P-glycoprotein functions as an immunomodulator in healthy human primary nasal epithelial cells

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Background: P-glycoprotein (P-gp) is an adenosine triphosphate (ATP)-dependent efflux pump that confers chemotherapeutic resistance in cancer cells. Recent studies suggest that P-gp may also function as an immunomodulator through regulation of cytokine transport. Sinonasal epithelial cells have been recognized as drivers of local innate and adaptive immunity and are known to overexpress P-gp in the setting of inflammation. The objective of this study is to therefore determine whether P-gp participates in the regulation of cytokine secretion in sinonasal epithelial cells.

Methods: Primary nasal epithelial cell cultures (PNECCs) were cultivated from 5 healthy patients. Membranous P-gp was quantified through quantitative fluorescent immunohistochemistry (Q-FIHC) and confirmed by enzyme-linked immunosorbent assay (ELISA). Sensitivity to inhibition was determined using a rhodamine 123 accumulation assay. Baseline and lipopolysaccharide (LPS)-stimulated cytokine secretion of interleukin 6 (IL-6), IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and thymic stromal lymphopoietin (TSLP) were quantified by ELISA and compared to LPS stimulated secretion in the setting of P-gp–specific inhibition. Differences in P-gp expression and cytokine secretion were compared using 2-tailed Student t tests with post hoc testing using the Bonferroni procedure.

Results: Membranous P-gp is detectable in PNECCs and upregulated following LPS exposure. P-gp is sensitive to inhibition by both PSC 833 and verapamil in a dose-dependent fashion. LPS stimulated secretion of normalized IL-6 (mean, 95% confidence interval [CI]) (79.67, 42.26–117.07), GM-CSF (39.92, 7.90–71.94), and TSLP (6.65, 5.35–7.96) was significantly reduced following P-gp inhibition (37.60, 11.54–63.65, $p = 0.023$; 7.64, 2.25–13.03, $p = 0.044$; and 5.13, 4.44–5.82, $p = 0.038$; respectively).

Conclusion: P-gp is functionally active in PNECCs. P-gp participates in modulation of epithelial secretion of LPS stimulated IL-6, GM-CSF, and TSLP. © 2013 ARS-AAOA, LLC.

Key Words:
P-glycoprotein; multidrug resistance; nasal epithelium; cell culture; cytokine secretion; immunomodulator; PSC 833; verapamil; rhodamine 123; sinusitis

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P-glycoprotein (P-gp) is a 170-kDa glycoprotein encoded by the MDR1 (ABCB1) gene located on chromosome 7q21.12.[1] It is a member of the ATP-binding cassette (ABC) transporter family and is capable of energy dependent transport of a variety of intracellular substrates.[2] Although P-gp has been primarily studied as a xenobiotic efflux pump conferring chemotherapeutic resistance in cancer cells, additional evidence has suggested that it may also be capable of mediating the export of intracellular proteins including cytokines.[3] Overexpression of P-gp has been reported in chronic sinonasal inflammation[4]; however, its ability to modulate nasal epithelial cytokine secretion has not been investigated.

While P-gp has been extensively studied in a variety of tissues including bronchial epithelium,[5] data on its presence and distribution in nasal mucosa has traditionally been limited. Wioland et al.[6] examined the expression of a variety of ABC transporter superfamily members in healthy inferior turbinate specimens and reported the presence of both apical and basolateral P-gp staining. Kandimalla and Donovan[7] examined P-gp expression in bovine nasal mucosa and found it to be present but underexpressed relative to the olfactory epithelium. Recent evidence examining P-gp in the setting of chronic sinonasal disease suggests that it is locally overexpressed in regions of active inflammation such as nasal polyps.[4]

While the efflux behavior of P-gp is well established, the potential for the role of P-gp as an immunomodulator is a new concept derived from a variety of in vitro and animal studies. Drach et al.[8] reported that P-gp inhibition of phytohemagglutinin stimulated T-cells using both verapamil and tamoxifen resulted in a reduction of interleukin-2 (IL-2), IL-4, and interferon (IFN)-γ secretion while messenger RNA (mRNA) levels remained unchanged. In a complementary line of evidence Kooij et al.[9] studied a P-gp knockout mouse model (Mdr1a/1b$^{-/-}$) of recombinant myelin oligodendrocyte glycoprotein (rMOG)-induced experimental autoimmune encephalomyelitis. They reported an impairment in MOG-specific T-cell proliferation and dendritic cell maturation with an associated reduction in IFN-γ, tumor necrosis factor (TNF)-α, IL-5, and IL-10 secretion relative to control mice.

The recent findings of P-gp overexpression in nasal polyps coupled with the evolving evidence that it can regulate cytokine secretion suggests that P-gp may participate in innate and adaptive immune responses within the nasal epithelium. The goal of this study is to investigate whether sinonasal epithelial P-gp is capable of modulating cytokine secretion from its host cell.

Patients and methods

Mucosal biopsy procurement

Procurement of the sinus mucosal biopsies from 5 patients utilized to generate the primary nasal epithelial cell cultures (PNECCs) was approved by the Massachusetts Eye and Ear Infirmary Institutional Review Board. All experiments were performed in duplicate. Subjects were all free of rhinosinusitis and undergoing surgery for either cerebrospinal fluid leak repair or tumor removal. Exclusion criteria included the following: diagnosis of chronic sinusitis; use of oral or topical steroids or immunotherapy within the preceding 4 weeks; aspirin sensitivity; ciliary dysfunction; autoimmune disease; cystic fibrosis; or any known immunodeficiency. All tissue was derived from Schneiderian mucosa within the middle meatus.

PNECC

Mucosal biopsies were washed and digested in Pronase for 90 minutes at 37°C. Cell suspensions were separated from particulate matter by centrifugation and resuspended in basal epithelial growth medium (BEGM) (Lonza, Basel, Switzerland). Cells were plated for 2 hours on standard tissue culture plates to remove contaminating fibroblasts. Cells were then expanded for 3 to 5 days on collagen-coated 75-cm^2 dishes (Corning Life Sciences, Corning, NY). Once confluent, the PNECCs were trypsinized and reseeded evenly on human collagen type IV–coated tissue 6-well culture plates. Cultures were grown to 80% confluence in BEGM prior to testing. Cells intended for immunohistochemistry were grown on tissue culture treated coverslips placed within the well.

Quantitative fluorescent immunohistochemistry

Quantitative fluorescent immunohistochemistry (Q-FIHC) for membranous P-gp expression was performed using previously described techniques.[4] Briefly cells were fixed in 4°C acetone. Following blocking, the primary antibody (monoclonal anti-p-glycoprotein clone F4, 1:250; Sigma Aldrich, St. Louis, MO) was applied for 24 hours at 4°C. The tissue was then rinsed followed by application of the secondary antibody (Anti-Mouse IgG [Fc-specific] F(ab′)2 fragment-FITC, 1:160; Sigma Aldrich) for 30 minutes at room temperature. The coverslips were then rinsed and mounted in Vectashield containing propidium iodide (Vector Laboratories, Burlingame, CA) for nuclear counterstaining. Negative control slides were considered those in which the primary antibody was omitted from the staining procedure. The mean corrected luminosity was considered the staining intensity (calculated using Image J v1.45s) divided by the total number of pixels subtended by the cells.

Rhodamine 123 accumulation assay

Specific inhibitors of P-gp (PSC 833 at 8 μM or 80 μM; Tocris Bioscience, Bristol, UK; verapamil 10 μM or 100 μM; Sigma) were added to the culture medium for 21 hours. PSC 833 is a derivative of cyclosporine which is a P-gp inhibitor with no independent immunosuppressive properties.[10] Rhodamine 123 (500 μM; Sigma), a P-gp specific substrate, was then added to each well for 2 hours. The rhodamine 123 was then removed and the P-gp inhibitor alone was added back to the wells for 1 hour. The media was then removed and the cells were lysed using Cell Lytic M (Sigma). The total intracellular rhodamine 123 concentration in each well was determined by spectrophotometry (excitation 510 nm, emission 534 nm) and normalized to the total cytoplasmic protein using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Retention of intracellular rhodamine 123 over baseline was considered proportional to degree of P-gp inhibition.

P-gp–mediated cytokine secretion testing

Wells were exposed to 23 hours of stimulation with lipopolysaccharide (LPS, 0.05 mg/mL), a toll-like receptor 4 agonist, with or without concomitant P-gp inhibition using PSC 833 (8 μM) applied 1 hour prior. Control wells were considered those exposed to culture medium alone (BEGM). A 0.4% trypan blue (Sigma, St. Louis, MO) cell survival assay was used to ensure the stimulant and inhibitor exposures were not cytotoxic. In all wells less than 20% of cells were stained blue indicating greater than 80% survival. Following the LPS and PSC 833 exposures, the media was removed from each well. Cytokine concentrations for IL-6, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and TSLP in each well were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer guidelines (eBioscience, San Diego, CA). Cytokine concentrations were normalized to total media protein concentrations using a Pierce BCA Protein Assay Kit.

Quantification of membrane-bound P-gp

Following removal of media from each well, the cytoplasmic and membranous protein fractions were isolated using a 2-step extraction assay (DualXtract; Bulldog Bio, Inc., Portsmouth, NH). Membrane bound P-gp was quantified by subjecting the membranous fraction to ELISA (USCN Life Sciences Inc., Wuhan, P.R. China) and normalized to the cytoplasmic protein concentration.

Statistics

The sample size of 5 patients was determined by a power analysis assuming a 1-β of 80% and a significance level of $p < 0.05$. The significance of differences between normalized cytokine secretion and membranous P-gp expression was determined using 2-tailed Student t tests with post hoc testing using the Bonferroni procedure (SigmaStat v4; Systat Software Inc, San Jose, CA).

Results

Membranous P-gp is expressed in PNECCs

Membranous P-gp was detected by Q-FIHC in submerged PNECCs grown in culture media alone (BEGM) with a mean corrected luminosity of 1.27 (95% CI, 0.41–2.13). Exposure of PNECCs to 23 hours of LPS resulted in a significant increase in expression with a mean corrected luminosity of 9.64 (95% CI, 4.30–14.98, $p = 0.017$) (Figs. 1 and 2). This increase in expression was confirmed by ELISA for P-gp following membrane extraction. Normalized membranous P-gp was significantly greater in LPS exposed PNECCs than in control (mean, 95% CI; 19.63, 11.62–27.64 vs 4.42, 2.88–5.97, respectively, $p = 0.016$). LPS stimulated membranous P-gp did not change significantly when an 8 μM PSC 833 solution was added as compared to LPS alone (70.40, 22.77–118.04; $p = 0.115$) (Fig. 3).
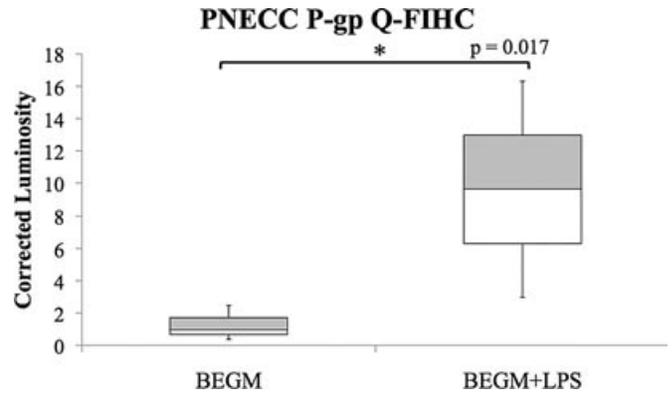

FIGURE 1. Q-FIHC staining intensity of membranous P-gp in PNECCs exposed to culture media alone (BEGM) vs BEGM with 0.05 mg/mL of LPS for 23 hours. Corrected luminosity refers to the total luminosity divided by the total cell area per field. Exposure to LPS results in a significant increase in staining intensity. BEGM = basal epithelial growth medium; LPS = lipopolysaccharide; P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell cultures; Q-FIHC = quantitative fluorescent immunohistochemistry.
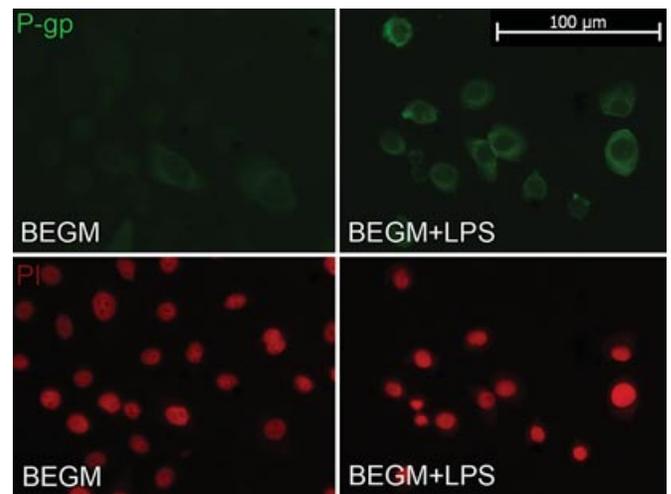

FIGURE 2. Fluorescent image depicting P-gp staining following exposure to BEGM or BEGM+LPS (upper windows). The lower windows depict the matched PI nuclear staining for cellular localization. P-gp staining intensity is quantified in Figure 1. BEGM = basal epithelial growth medium; LPS = lipopolysaccharide; P-gp = P-glycoprotein; PI = propidium iodide.

P-gp is responsive to selective inhibition in vitro

Using a rhodamine 123 accumulation assay, selective inhibition of P-gp led to a significant increase in intracellular rhodamine 123 over baseline in a dose-dependent fashion. Exposure to an 8 μM solution of PSC 833 resulted in a mean 118.12% $\pm$ 12.16% increase in accumulated P-gp while an 80 μM solution demonstrated a significant mean increase of 439.46% $\pm$ 117.59% ($p = 0.015$). These results were confirmed using verapamil, another known P-gp inhibitor. A similar dose-dependent accumulation was seen using a 10 μM and 100 μM verapamil solution (mean $\pm$ SD, 185.33% $\pm$ 6.59% vs 328.27% $\pm$ 66.98%, respectively; $p = 0.014$) (Fig. 4).
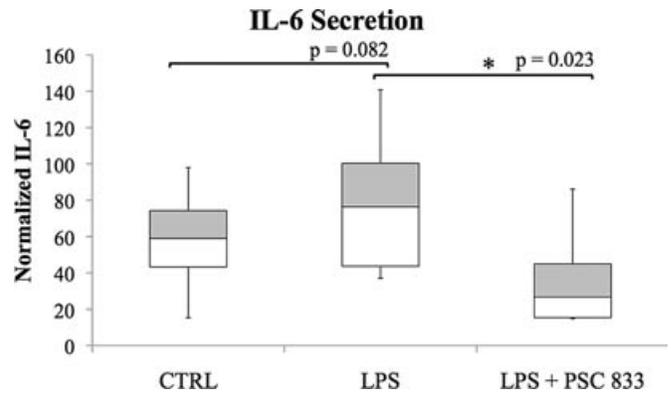
Membranous P-gp Expression
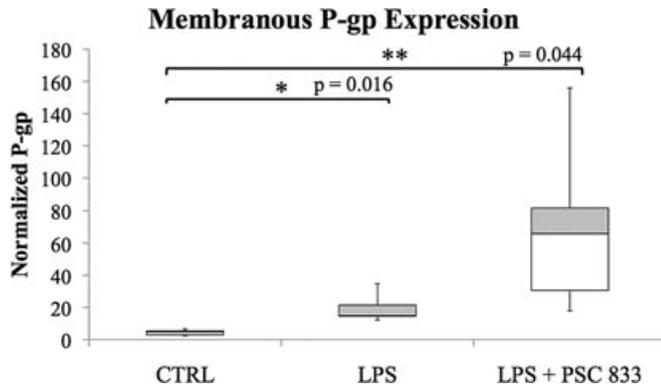
FIGURE 3. Membranous P-gp expression determined by ELISA following membrane protein extraction. Normalized P-gp is expressed as P-gp (ng/mL)/cytoplasmic protein (μg/mL) × 100. The significant increase in expression following LPS exposure is again seen confirming the findings by IHC. There is no significant difference in expression seen between cells exposed to LPS and those exposed to LPS along with a P-gp inhibitor (PSC 833, p = 0.115). ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry; LPS = lipopolysaccharide; P-gp = P-glycoprotein.

Rho 123 Accumulation
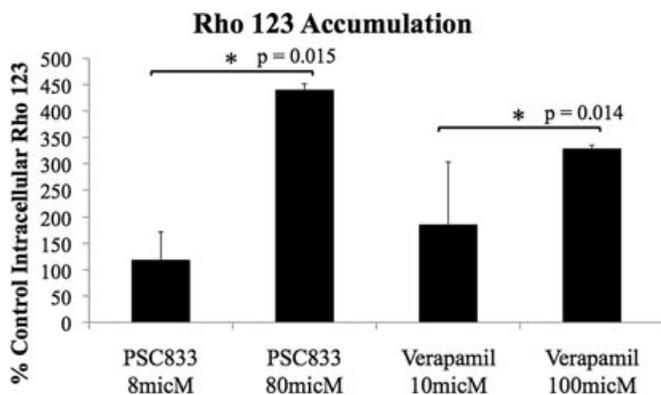
FIGURE 4. Rhodamine 123 accumulation assay demonstrating concentration of intracellular rhodamine as compared to baseline following inhibition of P-gp using either PSC 833 or verapamil. The evident dose response using 2 separate inhibitors confirms that the P-gp expressed in PNECCs is sensitive to inhibition. The accumulation in rhodamine 123 over baseline seen with 8 μM of PSC 833 suggests that the alterations in cytokine secretion seen following PSC 833 exposure at this concentration may be directly attributable to P-gp inhibition. P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell culture.

IL-6 Secretion

FIGURE 5. Secreted IL-6 concentration (normalized to total media protein IL-6 [pg/mL]/protein [μg/mL] × 100) in the control condition (CTRL) following exposure to culture media alone as compared to PNECCs exposed to media + LPS (0.05 mg/mL), and media + LPS (0.05 mg/mL) + PSC 833 (8 μM). Note the significant reduction in LPS-stimulated IL-6 secretion following inhibition of P-gp with PSC 833. IL-6 = interleukin-6; LPS = lipopolysaccharide; P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell culture.

GM-CSF Secretion
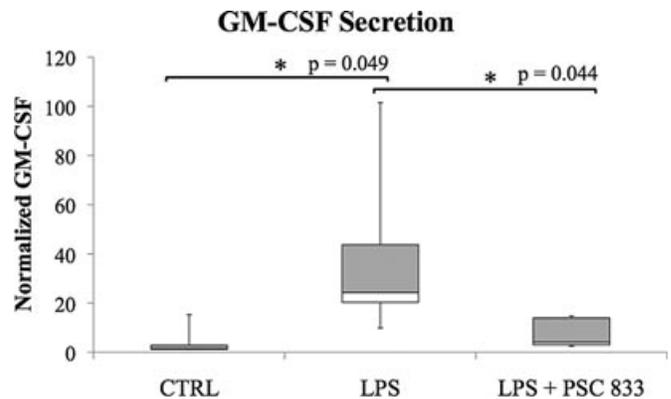
FIGURE 6. Secreted GM-CSF concentration (normalized to total media protein GM-CSF [pg/mL]/protein [μg/mL] × 100) in the control condition (CTRL) following exposure to culture media alone as compared to PNECCs exposed to media + LPS (0.05 mg/mL), and media + LPS (0.05 mg/mL) + PSC 833 (8 μM). Note the significant reduction in LPS-stimulated GM-CSF secretion following inhibition of P-gp with PSC 833. GM-CSF = granulocyte macrophage colony stimulating factor; LPS = lipopolysaccharide; P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell culture.

P-gp participates in regulation of stimulated epithelial cytokine secretion in vitro

With respect to normalized IL-6, PNECCs demonstrated a detectable baseline secretion that was nonsignificantly upregulated following LPS stimulation (mean, 95% CI; 57.95, 30.58–85.37 vs 79.67, 42.26–117.07, respectively, p = 0.082). Stimulated IL-6 secretion was significantly decreased following P-gp inhibition (mean 37.60, 95% CI, 11.54–63.65, p = 0.023) (Fig. 5). With respect to normalized GM-CSF, PNECCs demonstrated a detectable baseline secretion which was significantly upregulated following LPS stimulation (mean, 95% CI; 4.45, −0.88 to 9.77 vs 39.92, 7.90–71.94, respectively, p = 0.049). Stimulated GM-CSF secretion was significantly decreased following P-gp inhibition (mean 7.64, 95% CI, 2.25–13.03, p =

0.044) (Fig. 6). With respect to normalized TSLP, PNECCs demonstrated a detectable baseline secretion that was nonsignificantly upregulated following LPS stimulation (mean, 95% CI; 7.27, 6.23–8.30 vs 6.65, 5.35–7.96, respectively, p = 0.274). Stimulated TSLP secretion was significantly decreased following P-gp inhibition (mean 5.13, 95% CI, 4.44–5.82, p = 0.038) (Fig. 7). With respect to normalized IL-8, PNECCs demonstrated a detectable baseline secretion that was significantly upregulated following LPS stimulation (mean, 95% CI; 263.81, 157.22–370.40 vs 912.91, 466.89–1358.92, respectively, p = 0.018). Stimulated IL-8 secretion demonstrated a trend toward reduction following P-gp inhibition although this was not significant (mean 801.09, 95% CI, 596.88–1005.30, p = 0.313) (Fig. 8). Among the cytokines sensitive to PSC 833 exposure, the LPS-stimulated secretion following P-gp inhibition was

TSLP Secretion
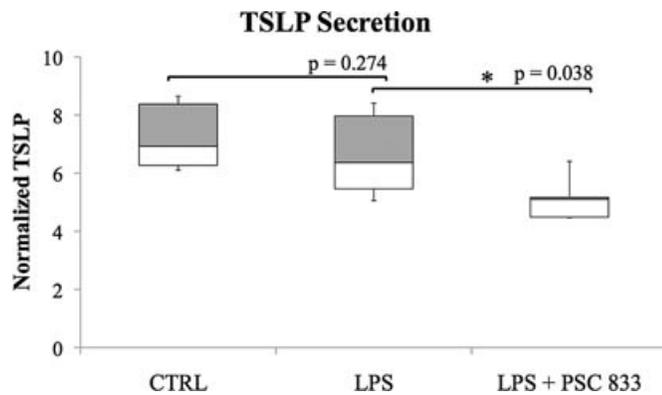
FIGURE 7. Secreted TSLP concentration (normalized to total media protein TSLP [pg/mL]/protein [μg/mL] $\times$ 100) in the control condition (CTRL) following exposure to culture media alone as compared to PNECCs exposed to media + LPS (0.05 mg/mL), and media + LPS (0.05 mg/mL) + PSC 833(8 μM). Note the significant reduction in LPS-stimulated TSLP secretion following inhibition of P-gp with PSC 833. LPS = lipopolysaccharide; P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell culture; TSLP = thymic stromal lymphopoietin.

IL-8 Secretion
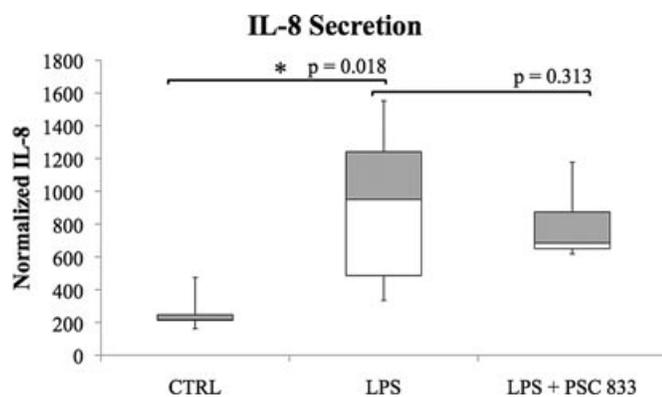
FIGURE 8. Secreted IL-8 concentration (normalized to total media protein IL-8 [pg/mL]/protein [μg/mL] $\times$ 100) in the control condition (CTRL) following exposure to culture media alone as compared to PNECCs exposed to media + LPS (0.05 mg/mL), and media + LPS (0.05 mg/mL) + PSC 833 (8 μM). In this case PSC 833 failed to significantly impair IL-8 secretion suggesting that P-gp–mediated secretion is selective to specific cytokines. IL-8 = interleukin-8; LPS = lipopolysaccharide; P-gp = P-glycoprotein; PNECC = primary nasal epithelial cell culture.

equivalent to or significantly less than baseline levels (IL-6, $p = 0.014$; GM-CSF, $p = 0.001$; TSLP, $p = 0.008$).

Discussion

The sinonasal epithelium functions as a barrier organ against the external environment and is endowed with an array of innate and adaptive immunologic mechanisms to combat extrinsic pathogens. Multiple lines of evidence have suggested that sinonasal epithelial cells may function as primary actors in the initiation and maintenance of chronic sinonasal inflammation through the elaboration of an array of cytokines and subsequent recruitment of professional immune cells.[11–14]

While these studies suggest that epithelial cells are capable of orchestrating an innate immune response, the post-translational mechanisms governing noncanonical cytokine secretion at the cellular level are not fully understood. Prior data demonstrating that P-gp expression persists in primary nasal epithelial cell cultures provides an opportunity to study its immunoregulatory capacity in vitro.[15] Our immunohistochemical and P-gp ELISA data confirm these findings indicating that P-gp was both present and functional within our PNECCs. Of note, the primary antibody used in our Q-FIHC study is specific to an extracellular loop of the P-gp protein providing further confirmation that our data reflects the activity of the membrane bound P-gp as opposed to the cytoplasmic fraction which does not participate in substrate transport out of the cell.

PSC 833 represents a "second-generation" P-gp specific inhibitor that is derived from cyclosporine D but lacks the associated immunosuppressive activity. PSC 833 is thought to impair both the ATPase activity as well as the transport function of P-gp as a high affinity competitive substrate.[10] Our accumulation assay results confirm that the low-dose PSC 833 used in this study was sufficient to inhibit P-gp–mediated transport as evidenced by increased retention of intracellular rhodamine 123 over baseline. While the dose response seen suggests that P-gp is not fully inhibited at 8 μM of PSC 833, we elected to use the lower dose in order to prevent the possibility of cytotoxicity.

Our cytokine assays demonstrated that inhibition of P-gp resulted in a significant reduction in LPS-stimulated IL-6, GM-CSF, and TSLP secretion. The lack of significant inhibition of IL-8 suggests that P-gp–mediated immunomodulation is selective and does not apply to all secreted cytokines. The stable P-gp expression in LPS-stimulated cells exposed to PSC 833 as compared to LPS alone suggests that the reduction effect cannot be attributed to a downregulation in epithelial P-gp. The accumulation assay confirms that PSC 833 is mediating its effect through P-gp–specific inhibition although this does not necessarily imply that these cytokines are actual substrates for the P-gp pump. Possible alternative mechanisms based on prior studies could include prevention of translocation of transcribed cytokines into secretory vesicles or inhibition of cytokine processing through P-gp–mediated alterations in intracellular chloride concentration.[16]

Conclusion

Our data confirms that membrane bound P-gp is present and functionally active in PNECCs derived from healthy patients. The significant reduction in stimulated cytokine secretion following PSC 833 exposure suggests that P-gp participates in modulating cytokine secretion at the nasal mucosal surface. In light of these findings, reports of local P-gp overexpression in regions of nasal inflammation may point to a role for P-gp in the etiopathogenesis of chronic sinonasal inflammatory disease. Future studies will be directed at examining the function of P-gp in primary cultures derived from patients with chronic sinusitis with or without nasal polyposis.

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