia.* 1997;11:1124.
 10. de Smet MD, Taylor SR, Bodaghi B, Miserocchi E, Murray PI, Pleyer U, et al. Understanding uveitis: the impact of research on visual outcomes. *Prog Retin Eye Res.* 2011;30:452-70.
 11. Nguyen AM, Rao NA. Oxidative photoreceptor cell damage in autoimmune uveitis. *J Ophthalmic Inflamm Infect.* 2011;1:7.
 12. Ramesh R, Kozhaya L, McKeivitt K, Djuretic IM, Carlson TJ, Quintero MA, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med.* 2014;211:89-104.
 13. Dimeloe S, Frick C, Fischer M, Gubser PM, Razik L, Bantug GR, et al. Human regulatory T cells lack the cyclophosphamide-extruding transporter ABCB1 and are more susceptible to cyclophosphamide-induced apoptosis. *Eur J Immunol.* 2014;44:3614–3620.
 14. Buck MD, O’Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, et al. Mitochondrial dynamics controls T cell fate through metabolic programming. *Cell.* 2016;166:63-76.

15. Park J, Choi H, Min JS, Park SJ, Kim JH, Park HJ, et al. Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. *J Neurochem.* 2013;127:221-32.
16. Agarwal V, Mittal SK, Misra R. Expression of multidrug resistance-1 protein correlates with disease activity rather than the refractoriness to methotrexate therapy in rheumatoid arthritis. *Clin Rheumatol.* 2009;28:427-33.
17. Tsujimura S, Saito K, Nakayamada S, Nakano K, Tsukada J, Kohno K, et al. Transcriptional regulation of multidrug resistance-1 gene by interleukin-2 in lymphocytes. *Genes Cells* 2004;9:1265–1273.
18. Klukovits A, Krajcsi P. Mechanisms and therapeutic potential of inhibiting drug efflux transporters. *Expert Opin Drug Metab Toxicol.* 2015;11:907-20.
19. Lowder C, Belfort R, Lightman S, Foster CS, Robinson MR, Schiffman RM, et al. Dexamethasone intravitreal implant for noninfectious intermediate or posterior uveitis. *Arch Ophthalmol.* 2011;129:545-53.
20. Nguyen QD, Merrill PT, Clark WL, Banker AS, Fardeau C, Franco P, et al. Intravitreal sirolimus for noninfectious uveitis: a phase III Sirolimus study Assessing double-masked Uveitis tReAtment (SAKURA). *Ophthalmology.* 2016;123:2413-23.

Legends

Figure 1: (A) Histogram of Rh-123 efflux in CD4⁺ T-cell isolated from blood and vitreous humor of the same patient. (B) Statistical analysis of P-glycoprotein function (Rh-123^{lo}) between blood and vitreous humor samples (n=4). (C) Intracellular cytokine staining of CD4⁺ T-cell isolated from blood and vitreous humor following activation with PMA-Ionomycin for 8 hours (D) Statistical analysis of pro-inflammatory cytokine (IL-17 and IL-17⁺IFN- γ) production in blood and vitreous humor samples (n=4). *p* values are indicated by **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

Figure 2: Live confocal microscopy of CD4⁺ T-cells stained with Mitotracker Red showing (A) Mitochondrial elongation suggestive of fusion in blood samples, and (B) Mitochondrial fragmentation suggestive of fission in vitreous humor samples.

Table 1: P-glycoprotein function and cytokine secretion data of individual patients

Patient no.	Diagnosis	Age/gender	% P-glycoprotein		% IFN γ /IL-17		% IL-17	
			Blood	Vitreous	Blood	Vitreous	Blood	Vitreous
1	Panuveitis	36/M	20	33	1	2.2	5	7
2	Sarcoidosis	45/M	19.6	47	1.6	2.9	4.5	9
3	Sarcoidosis	29/F	19	38	1.4	2.6	5.3	8.7
4	Panuveitis	61/F	11	29	0.9	2.1	4.7	7.2



