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Title: Identification of IL6 mediated molecular Mechanisms and Potential Role of lectin pathway activation in antibody-mediated rejection

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ABSTRACT

Acute and chronic antibody-mediated rejection (AMR), depending on pre-formed or de novo development of donor-specific antibodies (DSA), mainly anti-HLA, are the major problem in long-term allograft survival. The interaction between DSAs and renal endothelial cells is well studied, but less is known about the interaction with renal epithelial cells and its potential role in tubule-interstitial damage. We have previously demonstrated the positive effects of IL6 receptor (IL6R) antagonist Tocilizumab (TCZ) in chronic AMR, also noting a positive modulation of tight junction 1 (TJP1/ZO1) in the tubular compartment. TCZ has been associated with positive effects and the potential risk of IL6 rebound after discontinuation. We, therefore, investigated the *in vitro* effects of anti-HLA class I (W6/32) on immortalized proximal tubular epithelial cells (RPTEC/TERT1), evaluating the different impacts of contemporary direct IL6 inhibition (with Siltuximab [SLTX]) or indirect (TCZ). The incubation of RPTEC/TERT1 with W6/32 induced no significant effects on proliferation, but a significant reduction in TJP1-ZO1 expression was documented by PCR and immunofluorescence on cultured cells. Contemporary culture for 72h with TCZ and SLTX determined different effects, with increased IL6 concentration, IL6R expression, and restoration of TJP1/ZO1 by TCZ and no modification by SLTX. Increased IL6R expression with TJP1/ZO1 restoration was observed *in vivo* in patients TCZ-treated with chronic AMR, where anti-HLA DSAs could also be detected in urine.

Additionally, the principal activator of the lectin pathway (mannose-binding lectin [MBL]) gene could be detected in RPTEC/TERT1, and the addition of MBL to the cultured RPTEC/TERT1 induced reduced proliferation only with contemporary W6/32 and TCZ stimulation, suggesting a potential role of mannose-binding receptors in this scenario.

Taken together, our results suggested a direct effect of anti-HLA DSAs on the tubular cells (also through urinary excretion) and a different response pattern between direct and indirect IL6 inhibitions and lectin pathway with potential implications in the tubule-interstitial damage.

1. INTRODUCTION

Antibody-mediated rejection (AMR) is a major challenge in kidney transplantation. Despite advancements in immunological techniques for transplantation, AMR remains a significant cause of graft failure after kidney transplantation (1,2). This thesis aims to provide a comprehensive understanding of the pathophysiological processes contributing to antibody-mediated rejection in kidney transplantation and explore more possible features of this chronic pathology.

1.1 ROLE OF DONOR-SPECIFIC ANTIBODIES IN KIDNEY TRANSPLANTATION

Over the past two decades, there has been a shift in our understanding of rejection in kidney transplantation. Initially, rejection was primarily regarded as a T-cell-mediated process. However, it is now recognized that the inadequate control of the humoral arm of a recipient's immune system is the primary contributing factor to allograft dysfunction and loss. One of the most severe complications that frequently occurs after transplantation is acute ***antibody-mediated rejection (AMR)***. This condition can arise either due to pre-transplant *donor-specific antibodies* (DSA) or the development of de novo DSAs (3). The detection of DSAs in recipient serum and advancements in graft pathology assessment have allowed for the recognition and understanding of this entity in recent years. Up-to-time methods for treating antibody-mediated rejection involve a multi-step process, including desensitization of patients with pre-formed antibodies to prevent acute rejection. During acute antibody-mediated rejection, the focus is removing antibodies from the serum and using immunosuppressants targeting B cells (4). New therapies are being tested to control chronic antibody-mediated rejection, which is a frequent cause of late graft dysfunction.

1.2 ACUTE AND CHRONIC ANTIBODY-MEDIATED DAMAGE AND MANAGEMENT

DSAs are a critical factor in organ transplantation, which plays a prominent role in acute and chronic antibody-mediated rejection (cAMR). **Acute AMR** can occur due to pre-formed antibodies at high titers or develop after transplantation; it is observed in 5-7% of kidney transplant cases. It leads to endothelial damage, neutrophil infiltration of glomeruli and peritubular capillaries, interstitial edema, and hemorrhaging. The identification of AMR has become more accessible with the recognition of C4d in biopsies (5). In contrast, **chronic antibody-mediated rejection** is histologically characterized by transplant glomerulopathy. Transplant glomerulopathy is marked by mesangial expansion, basement membrane duplication, and multilayering of the basement membrane of peritubular capillaries (6). Typically asymptomatic in its early stages, it can later lead to nephrotic-range proteinuria, hypertension, and a gradual decline in renal function, leading to the loss of the organ. The prevalence of transplant glomerulopathy in protocol biopsies varies from 5% at one year to 20% at five years. Both acute and chronic antibody-mediated rejection pose significant challenges in organ transplantation. While acute AMR is characterized by early endothelial damage, cAMR rejection, represented by transplant glomerulopathy, progresses slowly and can lead to severe renal dysfunction over time. Understanding the role of donor-specific antibodies in these rejection processes is crucial for improving transplantation outcomes.

The management of chronic antibody-mediated rejection continues to pose a significant challenge, as conventional therapeutic modalities have shown limited success in improving long-term outcomes. Despite the use of immunosuppressive medications, AMR remains a major cause of kidney allograft failure and hinders the improvement of long-term allograft survival. In recent years, significant advancements in our understanding of the pathophysiological processes of antibody-mediated rejection have paved the way for developing novel therapeutic options. These advancements have led to the establishment surveillance protocols using donor-derived cell-free DNA and gene profiling testing, enabling early detection of antibody-mediated rejection. As a result, numerous clinical trials are currently underway, offering promising opportunities to enhance kidney transplant recipients' outcomes (4,7).

A multimodal treatment approach is necessary to manage antibody-mediated rejection in kidney transplantation effectively. The current guidelines, along with published consensus

guidance, recommend several therapeutic modalities for the treatment of AMR in kidney transplant recipients.

Firstly, the removal and neutralization of alloantibodies are crucial in managing AMR. Therapeutic plasmapheresis, a process that involves removing plasma from the patient and replacing it with a replacement fluid, is effective in reducing circulating alloantibody levels. Additionally, intravenous immunoglobulin can be administered to prevent the binding of alloantibodies to their target antigens. Furthermore, the optimization of the maintenance immunosuppression regimen is essential in preventing and treating antibody-mediated rejection. This typically involves using immunosuppressive medications that target B cells, such as anti-CD20 antibodies like rituximab. Moreover, lymphocyte-depleting antibody therapies can be utilized to suppress the immune response to alloantibodies. However, no therapy has been to date specifically approved for this condition, and the long-term outcomes of kidney transplant recipients with antibody-mediated rejection remain poor. Recently, some experiences, including our group, documented a stabilization of the eGFR after treatment with Tocilizumab, an IL6 receptor inhibitor, for chronic antibody-mediated rejection, paving the way for future research in this area (8).

1.3 AVAILABLE EXPERIMENTAL EXPERIENCE FOR ANTIBODY-MEDIATED DAMAGE

It was demonstrated that HLA class I antibodies activate endothelial cells and smooth muscular cells through various signaling pathways, which may contribute to AMR (9). In particular, crosslinking of HLA class I molecules by anti-HLA antibodies activates Rho signaling, triggering reorganization of the cytoskeleton and formation of F-actin stress fibers (10).

It was demonstrated that phosphorylation of the guanosine-50-triphosphate (GTP)-binding protein RhoA and its association with stress fibers following antibody ligation of class I molecules on endothelial cells. RhoA mediated phosphoinositide 3-kinase (PI3K) dependent endothelial cell proliferation (11). Valenzuela et al. also demonstrated the potential of Class I HLA antibodies to activate endothelium for monocyte recruitment, indicating it as a multi-step process dependent on the IgG subclass too (12). They also demonstrated that monocyte Adhesion to HLA I Antibody-Activated Endothelial Cells is enhanced by activation of the complement cascade (13).

1.4 IL-6: PHYSIOLOGIC ROLE AND POTENTIAL INVOLVEMENT IN ANTIBODY-MEDIATED DAMAGE

To address the unmet need to improve outcomes for patients at risk for antibody-mediated rejection, Tocilizumab has emerged as a potential therapeutic option. Tocilizumab is a monoclonal antibody that targets the interleukin-6 receptor, a key mediator of inflammation and immune response.

Several studies have investigated the use of Tocilizumab in the management of AMR in kidney transplantation. Choi et al. (14) and our group (15) separately evaluated the efficacy of Tocilizumab as a potential treatment for chronic antibody-mediated rejection and transplant glomerulopathy in HLA-sensitized renal allograft recipients.

Moreover, Pottebaum et al. examined the use of Tocilizumab in a single-center observational study of kidney transplant recipients who received at least one dose of Tocilizumab in addition to conventional therapies for acute AMR, claiming that Tocilizumab may be considered as an addition to conventional therapies for the treatment of acute active AMR (15). The analyses demonstrated that Tocilizumab significantly decreased circulating donor-specific antibodies and improved patient outcomes.

IL-6 plays a crucial role in inflammation and immune responses, and its signaling is involved in the activation and survival of B cells, which are central to the alloimmune response in AMR by producing DSA.

IL-6 signaling can occur through two distinct pathways: classic signaling and trans-signaling. Classic IL-6 signaling is mediated by membrane-bound IL-6 receptor (IL-6R), which is primarily expressed in hepatocytes, monocytes, and some lymphocytes. In this pathway, IL-6 binds to the IL-6R, leading to the activation of intracellular signaling cascades and subsequent immune responses. On the other hand, trans-signaling involves the binding of soluble IL-6R (sIL-6R) to IL-6, forming a complex that can bind to glycoprotein 130. This complex can activate signaling pathways in cells that do not express the membrane-bound IL-6R, expanding the range of cells affected by IL-6 signaling. The regulation of IL-6 signaling is crucial in maintaining immune homeostasis and preventing excessive inflammation. The role of IL-6 in antibody-mediated rejection in kidney transplantation has been extensively studied. The overexpression of IL-6 in antibody-mediated rejection suggests its involvement in the pathogenesis and progression of this complication in kidney transplantation (16–18). Despite the potential benefits of

Tocilizumab in the management of antibody-mediated rejection, there is still a need for further research to fully understand its efficacy, safety, and optimal use in kidney transplantation (19).

1.5 COMPLEMENT CASCADE AND ROLE IN ANTIBODY-MEDIATED DAMAGE

In the complex landscape of transplantation immunology, the interplay between donor-specific antibodies (DSAs), particularly IgG1 and IgG3 isotypes, and the complement cascade could play a pivotal role in mediating antibody-mediated damage. These IgG subclasses are potent activators of the classical complement pathway and exhibit high affinity for Fc gamma receptors on myeloid cells, making them central to the immune response in transplant recipients. Despite advancements in assay development to characterize the functional deposition of complement components, inconsistencies and contradictions persist in the predictive significance of complement-fixing DSAs, leading to a lack of correlation with lymphocytotoxicity results. The factors influencing complement activation and antibody-mediated damage are multifaceted, encompassing antigen density, antibody titer, the presence of immunoglobulin M, and possibly the epitope specificity and Fc glycan composition of the antibodies. The utility of C4d staining as a diagnostic marker has also been questioned, particularly in the context of *chronic antibody-mediated injury*, which does not always correlate with complement activation. Additionally, the evidence that C4d has not always been associated with C1q in kidney biopsies may suggest a potential involvement of the lectin pathway instead of the classical one. Endothelial cells, the primary targets of antibody-mediated alloimmunity, display a remarkable resistance to complement-mediated lysis, further complicating our understanding of the role of complement in transplant rejection (20).

2. MATERIAL AND METHODS

Cell Culture and Maintenance

The RPTEC/TERT1 cells were acquired from the American Type Culture Collection (ATCC® CRL-4031™). Upon receipt, the cells were stored in liquid nitrogen (vapor phase) for preservation. For cell thawing, a water bath at 37°C was prepared, and the cryovial containing frozen RPTEC/TERT1 cells was rapidly thawed. The thawed cells were then aseptically transferred into a 15 mL centrifuge tube containing pre-warmed RPTEC/TERT1 growth medium added with the appropriate growth kit.

For cell seeding and subculturing, the thawed RPTEC/TERT1 cells were seeded into T-75 culture flasks at appropriate cell seeding density in the RPTEC/TERT1 growth medium. The culture flasks were incubated at 37°C in a humidified atmosphere with 5% CO2. The culture medium was replaced every 48 hours until the cells reached approximately 80-90% confluency. Subculturing was performed by washing the cells with 1x PBS, adding trypsin-EDTA solution, and neutralizing trypsin with RPTEC/TERT1 growth medium before transferring the cells at the desired ratio into new culture flasks.

The RPTEC/TERT1 growth medium was prepared using Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) in a 1:1 ratio, supplemented with RPTEC/TERT1 growth kit (hTERT Immortalized RPTEC Growth Kit, ATCC® ACS-4007™), 1% Penicillin-Streptomycin solution, and 1% Glutamine.

Cell proliferation assay

The RPTEC/TERT1 were grown in appropriate culture medium in tissue culture plates or dishes and maintained in an incubator at 37°C with 5% CO2 until they reached the desired confluence or experimental conditions. BrdU Labeling:BrdU working solution were prepared by diluting the BrdU reagent from the Cell Proliferation Kit in the cell culture medium at the recommended concentration following the manufacturer's instructions (BrdU Cell Proliferation ELISA Kit, Abcam, Cambridge, UK). Then the culture medium was replaced with the BrdU working solution, and incubate the cells overnight to allow BrdU incorporation into proliferating cells, additionally adding with different concentrations of azide-free InVivoMab anti-human MHC Class I (HLA-A, HLA-B, HLA-C) (W6/32) (BioXCell, New Hampshire, USA), IL-6 receptor inhibitor

(Tocilizumab, RoActemra, Roche, Germany) or direct IL-6 inhibitor (Siltuximab, Sylvant, Janssen Biologics, Holland). RPTEC/TERT1 were fixed and permeabilized by adding Fixation Buffer to each well and incubating for the recommended time (usually 30 minutes). Anti-BrdU-POD Antibody working solution was used by diluting the provided antibody in Antibody Dilution Buffer according to the kit's instructions. Substrate Solution was added to each well and incubated for the recommended time (usually 10-30 minutes) to develop a colorimetric reaction then the reaction was stopped using the Stop Solution provided in the kit. Absorbance of the samples at 370 nm and 492 nm (reference wavelength) was measured using a microplate reader.

Gene expression analysis

RNA Extraction was performed using TRIzol then cDNA Synthesis using ThermoFisher Retrotranscription KIT containing reverse transcriptase enzyme, reaction buffer, dNTPs, and RNase inhibitor, qPCR was performed using Syber green. Primers for the target genes of interest (TJP1/ZO1, MBL, IL6, e-Cadherin) were performed using primer design software (NCBI). We prepare the qPCR master mix according to the manufacturer's instructions, including Syber Green, Taq DNA polymerase, dNTPs, and reaction buffer.

Immunocytochemistry

RPTEC/TERT1 were grown to confluence on sterile glass coverslips in 8-well plates, then treated with 10 ug/ml W6/32 or/and 100 ug/ml Tocilizumab or Siltuximab for 48 h and finally fixed in 4% para-formaldehyde solution for 15 min at room temperature. Cells were incubated with TJP1/ZO1 antibody (Cell Signaling, Massachusetts, USA) at 1:400 dilution and IL6R antibody at 1:200 (Cell Signaling, Massachusetts, USA) for 1 hour. Anti-rabbit Alexa-Fluor 488 antibody (Invitrogen, Massachusetts, USA) was used to label bound antibodies, and DAPI to label nuclei. Coverslips were mounted in an anti-fading mounting solution (Slow Fade Gold, Invitrogen) and examined under 40× magnification. Digital images were processed using Fiji-ImageJ software (21,22).

Immunofluorescence assay on kidney biopsies

Serial separated sections of frozen tissue from pre- and post-treatment kidney biopsies of renal transplanted patients with chronic antibody-mediated rejection derived from our previous study[31]were fixed in cold acetone for 10 minutes, washed with 0.1% PBS-BSA, blocked with 1% PBS-BSA and subsequently incubated with TJP1/ZO1 antibody (Cell Signaling, Massachusetts, USA) at 1:400 dilution and IL6R antibody at 1:200 (Cell Signaling, Massachusetts, USA) for 1 hour. The sections were washed again, nuclear stained with Hoechst dye 33 258 2.5 µg/mL (Sigma-Aldrich), mounted, and observed under a confocal microscope (Leica SP5, Leica Microsystems). Digital images were processed using Fiji-ImageJ software (21,22).

Urinary determination of anti-HLA DSA

We examined six patients of our Renal Transplant Centre with chronic antibody-mediated rejection, the presence of donor-specific HLA antibodies in sera, and proteinuria > 1 g/die.
Spot urine samples (30 ml) were collected and centrifuged at 3300 x g for 10 minutes to obtain the complete removal of the sediment. Then, 30 ml of the clarified samples were concentrated to 250 (120 fold) in Amicon Ultra Centrifugal Filters with a regenerated cellulose membrane of 50 kDa (Millipore Corporation, Billerica, MA, USA) by centrifugation at 3300 x g for 45 minutes.
Screening tests for anti-HLA class I and II antibodies were performed on samples of serum and concentrated urine of kidney transplanted recipients with multi-antigen bead and single antigen bead kits (One Lambda, Canoga Park, CA, USA) according to manufacturer's instructions. Fluorescence was measured with the Luminex 100 flow analyzer (Luminex, Austin, TX, USA), and the data were expressed as mean fluorescence intensity (MFI).

IL-6 Determination

IL-6 secretion in supernatants of RPTEC/TERT1 stimulated with W6/32, Tocilizumab, or Siltuxiamb was quantified by ELISA immunoassays (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Results were calculated after the generation of a standard curve with appropriate controls and given as averages ± 1 SD.

Statistical analysis

All data of different experimental procedures are expressed as average ± SD. Statistical analysis was performed by analysis of variance with the Newmann-Keuls multi-comparison test or Student's t-test where appropriate. The P values less than 0.05 were considered as the threshold for significance.

3. RESULTS

Direct interaction between DSA and RPTEC/TERT1

DSAs are likely the triggering factor for the macroscopic and microscopic changes observed in antibody-mediated rejection (AMR). As previously mentioned, their role in the pathogenesis of acute AMR has been well-characterized, particularly concerning endothelial damage and transformation in the glomerulus and tubular microvasculature. However, especially in cases of chronic disease, the role of DSAs appears to diverge, often determining alteration of the tubular structure and chronic interstitial damage with tubule-interstitial fibrosis, leading to progressive organ dysfunction, ultimately necessitating re-transplantation or dialysis for the patient.

In this context, we focused on the actual under-considered effect on tubular damage, analyzing the direct contribution of anti-HLA DSAs on tubular epithelium. To do so, we utilized the immortalized cell line RPTEC/TERT1 and a commercial-available antibody that mimics the effect of anti-class I HLA-DSAs (W6/32) with low affinity to Fc receptor based on the selected species (mouse) to limit every potential interaction.

To mimic the previous studies on endothelial cells (23), we focused on the potential proliferative effect of the antibody. Surprisingly, the Brdu assay did not show any dose-response curve regarding pro-proliferative or anti-proliferative effects (Figure 1).

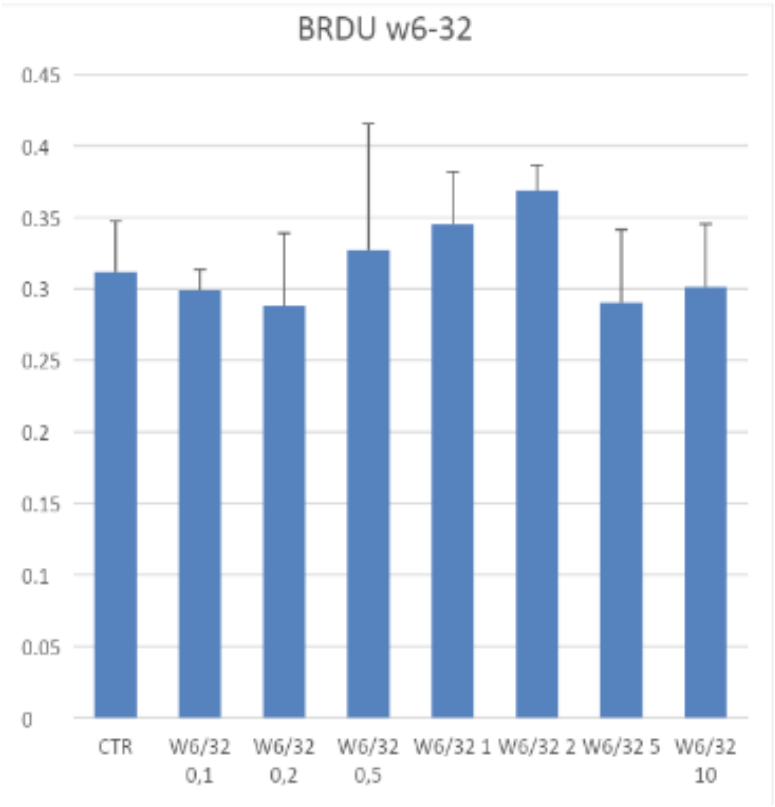


Figure 1. Direct stimulation of renal tubular cells by anti-HLA class I antibody (W6/32, concentrations range 0.1-10 mcg/ml) has no significant effect on cell proliferation.

We simultaneously embarked on a comprehensive assessment to elucidate the potential macroscopic impacts exerted by anti-HLA DSAs on the epithelial cells. For this reason, and based on the proven absence of significant modification of proliferation in our previous experiment, we chose the concentration with a proven effect on endothelial cells (10 mcg/ml).

On the in-vitro cultures after 72h co-culture with W6/32 treatment, we were able to observe a series of notable morphological alterations in the treated epithelial cells. Most prominently, RPTEC/TERT1 exhibited a significantly elongated shape, starkly contrasting with their untreated counterparts, which retained their original form. This observable change in cell morphology is indicative of potential underlying biological processes directly influenced by the antibody treatment.

Concomitantly, we noted a discernible alteration in the cellular adhesion properties. The treated cells demonstrated a less stringent adherence to the substrate, a phenomenon that could potentially be attributed to changes in the expression or functionality of cell adhesion

molecules. To further substantiate our observations and glean more profound insights, we extended our investigations to deep in vitro analyses.

Through these examinations, we were able to identify a notable reduction in the expression of the TJP1/ZO1 protein, a critical molecule implicated in the maintenance of cell-cell adhesion and the integrity of epithelial layers. Interestingly, we previously showed similar results in vivo in patients with chronic antibody rejection due to anti-HLA DSAs (24). More interestingly, cell morphology modifications are associated with increased expression of IL6R on the cell surface (Figure 2).

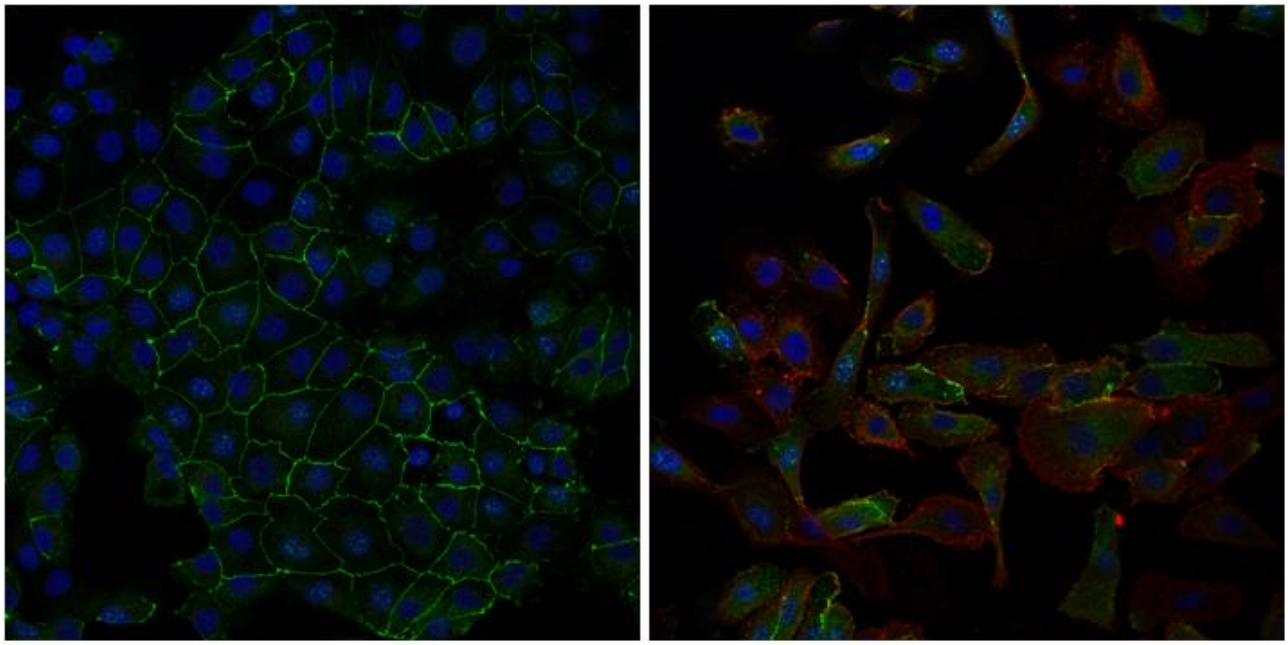


Figure 2. Direct stimulation of RPTEC/TERT1 cells by anti-HLA class I antibody (W6/32 10 µg/ml) altered cell morphology and interaction and reduced TJP1/ZO1 expression; conversely, IL6R could be more prominently detected on the cell surface (Z0-1 green; IL6R red).

Different gene expression and cell morphology after exposition to IL6-interfering drugs

The modification in TJP1/ZO1 expression aligns with our previous observations of altered cell morphology and adhesion properties, providing potential mechanistic insight into the direct effects of anti-HLAs DSAs. It also aims to unravel the complex interplay between antibody interactions and epithelial cell behavior, with a potentially significant role for IL6.

As previously mentioned, existing Literature strongly suggests that IL6 plays a pivotal role in the pathology, predominantly by directly influencing the production of DSA by B cells. It is within this scientific framework that Tocilizumab, an inhibitor of the IL-6R, becomes of paramount importance.

Our research was therefore directed towards scrutinizing the expression of the IL-6 itself and co-stimulating the RPTEC/TERT1 using both Tocilizumab and the W6/32 antibody.

As expected, we found that in vitro Tocilizumab increased IL6 gene expression and IL6 secretion; however, we already noted that co-stimulation with W6/32 and Tocilizumab bolster the higher expression of IL6 gene expression and secretion (Figure 3). Tocilizumab, per se, does not affect cell proliferation.

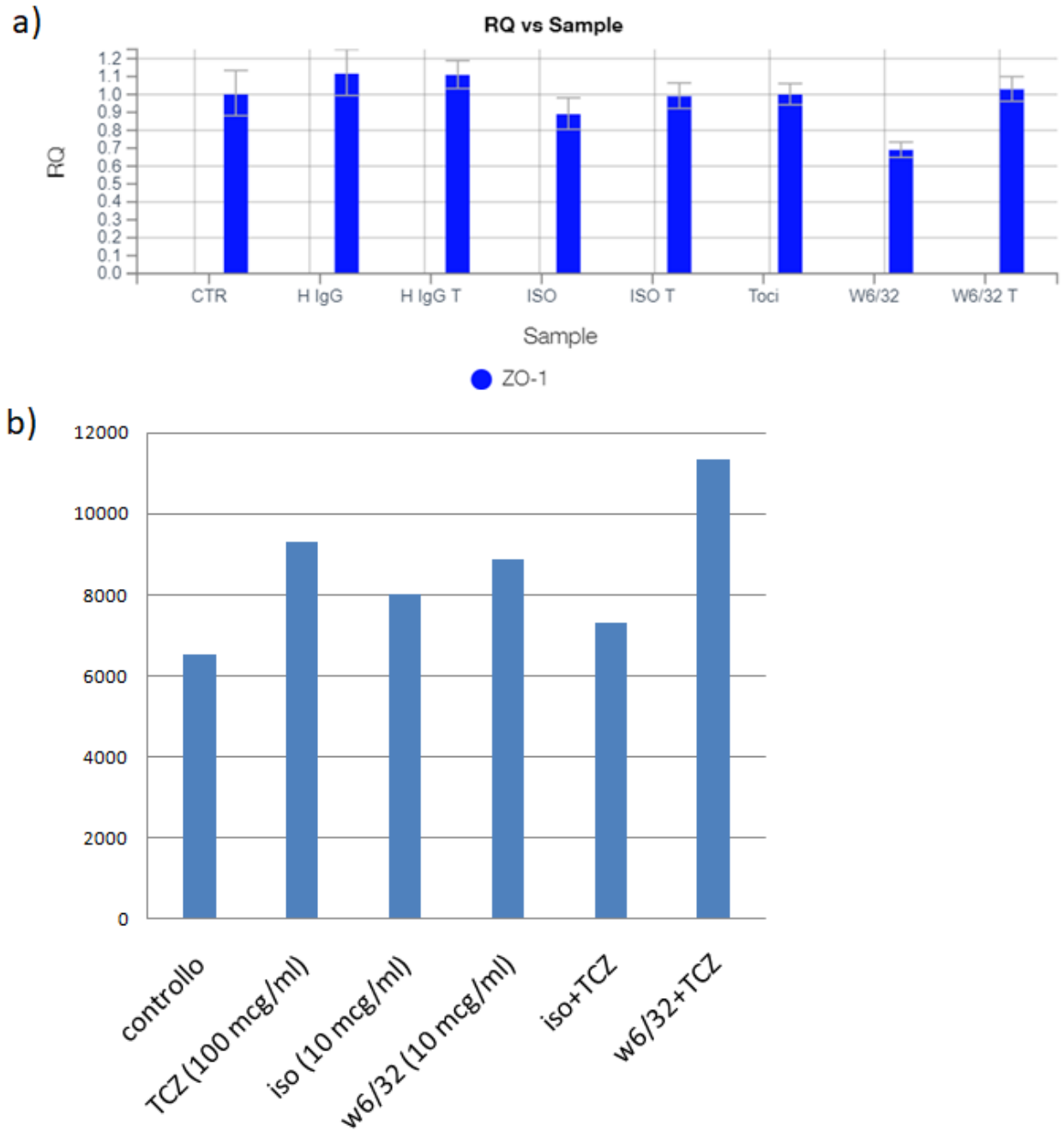
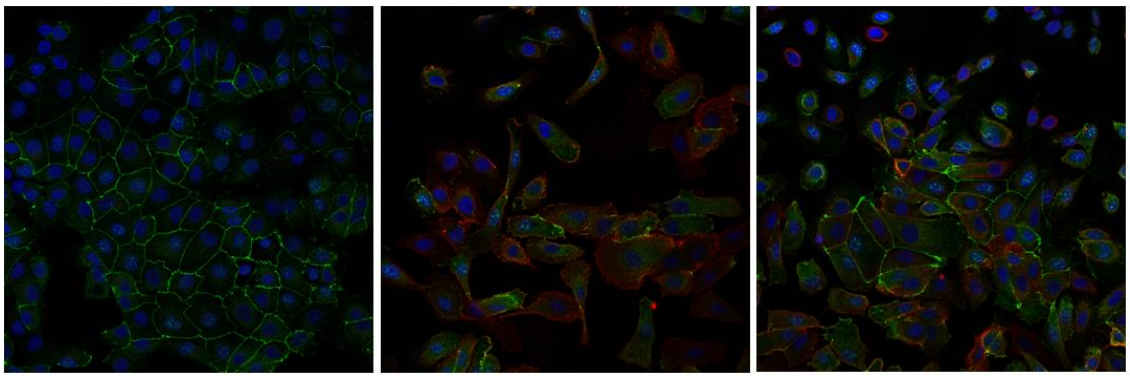


Figure 3. a) Direct stimulation of RPTEC/TERT1 cells by anti-HLA class I antibody (W6/32 10 µg/ml) reduces IL-6 transcription with limited effect on IL-6 secretion; b) treatment with IL-6R inhibitor (Tocilizumab 100 µg/ml) determine significant increase in IL-6 gene transcription and secretion (*p<0.005).

Based on these observations, we repeated the in-vitro cultures for 72h co-culture with W6/32 and Tocilizumab, noting that cells, when subjected to the combined treatment of Tocilizumab

and W6/32, demonstrated improved adherence properties and TJP1/Z01 and e-cadherin expression compared to W6/32 alone, combined with increased IL6R on the cell surface (Figure 4 and Figure 5).

a)



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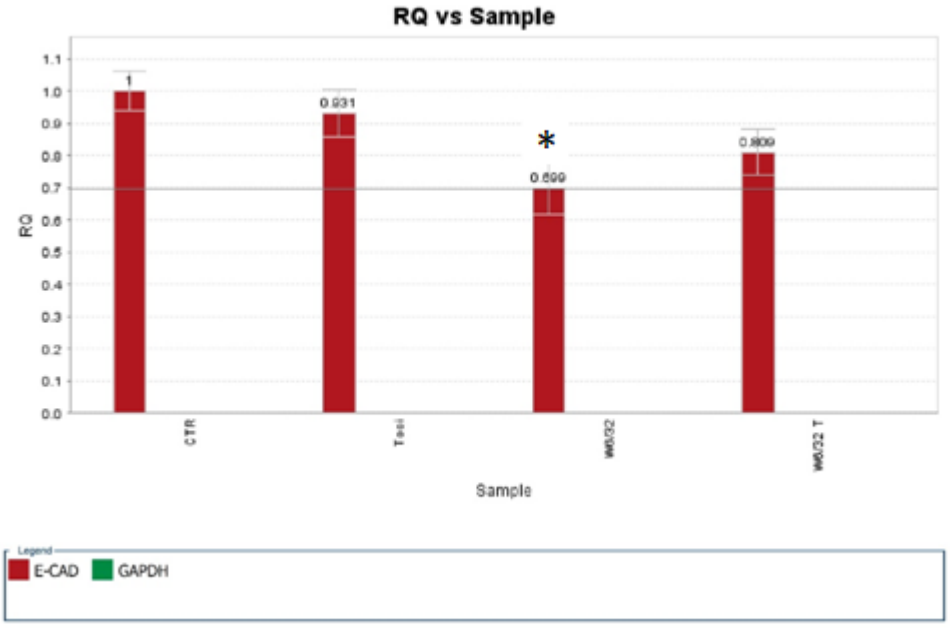

Figure 4. a) Contemporary stimulation with W6/32 and IL-6R inhibitor (Tocilizumab 100 µg/ml) for 72h increased IL6R and TJP1/ZO1 expression, restoring normal cell morphology (left: control; central: W6/32 10 mcg/ml; right: W6/32 and TCZ (100 mcg/ml). Green: TJP1/ZO1; Red: IL6R b) E-

CAD gene expression after 72h of w6/32 and TCZ treatment showed significant reduction with w6/32.

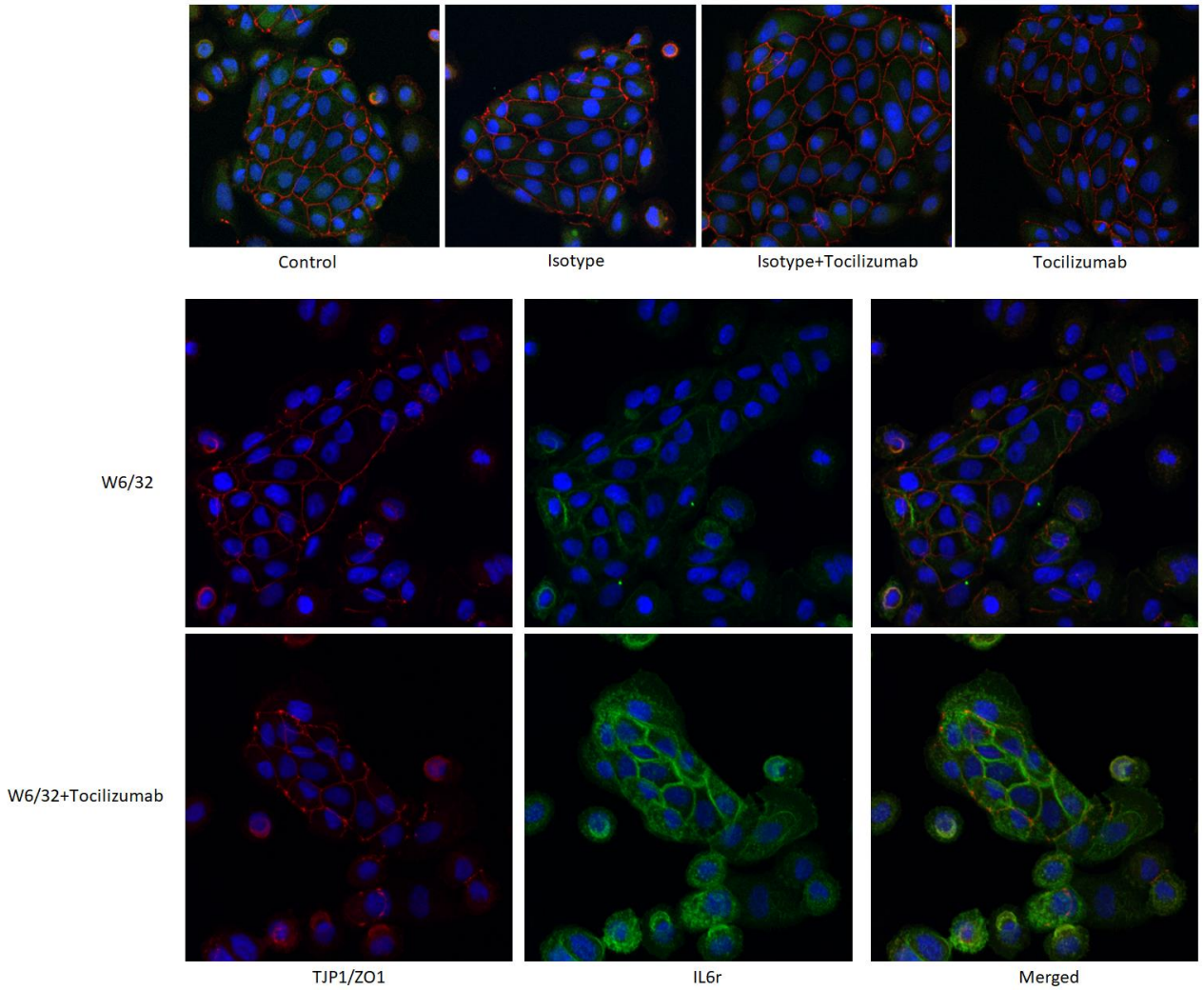

Figure 5a. Contemporary stimulation with W6/32 and IL-6R inhibitor (Tocilizumab 100 µg/ml). After W6/32 stimulation, cells create low contact interactions and clusters; when documented (as in the picture), they assume larger and shaped morphology with reduced TJP1/ZO1 and IL6R. In contrast, W6/32 and Tocilizumab stimulated cells create more clusters, with a morphology similar to control and increased expression of TJP1/ZO1 and IL6R. Green: IL6R; Red: TJP1/ZO1

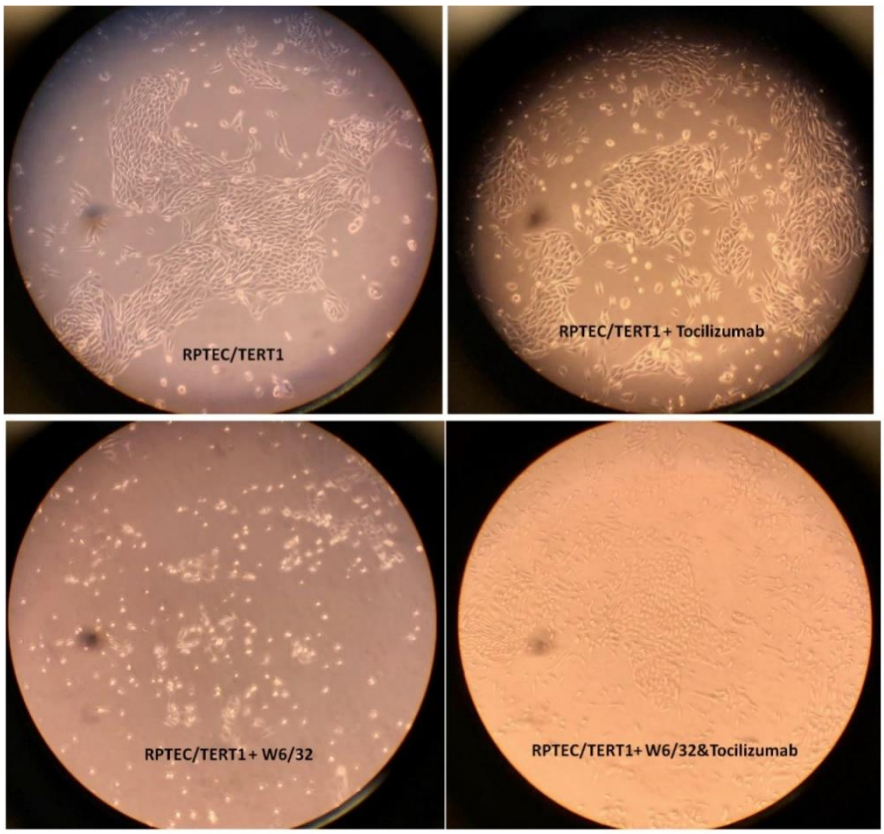

Figure 5b. Contemporary stimulation with W6/32 and IL-6R inhibitor (Tocilizumab 100 µg/ml). After W6/32 stimulation, cells create low contact interactions and clusters; when documented (as in the picture), they assume larger and shaped morphology, whereas Tocilizumab restored the typical pattern.

We then rechecked our kidney biopsies treated with Tocilizumab; patients treated with Tocilizumab showed, apart from the increase of TJP1/ZO1 previously described, an associated increase of IL6R (Figure 6).

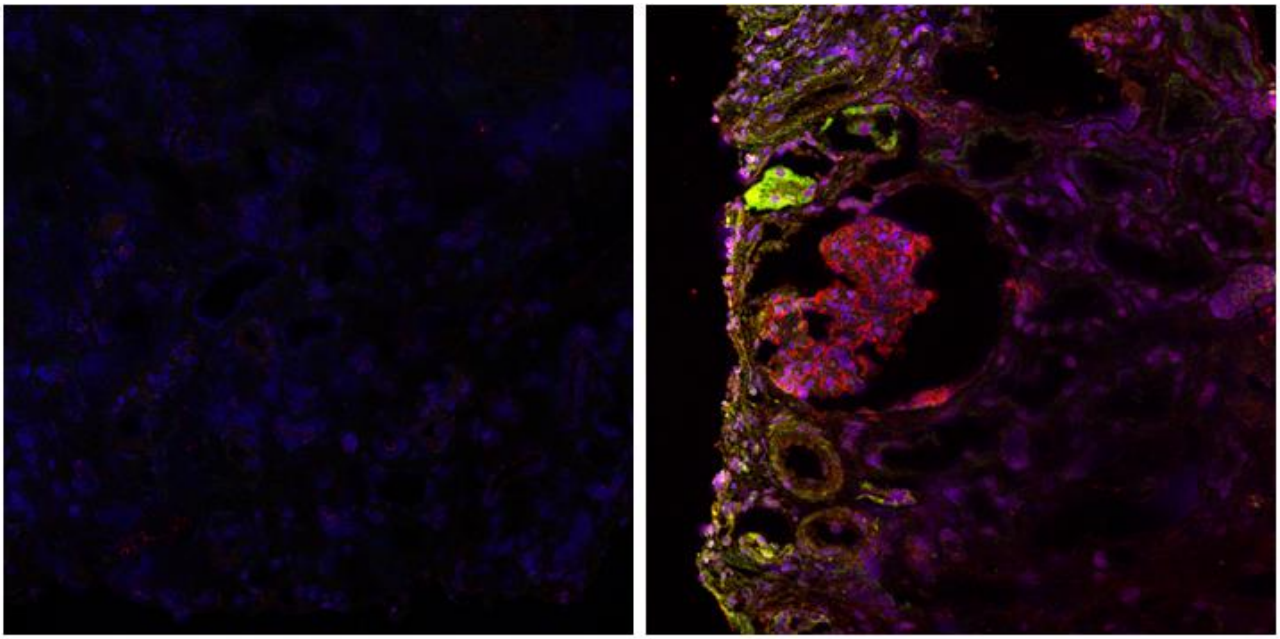

Figure 6. Patients with cAMR have altered morphology of tubular cells and reduced TJP1/ZO1 expression (especially in the tubular compartment) (left); both conditions were restored after IL-6R inhibitor treatment with contemporary increased IL6R expression (right). Green: IL6R; Red: TJP1/ZO1

To dissect the nuances of the IL-6 effect, we set out to draw a direct comparison between the outcomes of inhibiting IL-6 itself and the inhibition of its receptor. For this comparative analysis, we employed Siltuximab, a direct IL-6 inhibitor, alongside Tocilizumab, which is known to inhibit the IL-6 receptor. This dualistic approach allowed us to isolate and understand the unique contributions of each pathway to the observed cellular phenomena. Our analysis revealed a rather intriguing result: the inhibition of IL-6 using Siltuximab has substantially no effect on the TJP1/ZO1 gene or its expression on cultured RPTEC/TERT1. The comparatively lesser effect observed with Siltuximab suggested a differential mechanism at play when IL-6 activity is directly inhibited, as opposed to when its receptor is targeted (Figure 7).

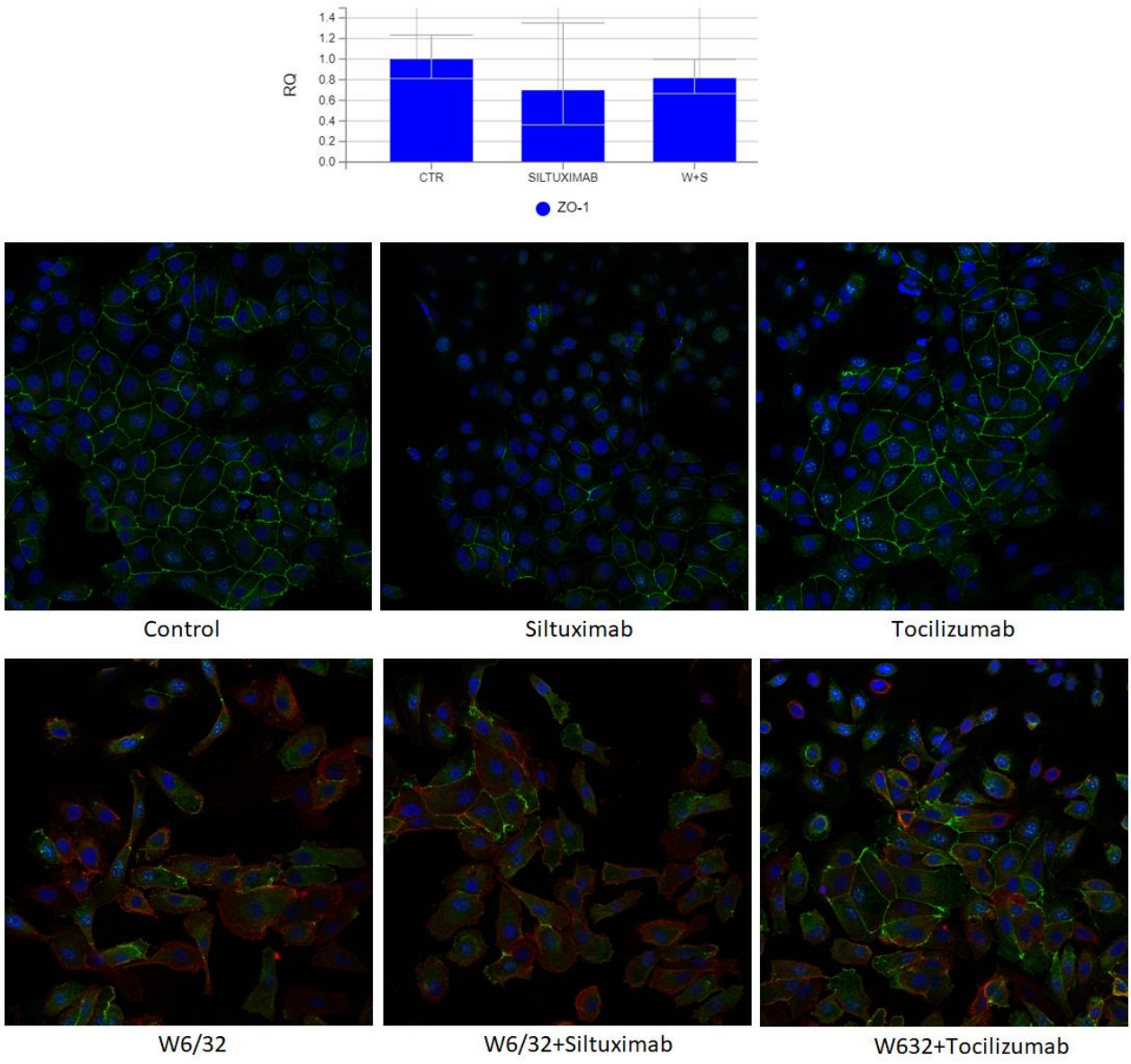

Figure 7. Direct stimulation of renal tubular cells by anti-HLA class I antibody (W6/32 10 µg/ml) altered cell morphology and interaction and reduced TJP1/ZO1 expression; treatment with Siltuximab (100 µg/ml) had no effect on restoring TJP1/ZO1 gene expression or cell morphology/interaction. Green: TJP1/ZO1; Red: IL6R

Potential interactions between antibody mediated-damage and lectin pathway

Within the complex terrain of chronic antibody-mediated rejection, the complement system emerges as a critical player. Given that the deposition of C4d at the tubular level stands as a hallmark of disease progression, we posed the question of whether the lectin pathway could be implicated in the generation of these deposits and the resultant damage to endothelial and

epithelial structures, also considering the putative role of MBL-mediated activation on tubular cells in some glomerulonephritis (25,26) (Mella A et al. Personal Communication).

To explore this avenue, we centered our attention on the expression of Mannose-binding lectin (MBL) by the epithelial cells of the renal tubules. MBL is a crucial activator of the lectin pathway, which can initiate a cascade leading to the deposition of C4d, thereby marking a critical step toward the chronic rejection process.

We documented an expression of the MBL gene in RPETC/TERT1 (Figure 8); at the same time, we reported that contemporary stimulation with MBL determined a significant decrease in cell proliferation if cells were stimulated by Tocilizumab, W632, and MBL (Figure 9).

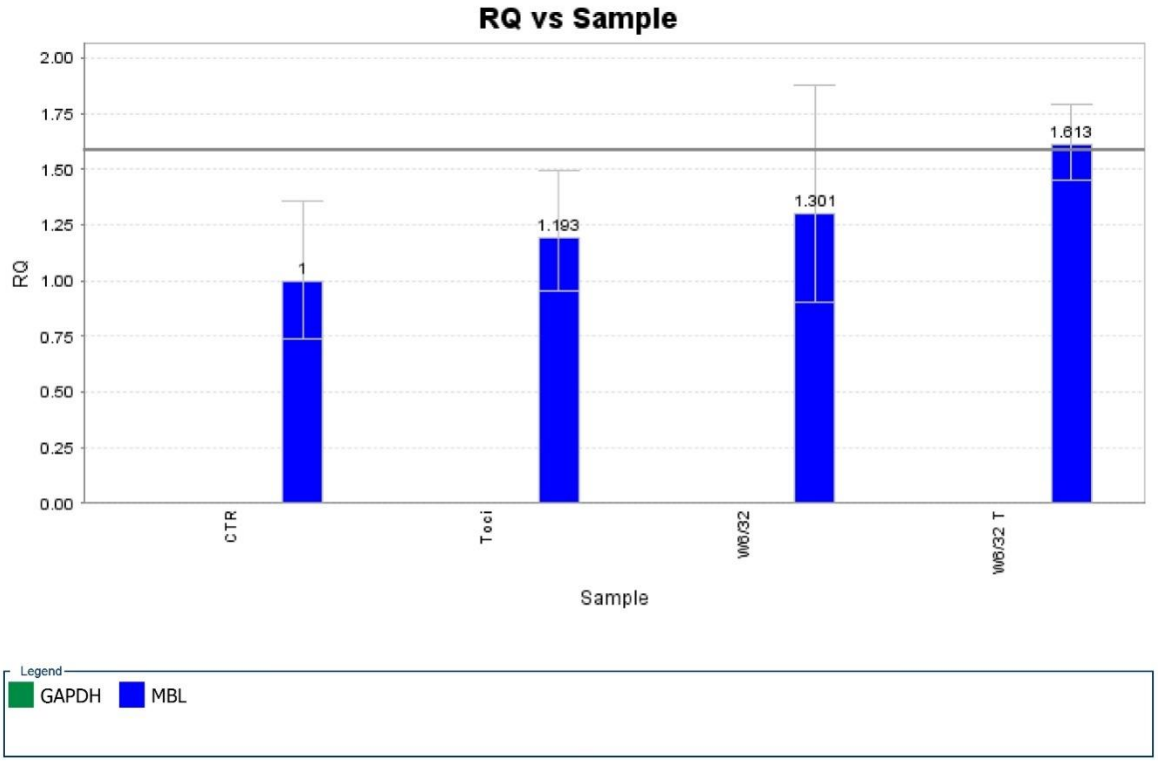

Figure 8. Gene expression analysis of MBL gene in RPTEC. RPTEC/TERT1 expressed the MBL gene with a progressive increase of gene expression after stimulation with Tocilizumab (Toci), W6/32 or W6/32+Tocilizumab (W6/32 T)

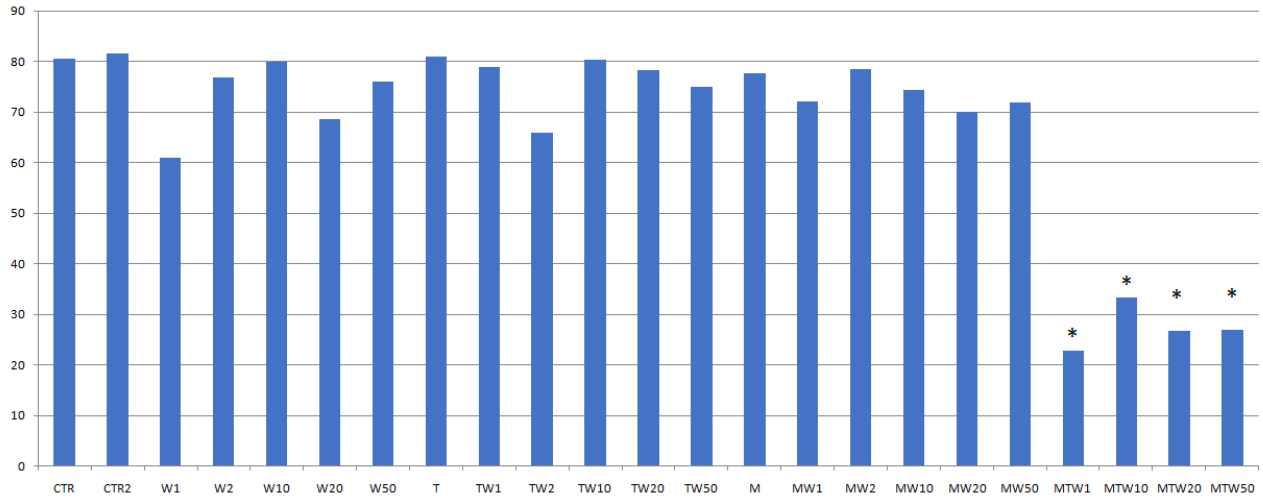

Figure 9. Direct stimulation of renal tubular cells by anti-HLA class I antibody W6/32 (W, concentrations range 1-50 mcg/ml), Tocilizumab (T, concentration 100 mcg/ml), MBL (M, concentration 2 mcg/ml), MBL+W6/32 (MW) for 72h has no significant effect on cell proliferation; contemporary stimulation with MBL+W6/32+Tocilzumab induced potent reduction of cell proliferation independently on the W6/32 dose (*$p<0.005$ vs. control).

Detection of anti-HLA antibodies in serum and urine of patients with AMR

To better understand the interaction dynamics between antibodies and tubular cells, we evaluated the possible presence of anti-donor HLA antibodies in kidney transplant patients with AMR and proteinuria.

In all screened patients, the anti-donor antibodies present in serum were detected in spot urine, suggesting their endoluminal interaction with tubular cells (Table 1). Overall, the pattern of anti-HLA antibodies and DSA detected in urine reflected that observed in serum analysis with few differences. However, a urinary DSA not detected in serum and vice versa was observed in one patient, potentially suggesting that the tubular compartment may absorb DSA and eventually release it in the urine.

	DONOR HLA TYPING	RECIPIENT HLA TYPING	HLA ANTIBODIES SERA	MFI DSA SERA	TOTAL MFI	HLA ANTIBODIES URINE SPOT	MFI DSA URINE SPOT	TOTAL MFI URINE SPOT
Patient 1	A32, A33, B8, B55, Cw1, Cw7, DR3, DR14,DR52, DQ5	A11, A30, B8, B49, DR3, DR13, DR52, DQ2, DQ6	A1, A2, A9, A25, A28, A34, A36, A80, B12, B17, B76, B82, Cw2, Cw4, Cw5, Cw6, Cw12, Cw15, Cw17, Cw18, DR1, DR4, DR51, DR103, **DQ6**, DQ6	DQ5 - 5700	HLA I – 17000, HLA II – 5700	A1, A2, A9, A25, A28, **A32,** A34, A36, A66, A80, B12, B17, B27, B76, Cw2, Cw4, Cw5, Cw6, Cw15, Cw12, Cw17, Cw18	A32 – 1500	HLA I – 25000
Patient 2	A24, A11, B35, Cw4, DR7, DR11, DR52, DR53, DQ7	A1, A30, B35, B58, DR7, DR11, DR52, DR53	A2, **A24**, A28, B8	A24 – 6300	HLA I – 12300	A2, **A24**, A28, A29, B45, B76	A24 – 3500	HLA I – 13300
Patient 3	A24, A31, B62, B57, DR7, DR13, DR52, DR53, DQ6, DQ9	A11, A68, B8, B58, DR3, DR13, DR52, DQ2, DQ6	**DQ9**, DR13	DQ9 – 3400	HLA II - 3700	**DQ9,** DR13	DQ9 – 3400	HLA II - 3700
Patient 4	A2, A24, B8, B35, DR4, DR7, DR53, DQ2	A24, A26, B44, B61, Cw3, DR4, DR53, DQ8	**B8,** B16, **B35,** B42, B51, B53, B54, B71, B75, B76, B77, B78, DQ2	B8 – 9400, B35 – 8200	HLA I – 9400	**B8, B35**, B39, B51, B53	B8 – 500, B35 – 700	HLA I – 1000
Patient 5	A1, A24, B7, B8, DR3, DR15, DR51, DR52, DQ2, DQ6	A1, A32, B7, B8, DR3, DR15	A2, A11, **A24**, A28, A29, Bw4, B12, B21, B40, B41, Cw2, Cw5, Cw6, Cw15, Cw17, Cw18	A24 – 11600	HLA I – 16600	**A24,** A28, A29, Bw4, B12, B21, B35, B40, B41, Cw2, Cw5, Cw6, Cw15, Cw17, Cw18	A24 – 15000	HLA I – 20000
Patient 6	A2, A3, B7, B60, Cw7, DR8, DR15, DR51, DQ1, DQ4	A2, B51, B39, DR15, DR13, DR51, DR52, DQ6, DQ9	DQ2, **DQ4**, DQ7	DQ4 – 9000	HLA II – 11900	DQ2, **DQ4**, DQ7	DQ4 – 24500	HLA II – 24900

Table 1. Detection of anti-HLA antibodies in the urine of proteinuric patients with AMR. MFI is the mean fluorescence intensity; DSA is the donor-specific antibodies. Anti-donor specificity is indicated in bold and underlined.

4. DISCUSSION

Chronic antibody-mediated rejection (AMR) emerges as a significant post-transplant complication, characterized by the interaction between the recipient's anti-HLA antibodies and the endothelial cells (ECs) of the transplanted organ (27). This pathological dynamic, stemming from the binding of anti-HLA antibodies to ECs, leads to functional impairment of the transplanted organ (28).

ECs, representing the primary interface between the transplanted organ and the recipient's immune system, play a pivotal role in this process. Their cell surface, densely populated with HLA molecules, becomes a primary target for anti-HLA antibodies (29). The binding of these antibodies to class I and II HLA molecules triggers the activation of ECs, transforming them into highly pro-inflammatory and immunogenic entities. This activation occurs through complex intracellular signaling pathways, such as those mediated by tyrosine kinase receptors and the mTOR pathway, influencing not only EC proliferation but also the expression of adhesion molecules like P-selectin and ICAM-1 thereby facilitating leukocyte adhesion (30).

In response to this interaction, activated ECs produce an array of chemokines (such as CXCL1 and CXCL8) and adhesion molecules, intensifying inflammation at the transplant site and further attracting leukocytes. In this context, ECs can also express a range of pro-immunogenic factors, including costimulatory molecules, significantly modulating immune cell function and contributing to the persistence of alloimmunity and chronic damage to the transplanted organ.

Pathological vascular remodeling, known as transplant vasculopathy (TV), is a long-term consequence of this interaction. The endothelial-to-mesenchymal transition (EndoMT) process is a key mechanism in this pathology, where ECs lose their specific markers and acquire traits typical of myofibroblasts. Key molecules such as TGF-β, Notch, WNT, and BMP are implicated in this process (31). TGF-β, in particular, plays a crucial role in inducing EndoMT, contributing to fibrosis and neointimal occlusion.

Our experimental endeavor sought to extend the investigations, akin to previous studies on the endothelium, into the interaction between anti-HLA antibodies and the epithelial tissue of the tubule, represented by Renal Proximal Tubular Epithelial Cells (RPTECs). We focused on studying class I anti-HLA antibodies, facilitated by the availability of a commercial antibody known as W6/32. Although we acknowledge the nascent stage of this study, our approach

yielded some initial insights, notably highlighting the stark contrasts between the antibody's interaction with the tubule and the endothelium.

A primary observation was the absence of effects related to cell cycle and proliferation, a finding that underscores how the effects of antibody binding to the cell might vary depending on the cell's internal signal transduction mechanisms or changes in the surrounding cellular context. In our case, drawing parallels with the aforementioned studies on the endothelium, even though we did not observe an effect on proliferation, we witnessed a cellular transition. In the case of endothelial cells, this is classifiable as an endothelial-to-mesenchymal transition; however, in our context, we might hypothesize an epithelial-to-mesenchymal transition, although this would clearly require verification under specific experimental conditions.

The hints to which I refer can be found in the loss of adhesion molecules, one of the hallmarks of epithelial-to-mesenchymal transition. Indeed, we observed that our cells, following binding with the antibody, tended to alter their shape and express the TJP1/ZO1 molecule to a lesser degree. This transition could be one of the contributing factors to the progressive loss of function in the tubular structure. In our experimental setting, we noted that the use of an interleukin-6 (IL-6) receptor inhibitor, namely Tocilizumab, mitigated the observed effect . It is well-established that IL-6 plays a pivotal role in the regulation of inflammatory responses and is targeted in various autoimmune diseases. In our experiments, Tocilizumab appeared to protect the cells from antibody-mediated damage, particularly regarding TJP1/ZO1 expression. This leads to the question: Is IL-6 an intermediary in antibody-induced damage? Our data suggest otherwise (Figure 10).

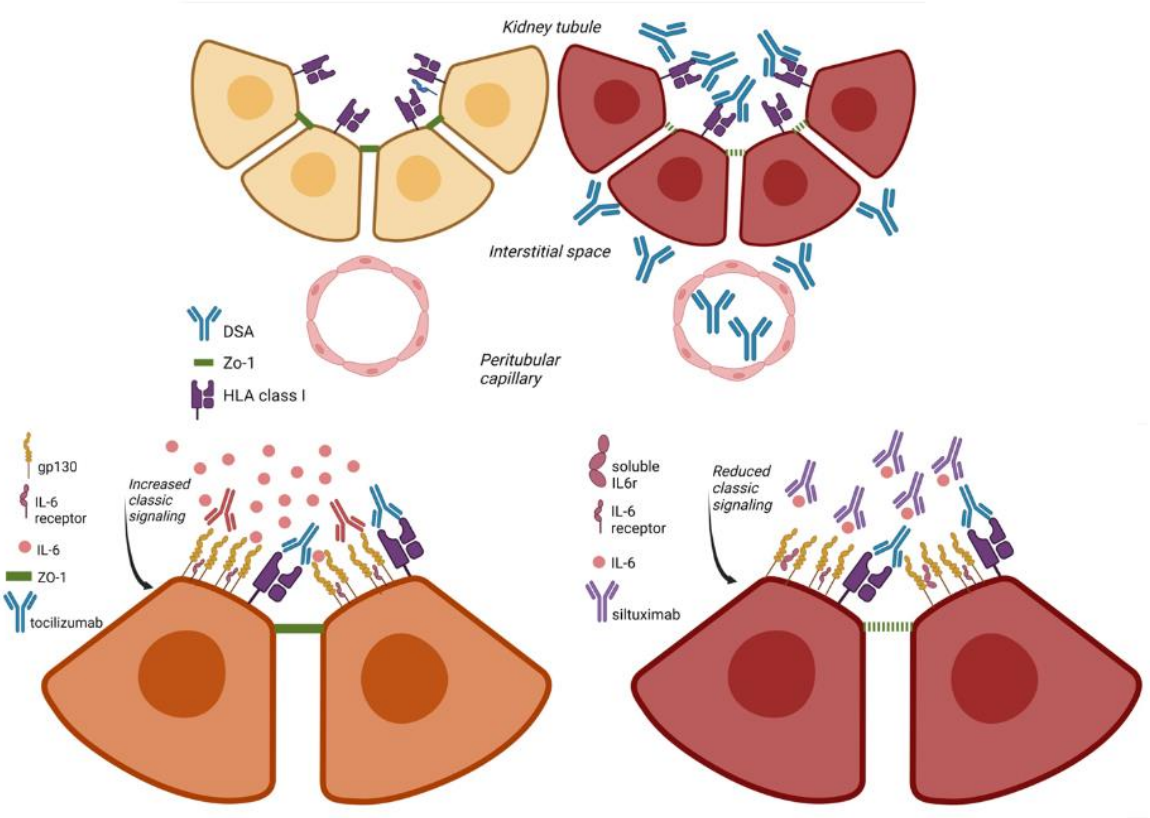

Figure 10. The interaction of anti-HLA DSAs caused direct cell damage and TJP1/ZO1 reduction. Below, we see a representation of the two main signaling pathways of interleukin-6 (IL-6). On one side, the focus is on tubular cells that express IL-6R receptors on their surface. Here, IL-6 directly binds to these membrane receptors, closely interacting with the gp130 subunit, initiating intracellular signaling typically associated with anti-inflammatory responses. Notably, this process, known as classical IL-6 signaling, may promote the expression of adhesion molecules like TJP1/ZO1, playing a vital role in maintaining the integrity and function of tubular cells. Tocilizumab, promoting higher IL6 concentration and classic signalling activation may induce more pronounced cell stabilization (expressed by TJP1/ZO1) whereas the complete inhibition by Siltuximab reduce this positive effect. The entire image thus tells the story of how IL-6 can act in different ways, depending on the presence or absence of the IL-6R receptor on the cell surface, highlighting the significance of these diverse signaling pathways in the context of inflammatory responses and the regulation of cellular functions.

By juxtaposing the impact of Tocilizumab on cells in contact with the antibody with that of a direct IL-6 inhibitor, Siltuximab, we observed that the protective effect was not replicated. This

led us to hypothesize that Tocilizumab, by inhibiting the IL-6R, predominantly acts on trans-signaling of the signal, also stimulating the expression of the receptor on the membrane of our cultured cells, unlike Siltuximab, which, by directly inhibiting IL-6, can only impede both signaling modes, including the classic signaling pathway. It is particularly noteworthy that the tubular epithelial cell expresses the IL-6R, which may underlie the difference between the pro-inflammatory and anti-inflammatory effects inherent to the pleiotropy of IL-6.

Interleukin-6 (IL-6) is a multifunctional cytokine that exhibits divergent effects contingent upon the cell type it encounters, a dynamic particularly pertinent in the context of antibody-mediated chronic rejection. While IL-6 interacts with its receptor IL-6R, which lacks intrinsic kinase activity, it necessitates the gp130 receptor subunit for intracellular signaling initiation. Notably, only a select array of cells, including tubular cells and hepatocytes, express IL-6R on their membrane, facilitating an anti-inflammatory effect of IL-6 through classical cytokine signaling in these cells, contingent upon its binding to the membrane-bound receptor. Conversely, other cell types, such as endothelial cells and various immune cells, are exclusively subject to trans-signaling mediated by the soluble IL-6 receptor (sIL6R), which tends to contribute to pro-inflammatory outcomes and pathological processes like endothelial dysfunction and immune dysregulation. This dichotomy between classical signaling and trans-signaling modalities has been elucidated in recent studies, where the pro-inflammatory trans-signaling of IL-6 in endothelial cells was effectively countered by the application of sgp130Fc, a selective inhibitor. Understanding the nuances of these signaling pathways, especially their differential effects on diverse cell types such as tubular cells, hepatocytes, endothelial cells, and immune cells, is paramount in devising selective therapeutic strategies in the milieu of antibody-mediated chronic rejection. The presence of sGP130, an endogenous soluble form of the IL-6/IL6R signal transducer, acts as an inhibitor of trans-signaling, yet without initiating an intracellular signaling cascade, suggesting that targeted inhibition of trans-signaling could be a viable therapeutic approach to mitigate risks associated with chronic rejection, whilst preserving the protective functions of IL-6 classical signaling in cells where it exerts beneficial effects (32,33).

Ultimately, we initiated an analysis of the lectin pathway of complement's contribution in the context of antibody-mediated chronic rejection. Our data indicate significant mannose-binding lectin (MBL) presence in the tubule, both due to tubular epithelial cells expressing the MBL

gene and the observed deposition of complement cascade elements in autoimmune diseases such as glomerulonephritis, which some cases of chronic rejection exhibit. MBL, in these instances, is also correlated with proteinuria and hematuria in patients suffering from these conditions (34). The evidence of a potential synergistic role between DSA, increased IL-6 and MBL suggest a potential involvement of mannose receptor in this picture as observed in some conditions (35). Overall, while we cannot definitively elucidate the mechanisms by which MBL contributes to antibody-mediated damage, our findings suggest a potential role for MBL in sustaining and exacerbating autoimmune disease, in our case, directed at the graft.

In summary, our results suggest a direct impact of anti-HLA DSAs on tubular cells and reveal distinct response patterns between direct and indirect IL-6 inhibition. These findings may have implications for antibody-mediated rejection treatment in organ transplantation.

Additionally, based on these results, an expansion of our findings would be possible by considering the involved molecular mechanisms looking at MAPK and mTOR pathways. The Wnt/B-catenin pathway has to be assayed since, in Literature, the phenotypical changes in tubular epithelial cells suggest reactivation of the pathway leading to cellular senescence.
It would also be great better explain the potential role of the Lectin Pathway on damage to epithelial cells using a recombinant MBL or patient-derived serum blocking Classical pathway, with a focus on Wnt/B-catenin pathway, phenotypical changes (morphology, secretome and senescence, and EMT markers expression), and proliferation. Analyze the co-culture of tubular cells and PBMC, focusing on media content (proteins, extracellular vesicles, and their mRNA content) and PBMC phenotypical and functional changes due to the co-culture in the presence of W6/32 may allow us to ultimately correlate the interplay between tubule interstitial damage and immunological activation.

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