tvst

Article

Role of Multidrug Resistance Proteins in Nonresponders to Immunomodulatory Therapy for Noninfectious Uveitis

Ravichandra Tagirasa[1,2], Khokan Rana[1], Hrishikesh Kaza[3], Debaprasad Parai[1], Santhoshkumar Damera[1], and Soumyava Basu[1,3]

[1] Ocular Immunology, Brien Holden Eye Research Centre, Mithu Tulsi Chanrai Campus, L V Prasad Eye Institute, Bhubaneswar, India
[2] National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
[3] Retina and Uveitis Services, Brien Holden Eye Research Centre, Mithu Tulsi Chanrai Campus, L V Prasad Eye Institute, Bhubaneswar, India

Correspondence: Soumyava Basu, Retina and Uveitis Services, Mithu Tulsi Chanrai Campus, L V Prasad Eye Institute, PO KIITS, Bhubaneswar, 751024, India. e-mail: basu@lvpei.org

Received: October 24, 2019
Accepted: February 3, 2020
Published: April 18, 2020

Keywords: uveitis; multidrug resistance; cytokines; immunomodulatory therapy; cyclosporine

Citation: Tagirasa R, Rana K, Kaza H, Parai D, Damera S, Basu S. Role of multidrug resistance proteins in nonresponders to immunomodulatory therapy for noninfectious uveitis. Trans Vis Sci Tech. 2020;9(5):12, https://doi.org/10.1167/tvst.9.5.12

Purpose: Nearly a third to half of patients with noninfectious uveitis (NIU) fail to achieve control with immunomodulatory therapy (IMT). Multidrug resistance (MDR) proteins are transmembrane proteins that allow efflux of intracellular drugs, leading to drug resistance. The aim of our study was to compare MDR protein function in blood $CD4^+$ cells between responders and nonresponders to IMT.

Methods: We included NIU patients on IMT for $\geq$6 months and corticosteroid dose $\leq$10 mg/d. Nonresponders to treatment were those with worsening (two or more steps) of inflammation in the past 3 months on full-dose immunosuppressive therapy. MDR function was assessed by Rhodamine-123 dye retention in blood $CD4^+$ cells. Three nonresponders were treated with adjunctive oral cyclosporine A (CSA, MDR inhibitor) therapy for 2 months and reevaluated.

Results: Fourteen NIU patients were recruited. Most ($n = 8$) had Vogt-Koyanagi-Harada disease. These included nine nonresponders and five responders to IMT. Nonresponders produced significantly higher MDR function and proinflammatory cytokines (interferon γ, tumor necrosis factor α, interleukin 17, and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)) than responders. In vitro CSA treatment of $CD4^+$ cells inhibited MDR expression and proinflammatory cytokine production while increasing Foxp3. Finally, adjunctive oral CSA therapy led to improvement in clinical inflammatory scores with a concurrent decrease in MDR function and proinflammatory cytokine secretion.

Conclusions: MDR function is significantly higher in $CD4^+$ T cells of nonresponders to IMT. Adjunctive CSA therapy may decrease MDR function and allow improvement in treatment response to IMT.

Translational Relevance: Our study highlights the need for MDR inhibition strategies in NIU patients not responding to IMT for improving the efficacy of anti-inflammatory therapy.

Introduction

Uveitis is a broad term used to describe inflammation of internal structures of the eye. Long-standing uveitis can lead to visual impairment, comparable in magnitude to diabetic retinopathy.[1] Uveitis can be of noninfectious or infectious etiology. Noninfectious uveitis (NIU) is treated primarily with corticosteroids (local and/or systemic) often in conjunction with immunomodulatory therapy (IMT). The latter includes conventional drugs (antimetabolites, calcineurin inhibitors, and alkylating agents) and biologics. While corticosteroids remain the mainstay of therapy in most patients with NIU, they are generally not tolerated for long-term treatment because of their side effect profile. IMT is essential for reducing the incidence of structural and functional complications of different forms of uveitis at tolerable doses of corticosteroids.[2–4] The recent Multicenter Uveitis Steroid Treatment Trial 7-year follow-up study also demonstrated the utility of IMT (conventional and biologic)

in controlling inflammation, reducing macular edema, and improving visual outcomes.[5] Nonetheless, only 36% to 61% of patients on conventional IMT achieve corticosteroid sparing (prednisolone <10 mg/d).[3] This is significant since recurrent episodes of inflammation due to inadequate IMT can result in cumulative damage to the eye and subsequent visual loss.[5] Thus, there is an urgent need to identify mechanisms of resistance to IMT in patients with NIU and strategies to overcome such resistance.

Multidrug resistance (MDR) can be caused by a wide range of mechanisms, including reduced uptake and increased efflux by drug transporters and alterations in a variety of cellular functions.[6] Among the transporter proteins are 2 major superfamilies: the solute carrier transporters and the adenosine triphosphate (ATP)–binding cassette (ABC) transporters. ABC transporter proteins are transmembrane efflux proteins that extrude both endogenous and exogenous molecules (including therapeutic drugs) from cells through ATP hydrolysis.[7,8] This reduces the intracellular concentration and thereby clinical efficacy of therapeutic agents. The most clinically significant substrates of these transporter proteins are anticancer, antiviral, and anti-inflammatory drugs. At least 48 members of ABC transporters have been identified in humans, although only select ones are generally linked to MDR.[9] These include P-glycoprotein (P-gp or MDR1), multidrug resistance associated protein 1 (MRP1 or ABCC1), MRP3, MRP4, and breast cancer resistance protein (BCRP or ABCG2). Although conventionally, each transporter is linked to a specific set of substrates and vice versa, recent studies have revealed overlapping substrate specificities between individual transporters not only within a given superfamily (eg, ABC) but also between superfamilies.[7] The most commonly studied among these transporters is P-gp, and its most prominent anti-inflammatory substrates are corticosteroids. The drugs used for conventional IMT in patients with uveitis, such as methotrexate, azathioprine, mycophenolate, and cyclosporine, have been linked to different MDR proteins, although not all are substrates of P-gp.[10] Methotrexate is a substrate of MRP1 and MRP3,[11] although it has also been linked to P-gp (MDR1) in leukemic cell lines.[12] Azathioprine sensitivity has been linked to both MDR1 and MRP4 polymorphisms.[13,14] Cyclosporine A (CSA) is not only a substrate of P-gp and other MDR proteins[8] but also a broad-spectrum inhibitor of different MDR proteins, including P-gp and MRP1.[15] Despite such extensive knowledge on the link between MDR proteins and therapeutic efficacy of anti-inflammatory drugs, to our knowledge, very limited attempts have been made to evaluate their role in the management of uveitis or ocular inflammation.[16]

The expression of MDR proteins on immune cells is measured by staining with specific antibodies and flow cytometry, while their function is measured with Rhodamine-123 (Rh-123), a fluorescent dye that passively enters cells and is extruded by the MDR drug efflux pumps.[17] Lower is the intracellular concentration of Rh-123 (as measured on flow cytometry), and higher is the function of drug efflux pumps. While most Rh-123 studies have been done for P-gp function, the dye also binds to multiple sites on MRP1,[18] and it has been used for kinetic studies of MRP1 function as well.[19] Thus, Rh-123 efflux studies provide a broad functional overview of multiple MDR/efflux transporter proteins. In this study, we have used Rh-123 efflux to compare MDR function in $CD4^+$ T cells between clinical responders and nonresponders to conventional IMT for NIU and demonstrated the utility of MDR inhibitor CSA in inhibiting MDR function and improving the therapeutic efficacy of IMT in patients with NIU.

Materials and Methods

Patients and Samples

The study was approved by the institutional review board of L V Prasad Eye Institute, Bhubaneswar, India (Study #2017-110-IM-20), and conducted in compliance with the Declaration of Helsinki. Written, informed consent was obtained from the patients after explanation of the nature and possible consequences of the study. This was a pilot study since there is no previous literature on role of MDR proteins in uveitis. We included patients with NIU on IMT for ≥6 months and corticosteroid dose ≤10 mg/d. Patients were evaluated in the uveitis clinic of the institute. All had been thoroughly investigated for ocular and systemic manifestations of uveitis (including internist evaluation) and undergone tailored laboratory investigations depending on the clinical manifestations. All patients were initially treated with corticosteroids (local and/or systemic), depending on the anatomical location and severity of the disease. IMT was initiated based on severity, chronicity, systemic manifestations, and prolonged need for or intolerance to corticosteroid therapy.[20] In this study, we only included patients who received treatment with either methotrexate or azathioprine, the two most common IMTs in our clinic. We excluded patients on mycophenolate, cyclosporine, or cyclophosphamide IMT since these are used infrequently in our clinic due to high cost,

relatively poor efficacy, and high toxicity, respectively. Nonresponders to treatment were those with worsening (increase in two or more steps) aqueous or vitreous inflammation or the appearance of new chorioretinal lesions in the past 3 months while on full-dose immunosuppressive therapy (20 mg/wk for methotrexate and 2–3 mg/kg/d for azathioprine). The responders were also on their highest dose of IMT at the time of sampling. The highest dose was at least 15 mg/wk for methotrexate and 2 mg/kg/d for azathioprine. Those meeting the inclusion criteria (responders and nonresponders) and consenting to the study (written, informed) were referred to the biochemistry service where peripheral venous blood was collected in EDTA.

T-Cell Isolation

Whole blood was used to collect peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation. Total memory $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs by negative selection, according to the manufacturer's protocol (Stem Cell Technologies, Vancouver, Canada). $CD14^+$ and $CD16^+$ cells were separated using commercially available monocyte isolation kits (Stem Cell Technologies).

MDR1 Functional Assay

PBMCs from the patients were washed in phosphate-buffered saline (PBS) and then incubated for 30 minutes in ice in the dark with Rh-123 (1 µM/mL). The cells were then washed twice with RPMI-1640 and incubated at 37°C for 2 hours. Then, cells were again washed with complete medium and PBS. Washed cells were stained for various cell surface markers such as CD3 PeCy7, CD4 APC, CD8 V500, CD14 BV421, and CD APC eflour 780 (all BD BD Biosciences, San Jose, CA). After staining, cells were washed twice in FACS buffer (1% fetal bovine serum–PBS) and acquired immediately by CytoFLEX S (Beckman Coulter, Indianapolis, IN) and analyzed by CytExpert Software, Beckman Coulter, Indianapolis, IN.

T-Cell Intracellular Cytokine Assay and CSA Treatment

Memory $CD4^+$ T cells were stimulated with phorbol 12-myristate acetate (PMA) (50 ng/mL) and ionomycin (1 µg/mL) for about 10 hours, with the last 4 hours with 10 µg/mL Brefeldin A and 2 µM monensin at 37°C and 5% CO_2. In some instances, cells were first treated with CSA (1 µM) for 1 hour before PMA/Ionomycin treatment. After 10 hours, cells were fixed and permeabilized with the Foxp3 staining kit (eBioscience, San Diego, CA), according to the manufacturer's protocol. The following antibodies were used: interferon-γ (IFN-γ) FITC, interleukin (IL) 17–Alexa Fluor 647, tumor necrosis factor α (TNFα)–Alexa Fluor700, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) PECF594, IL-2 BV510, IL-10 PE, Foxp3 APC, RORγc PE, and PD1 BV605 (all eBioscience). Stained cells were acquired by CytoFLEX S (Beckman Coulter) and analyzed with the CytExpert Software.

Standard-Dose Adjunctive Cyclosporine Therapy

CSA is known to be a broad-spectrum inhibitor of MDR proteins and is also a commonly used drug for IMT in the management of NIU. Three of the nonresponders enrolled into the study were managed by additional treatment with oral CSA (Psorid, Biocon Ltd., Bangalore, India) 100 mg twice daily for at least 2 months while being continued on treatment with the maximal dose of their earlier IMT (methotrexate or azathioprine). After 2 months of oral CSA therapy, peripheral venous blood was collected and subjected to Rh-123 and intracellular cytokine assays, as described above.

Statistical Analysis

Statistics were performed using paired and unpaired Student's *t*-tests to compare two groups. All statistics were performed on Prism 5.0 (GraphPad, La Jolla, CA) software. Results were expressed as mean ± SEM. $P < 0.05$ was considered significant.

Results

Patient Profiles

We recruited 14 patients with NIU. All patients were diagnosed with different forms of panuveitis. The patient details are available in the Table. Most of these patients ($n = 8$) were diagnosed Vogt-Koyanagi-Harada (VKH) disease. The remaining were diagnosed with sarcoidosis ($n = 3$), HLA-B27–associated anterior uveitis ($n = 2$), and idiopathic multifocal choroiditis with panuveitis ($n = 1$). Eight of these patients were under treatment with methotrexate and six with azathioprine. The mean duration of treatment for methotrexate was 9.6 ± 5.8 months and that of azathioprine was 13.2 ± 8.8 months. Nine patients were

Table. Patient Profiles and Treatment Details of Responders and Nonresponders to Immunomodulatory Therapy

Characteristic	Responders ($n = 5$)	Nonresponders ($n = 9$)
Age, median (range), y	45 (27–61)	46 (24–66)
Clinical diagnosis		
Vogt-Koyanagi-Harada disease	3	5
HLA-B27 anterior uveitis	0	2
Sarcoidosis	2	1
Idiopathic multifocal choroiditis with panuveitis	0	1
Immunomodulatory therapy		
Methotrexate	2	4
Azathioprine	3	5
Duration of treatment, median (range), mo	9.5 (6–48)	11 (6–25)

diagnosed as nonresponders and five as responders based on criteria described above.

Nonresponders Express High MDR on Peripheral CD4$^+$ T Cells

We first compared the MDR function between CD4$^+$, CD8$^+$, CD14$^+$, and CD16$^+$ cells (Supplementary Fig. S1) in peripheral circulation of patients with NIU. We found a significant difference in MDR function only on CD4$^+$ T cells but not on other cells of PBMCs. Next, we correlated the MDR function on CD4$^+$ T cells to the clinical response to IMT in NIU patients. The patients were classified as responders or nonresponders, as per criteria described above. We found that nonresponders ($n = 9$) had significantly higher MDR function on their CD4$^+$ T cells compared with responders ($n = 5$) (Fig. 1A). However, no significant difference was noted among CD8$^+$ T cells between the two groups (Fig. 1B). We also tested a control group without uveitis ($n = 3$) and another group with uveitis ($n = 3$, all HLA-B27–associated uveitis) but no treatment. We found comparable MDR function in both groups, suggesting that the baseline MDR function prior to initiation of IMT is low in patients with uveitis (Supplementary Fig. S2). As discussed earlier, the Rh-123 assay measures functions of not only P-gp but also MRP1, thus providing us a broad overview of the MDR function in the given cell population.

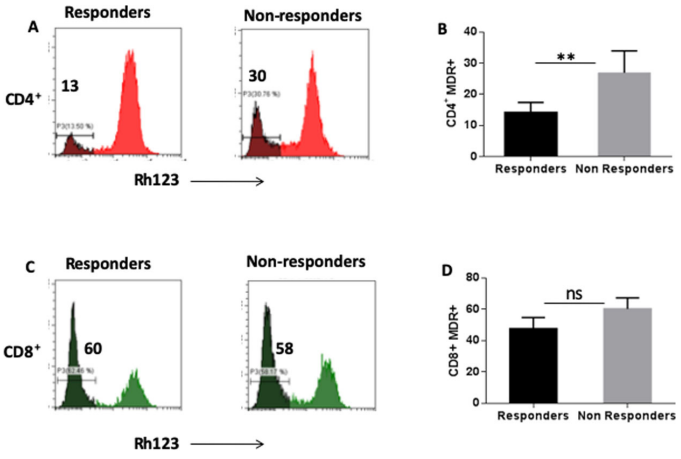

Figure 1. CD4$^+$ T cells of nonresponders have high MDR protein function. T cells of patients with NIU from responders ($n = 5$) and nonresponders ($n = 9$) were stained with Rhodamine 123(Rh-123), and after 2 hours, cells were analyzed for the Rh123$^+$(MDR$^-$) and Rh123$^-$(MDR$^+$). (A, B) Nonresponder CD4$^+$ T cells have higher MDR1 function as compared to responders. (C, D) Even though CD8$^+$ T cells had a higher MDR function than the CD4$^+$ T cells, there was no significant difference in the CD8$^+$ T cells from both groups. $P < 0.05$ was considered significant. $^{**}P < 0.01$.

CD4$^+$ T Cells of Nonresponders Are Highly Proinflammatory in Nature

Next, we investigated if nonresponders to IMT also differ from responders in the intracellular cytokine secretion in peripheral CD4$^+$ T cells. We analyzed the cytokine profile of ex vivo stimulated memory CD4$^+$ T cells from patients with NIU. This revealed that both IL-17A– and IFNγ-producing as well as IL-17A/IFNγ dual-producing cells were significantly elevated in nonresponders ($n = 6$) compared to the responders ($n = 4$) (Figs. 2A, 2B). Not surprisingly, the regulatory cytokine IL-10 was higher among the responder CD4$^+$ T cells, although not statistically significant ($P = 0.062$) (Figs. 2C, 2D). To summarize, the IMT nonresponder memory CD4$^+$ T cells not only have high MDR function but are also highly proinflammatory.

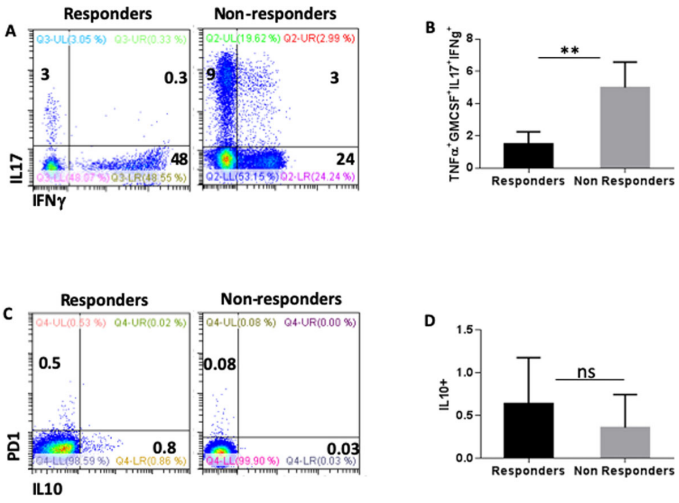

Figure 2. Proinflammatory CD4$^+$ T cells exist predominantly in nonresponders to IMT. CD4$^+$ T cells were stimulated with PMA/ionomycin for 10 hours and then stained for surface and intracellular cytokines. First, cells were gated on TNFα^+ GM-CSF$^+$ (data not shown) and then analyzed for IL-17 and IFNγ. (A, B) Nonresponders ($n = 6$) had very high IL-17$^+$ and IL-17$^+$ IFNγ^+ double-positive cells, although surprisingly, responders ($n = 4$) had high IFNγ^+ cells. (C, D) Even though there was a difference in anti-inflammatory cytokine IL-10, it was not statistically significant ($P = 0.06$). $P < 0.05$ was considered significant. **$P < 0.01$.

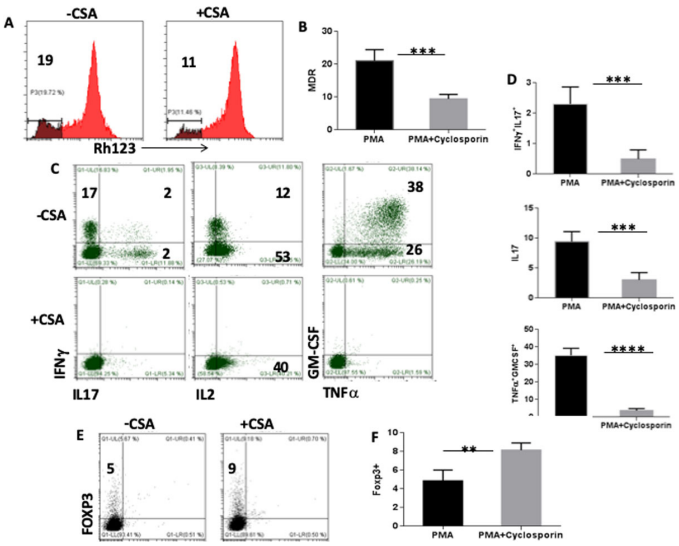

Figure 3. CSA selectively inhibits proinflammatory cytokines and MDR1 function in vitro. (A, B) A total of 1 µg/mL CSA treatment to memory CD4$^+$ T cells from nonresponders ($n = 3$) to IMT inhibited MDR1 function significantly. (C, D) CSA significantly inhibited the proinflammatory cytokines such as IFNγ, IL-17, TNFα, and GM-CSF but not the survival cytokine IL-2. (E, F) Not surprisingly, FOXP3 levels increased with CSA treatment. $P < 0.05$ was considered significant. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

CSA Selectively Inhibits the MDR and Proinflammatory Cytokines but Increases FOXP3 in Peripheral CD4$^+$ T Cells of Patients with Uveitis

To study whether CSA inhibits the proinflammatory cytokines by inhibiting the MDR overactivity, we treated the memory CD4$^+$ T cells from the nonresponders ($n = 6$) with CSA (1 µg/mL for 1 hour) following activation with PMA-ionomycin. CSA significantly decreased the MDR function in CD4$^+$ T cells (Figs. 3A, 3B). Concurrently, the intracellular proinflammatory cytokines IFNγ, IL-17, GM-CSF, and TNFα were also reduced upon CSA treatment (Figs. 3C, 3D), but we did not see a significant reduction in the basic T-cell cytokine, IL-2. Further, the transcription factor for T-regulatory cells, FOXP3, was significantly increased in the CSA-treated population (Figs. 3E, 3F). This strongly suggests that CSA not only inhibits inflammatory cytokine synthesis but also inhibits the MDR function and makes T cells sensitive to IMT treatment.

Adjunctive Oral CSA Can Reduce MDR Function and Improve Clinical Response to IMT Therapy

To study the adjunctive effect of CSA in nonresponders to IMT, we isolated and stimulated the CD4$^+$ T cells before and after treatment with oral CSA 100 mg twice daily for at least 2 months. All three patients were diagnosed with VKH disease and were under azathioprine therapy (2–3 mg/kg body weight daily) at the time of initiation of adjunctive CSA therapy. Following adjunctive CSA therapy, they had complete resolution of intraocular inflammation (as recognized by anterior chamber reaction in recurrent VKH disease) at the end of 2 months. In line with our clinical observations, a significant decrease in MDR function (Figs. 4A, 4B), as well as in intracellular proinflammatory cytokines, individually (IL-17) or in combinations (IFNγ–IL-17 and TNFα–GM-CSF), was also noted (Figs. 4C, 4D).

Discussion

In this study, we have investigated one of the key mechanisms that can affect the clinical response to IMT in the management of NIU—namely, transmembrane efflux proteins that decrease intracellular concentrations of drugs in the CD4$^+$ T-lymphocytes. We demonstrated the higher function of efflux transporter MDR proteins in patients with NIU who are nonresponders to IMT. The high MDR expression was associated with increased proinflammatory cytokine production

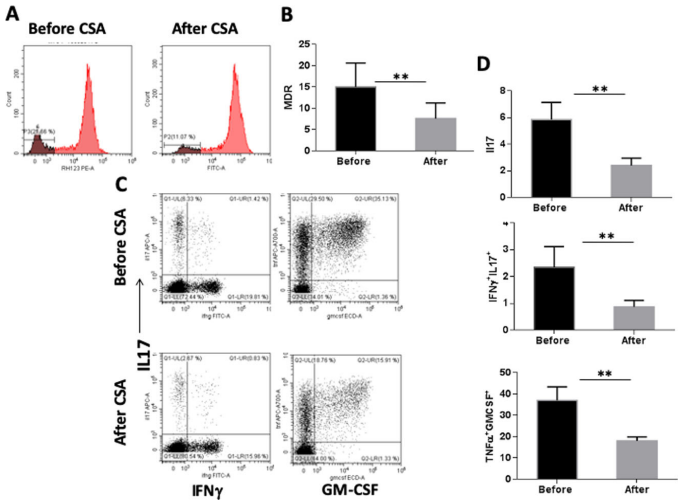

Figure 4. CSA as an adjuvant therapy decreased MDR1 function as well as proinflammatory CD4$^+$ T cells in IMD nonresponder ($n = 3$) patients. (A, B) MDR1 function decreased and (C, D) proinflammatory CD4$^+$ T cells also decreased after CSA treatment (100 mg twice daily for 2 months). $^{**}P < 0.01$.

in nonresponder samples. In a small cohort of patients, we also demonstrated the therapeutic efficacy of generalized MDR inhibitor CSA in reducing MDR function and proinflammatory cytokine secretion and improving clinical inflammation scores.

Several important issues need further explanation to establish the clinical relevance of our results in the management of NIU. These include the range of efflux transporter proteins that are covered by the Rh-123 assay, the relationship between those proteins and the two drugs used for IMT in our study (methotrexate and azathioprine), factors confounding the correlation between MDR protein function and refractoriness to therapy, and, finally, possible clinical application of adjunctive CSA therapy in the management of NIU. The central theme that can possibly explain all the above concerns is the overlapping substrate specificities of different transport proteins.[7] This applies not only to the drugs but also to the dyes used for functional assay of these proteins. To start with, Rh-123 is a classical substrate for MDR1 (or P-gp) and has been widely used for assessing MDR1 function in anticancer as well as anti-inflammatory therapies.[17] The dye also binds to multiple sites on MRP1,[18,19] although not as strongly as MDR1, and therefore other fluorescent dyes such as calcein and glutathione have been recommended for MRP1 assays. Nevertheless, Rh-123 assay possibly provides the broadest indication of the cellular efflux function, among all available dye efflux assays.

MDR1 (P-gp) has a wide array of substrates that are generally either neutral or cationic hydrophobic compounds with the ability of passive diffusion into cells.[10] Methotrexate, being anionic and hydrophilic, should not be a substrate of MDR1. Indeed, MRP3 and MRP1 are known to be the main efflux transporters for methotrexate.[11] However, methotrexate-resistant cell lines showed MDR1 (*ABCB1*) gene function that could be partially reversed with MDR1-specific monoclonal antibodies.[12] It is conceivable that local tissue environments that facilitate passive diffusion of the drug into cells would allow a significant role of MDR1 in methotrexate efflux. In contrast to methotrexate, limited data are available on drug efflux mechanisms in azathioprine. These include *MRP4* and *MDR1*, since polymorphisms in both these genes have been found to correlate with susceptibility to azathioprine in inflammatory bowel disease.[13,14] Together, published data suggest that while different efflux transporters have varying affinities for various drugs used for IMT, none of the drugs are specific for a unique transporter and may be using different transporters in different tissue environments. Among the efflux transporter proteins, MDR1 seems to the most versatile with the widest variety of substrates, and therefore a reasonable target for inhibition, as discussed later.

The other major confounder to evaluate MDR proteins is their association with inflammation. MDR1 has been associated with the production of several cytokines such as IL-2, IL-4, and IFNγ.[21] In line with this observation, MDR1 protein expression was found to correlate with disease activity in rheumatoid arthritis (RA) and not with refractoriness to methotrexate therapy.[22] In the present study too, increased MDR1 function in nonresponders to IMT was associated with increased secretion of proinflammatory cytokines, while the reverse was noted in responders. However, we also found that the MDR function was comparable between patients with uveitis not on systemic therapy and healthy controls, even though proinflammatory cytokines were significantly higher in the uveitis group (Supplementary Fig. S2). One possible reason for the association of MDR1 function with inflammation is that MDR1 is mostly expressed by the proinflammatory memory (effector and central) T cells but not regulatory T cells.[23] These memory T cells produce both Th1 and Th17 cytokines (Th1.17) and are enriched locally at the site of inflammation.[24] Our earlier study showed that in the eye, too, significant numbers of IFNγ and IL-17 dual-positive CD4$^+$ T cells were present, at least in tuberculosis-associated uveitis.[25] Moreover, we have recently found that vitreous CD4$^+$ T cells in NIU also have significantly higher MDR function than peripheral blood mononuclear cells (manuscript under review). However, published data on the association of MDR protein function with

inflammation also provide contradictory evidence. For example, one study found decreased functional activity of both MDR1 and MRP1 in peripheral blood leukocytes of RA patients compared to non-RA and healthy controls.[26] Another study found downregulation of MDR1 expression in during early stages of methotrexate treatment in patients with RA.[27] Part of this incongruence between MDR function and grade of inflammation could also be ascribed to the various endogenous functions of these transporters that include transport of several metabolites and signaling molecules, besides inflammatory cytokines.[7] Overall, it appears that the relationship between MDR expression/function and inflammation or disease activity is multifactorial and needs further analysis.

Since our experimental data suggested a positive association between MDR function and refractoriness to IMT in NIU, we investigated standard-dose cyclosporine as a possible therapeutic intervention for inhibiting MDR activity and improving clinical efficacy of IMT. Most clinical trials investigating MDR inhibition, specifically P-gp inhibition, have yielded disappointing results.[8] The first-generation inhibitors (verapamil, CSA) showed low therapeutic efficacy and high cell toxicity, while second-generation inhibitors (dexverapamil and valspodar) had frequent drug-drug interactions due to cytochrome P450 inhibition. Despite this information, we decided to test the efficacy of CSA in inhibition of MDR since it is part of the conventional IMT armamentarium for the management of NIU. It has been used in combination with other IMTs for management of NIU.[28] In vitro studies, including ours, suggest a high concentration of cyclosporine (0.1–1 µg/mL) is required for effective P-gp inhibition.[29] However, this would require very high and potentially toxic doses of cyclosporine to be used in patients. Instead, we used the standard dose of cyclosporine, 100 mg twice daily (though up to 10 mg/kg/d is used in renal transplant patients), as an adjunct to the existing IMT—methotrexate or azathioprine.

We could demonstrate that even the standard dose of CSA administered for 6 weeks could significantly reduce MDR function and improve clinical scores in patients with NIU. In this manner, we could repurpose combination therapy of CSA with another IMT for management of possible MDR protein-mediated resistance to IMT. This is comparable to earlier studies, where standard-dose tacrolimus had been used to overcome P-gp function in refractory RA.[30] However, the possibility of decreased cytokine secretion following combination therapy, alone being responsible for the decreased MDR function, cannot be ruled out. Also, pharmacokinetic studies have revealed that aqueous concentrations of CSA (28 ng/mL) following systemic treatment at a dose of 5 mg/kg/d are way below the therapeutic doses (50–100 ng/mL) required for immunosuppression or P-gp inhibition.[31] Thus, any benefit achieved by oral CSA therapy could only be through its systemic effect on lymphocytes. Interestingly, CSA has been shown to selectively attenuate glucocorticoid-resistant Th17 cells in mouse models of experimental autoimmune uveitis and in human Th17 cells generated in vitro.[32] We did not evaluate MDR function in intraocular T cells since none of the patients recruited in this study required therapeutic vitrectomy for management of uveitis and therefore no vitreous samples could be retrieved.

Notwithstanding the underlying mechanism, our study provides the first correlation between MDR function and responsiveness to IMT in NIU. It also offers the possibility of repurposing standard-dose cyclosporine therapy as a strategy to overcome MDR protein-mediated resistance to IMT. Future studies could be aimed at dissecting systems-level function of various drug transporter proteins and their role in the development and resolution of NIU. Our results should also stimulate larger studies to validate the role of oral CSA and other MDR inhibitor molecules in the management of NIU.

Acknowledgments

Supported by the Department of Biotechnology (BT/PR5539/MED/29/521/2012), Government of India (SB), and by Hyderabad Eye Research Foundation (SB).

Author contributions: RT and SB conceived and designed the experiments. SB and HK supervised and coordinated the work. RT performed most experiments and analyzed data. KR and SD provided technical assistance and performed experiments. SB drafted and finalized the manuscript.

Disclosure: **R. Tagirasa**, None; **K. Rana**, None; **H. Kaza**, None; **D. Parai**, None; **S. Damera**, None; **S. Basu**, None

References

1. de Smet MD, Taylor SR, Bodaghi B, et al. Understanding uveitis: the impact of research on visual outcomes. *Prog Retin Eye Res*. 2011;30:452–470.

2. Thorne JE, Wittenberg S, Jabs DA, et al. Multifocal choroiditis with panuveitis: incidence of ocular complications and of loss of visual acuity. *Ophthalmology*. 2006;113:2310–2316.
3. Jabs DA. Immunosuppression for the uveitides. *Ophthalmology*. 2018;125:193–202.
4. Forooghian F, Gulati N, Jabs DA. Restoration of retinal architecture following systemic immunosuppression in birdshot chorioretinopathy. *Ocul Immunol Inflamm*. 2010;18:470–471.
5. Kempen JH, Altaweel MM, Holbrook JT, Sugar EA, Thorne JE, Jabs DA. Association between long-lasting intravitreous fluocinolone acetonide implant vs systemic anti-inflammatory therapy and visual acuity at 7 years among patients with intermediate, posterior, or panuveitis. *JAMA*. 2017;317:1993–2005.
6. Moscow JA, Cowan KH, Sikic BI. Drug resistance and its clinical circumvention. In: Bast RC, Hait WN, Kufe DW, eds. *Holland-Frei Cancer Medicine*. Hamilton, ON: BC Decker; 2016:1–7.
7. Nigam SK. What do drug transporters really do? *Nat Rev Drug Discov*. 2015;14:29.
8. Li W, Zhang H, Assaraf YG, et al. Overcoming ABC transporter-mediated multidrug resistance: molecular mechanisms and novel therapeutic drug strategies. *Drug Resist Updat*. 2016;27:14–29.
9. Jaramillo AC, Al Saig F, Cloos J, Jansen G, Peters GJ. How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance. *Cancer Drug Resist*. 2018;1:6–29.
10. 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;15:4965–4976.
11. Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res*. 2001;61:7225–7232.
12. Norris MD, Graaf DD, Haber M, et al. Involvement of MDR1 P-glycoprotein in multifactorial resistance to methotrexate. *Int J Cancer*. 1996; 65:613–619.
13. Ban H, Andoh A, Imaeda H, et al. The multidrug-resistance protein 4 polymorphism is a new factor accounting for thiopurine sensitivity in Japanese patients with inflammatory bowel disease. *J Gastroenterol*. 2010;45:1014–1021.
14. Mendoza JL, Urcelay E, Lana R, et al. MDR1 polymorphisms and response to azathioprine therapy in patients with Crohn's disease. *Inflamm Bowel Dis*. 2007;13:585–590.
15. Peterson BG, Tan KW, Osa-Andrews B, Iram SH. High-content screening of clinically tested anticancer drugs identifies novel inhibitors of human MRP1 (ABCC1). *Pharmacol Res*. 2017;119:313–326.
16. Chen P, Chen H, Zang X, et al. Expression of efflux transporters in human ocular tissues. *Drug Metab Dispos*. 2013;41:1934–1948.
17. Petriz J, Garcia-Lopez J. Flow cytometric analysis of P-glycoprotein function using rhodamine 123. *Leukemia*. 1997;11:1124–1130.
18. Daoud R, Kast C, Gros P, Georges E. Rhodamine 123 binds to multiple sites in the multidrug resistance protein (MRP1). *Biochemistry*. 2000;39:15344–15352.
19. Saengkhae C, Loetchutinat C, Garnier-Suillerot A. Kinetic analysis of rhodamines efflux mediated by the multidrug resistance protein (MRP1). *Biophys J*. 2003;85:2006–2014.
20. Dick AD, Rosenbaum JT, Al-Dhibi HA, et al. Guidance on noncorticosteroid systemic immunomodulatory therapy in noninfectious uveitis: Fundamentals Of Care for UveitiS (FOCUS) Initiative. *Ophthalmology*. 2018; 125:757–773.
21. Drach J, Gsur A, Hamilton G, et al. Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. *Blood*. 1996;88:1747–1754.
22. 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;28:427–433.
23. Dimeloe S, Frick C, Fischer M, 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.
24. Ramesh R, Kozhaya L, McKevitt K, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med*. 2014;211:89–104.
25. Tagirasa R, Parmar S, Barik MR, Devadas S, Basu S. Autoreactive T cells in immunopathogenesis of TB-associated uveitis. *Invest Ophthalmol Vis Sci*. 2017;58:5682–5691.
26. Micsik T, Lőrincz A, Gál J, Schwab R, Peták I. MDR-1 and MRP-1 activity in peripheral blood leukocytes of rheumatoid arthritis patients. *Diagn Pathol*. 2015;10:216.

27. Hider SL, Owen A, Hartkoorn R, et al. Down regulation of multidrug resistance protein-1 expression in patients with early rheumatoid arthritis exposed to methotrexate as a first disease-modifying antirheumatic drug. *Ann Rheum Dis*. 2006;65:1390–1393.
28. Cervantes-Castañeda RA, Gonzalez-Gonzalez LA, Cordero-Coma M, Yilmaz T, Foster CS. Combined therapy of cyclosporine A and mycophenolate mofetil for the treatment of birdshot retinochoroidopathy: a 12-month follow-up. *Br J Ophthalmol*. 2013;97:637–643.
29. 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;52:1676–1683.
30. Suzuki K, Saito K, Tsujimura S, et al. Tacrolimus, a calcineurin inhibitor, overcomes treatment unresponsiveness mediated by P-glycoprotein on lymphocytes in refractory rheumatoid arthritis. *J Rheumatol*. 2010;37:512–520.
31. Diaz-Llopis M, Menezo JL. Penetration of 2% cyclosporin eyedrops into human aqueous humour. *Br J Ophthalmol*. 1989;73:600–603.
32. Schewitz-Bowers LP, Lait PJ, Copland DA, et al . Glucocorticoid-resistant Th17 cells are selectively attenuated by cyclosporine A. *Proc Natl Acad Sci USA*. 2015;112:4080–4085.