Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

Syk inhibitor R406 down-regulates inflammation in an in vitro model of Pseudomonas aeruginosa infection

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2017-0307.R1
Manuscript Type:	Article
Date Submitted by the Author:	22-Sep-2017
Complete List of Authors:	Alhazmi, Alaa; Lakehead University, Department of Biology Choi, Joshua; Northern Ontario School of Medicine, Medical Sciences Ulanova, Marina; Northern Ontario School of Medicine, Medical Sciences; Lakehead University, Department of Biology
Is the invited manuscript for consideration in a Special Issue?:	N/A
Keyword:	<i>Pseudomonas aeruginosa</i> , Syk, small molecule inhibitor, R406, inflammation

SCHOLARONE™
Manuscripts

Alaa Alhazmi¹, Joshua Choi², Marina Ulanova^{1,2*}

Tel: 807-766-7340 Fax: 807-766-7362

Abstract

As *Pseudomonas aeruginosa* infections are characterized by strong inflammation of infected tissues anti-inflammatory therapies in combination with antibiotics have been considered for the treatment of associated diseases. Syk tyrosine kinase is an important regulator of inflammatory responses, and its specific inhibition was explored as a therapeutic option in several inflammatory conditions; however, this has not been studied in bacterial infections. We used a model of *in vitro* infection of human monocytic cell line THP-1 and lung epithelial cell line H292 with both wild type and flagella-deficient mutant of *P. aeruginosa* strain K, as well as with clinical isolates from cystic fibrosis patients, to study the effect of a small molecule Syk inhibitor R406 on inflammatory responses induced by this pathogen. One-hour long pretreatment of THP-1 cells with 10 μ M R406 resulted in a significant down-regulation of the expression of the adhesion molecule ICAM-1, pro-inflammatory cytokines TNF α and IL-1 β , and phosphorylated signaling proteins ERK2, JNK, p-38, and I κ B α , as well as significantly decreased TNF- α release by infected H292 cells. The results suggest that Syk is involved in the regulation of inflammatory responses to *P. aeruginosa*, and R406 may potentially be useful in dampening the damage caused by severe inflammation associated with this infection.

Key Words: *Pseudomonas aeruginosa*, cystic fibrosis, Syk, small molecule inhibitor, R406, inflammation, cytokine

Introduction

Pseudomonas aeruginosa is the major cause of chronic pulmonary infection in cystic fibrosis (CF) patients as well as of other serious conditions in immunocompromised individuals (Saiman and Siegel 2004; Hakki et al. 2007; Crouch Brewer et al. 1996; Lieberman and Lieberman 2003). *P. aeruginosa* is a Gram-negative opportunistic pathogen armed with potent virulence factors including the type III secretion and quorum sensing systems, lipopolysaccharide, several powerful exotoxins, and various enzymes that contribute to disease pathogenesis via severe tissue damage and inflammation as well as immune evasion (Kipnis 2006). As *P. aeruginosa* infection is characterized by exaggerated inflammatory responses, anti-inflammatory therapy is considered important for treatment of *P. aeruginosa*-associated conditions (Cheng et al. 2013). In particular, intracellular protein kinases involved in the regulation of pro-inflammatory signaling pathways may represent potential therapeutic targets. We have recently found that an inhibitor of Syk tyrosine kinase piceatannol is able to down-regulate inflammatory responses in *P. aeruginosa*-infected lung epithelial cells (Aval et al. 2013). However, the effect of piceatannol in this model extended beyond inhibition of Syk, i.e. via potential modulation of Syk-independent signaling pathways (Aval et al. 2013). A small molecule inhibitor, N4-(2,2-dimethyl-3-oxo-4H-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine (R406) was demonstrated to selectively inhibit Syk kinase activity in an ATP-competitive manner both *in vitro* and *in vivo* (Cha et al. 2006; Braselmann et al. 2006; Spalton et al. 2009; McAdoo and Tam 2011). R406 is the active metabolite of an orally available drug Fostamatinib, which had undergone several clinical trials for treatment of some autoimmune and allergic diseases and hematological malignancies (Riccaboni et al. 2010). However, it is unknown whether R406 can modulate inflammatory responses in infections. In this study, we sought to assess the effect of R406 on inflammatory markers associated with *P. aeruginosa* infection of human monocytic and lung epithelial cells.

Materials and Methods

Cell culture conditions

The THP-1 human acute monocytic leukemia cell line (ATCC, Manassas, VA) was used at the passage numbers of 6-20. These cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat inactivated fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS) and 1% antibiotic-antimycotic (Invitrogen, Burlington, ON, Canada). Cells were grown at 37°C with 5% CO₂ and seeded every 3-4 days when cell counts neared 1×10⁶ cells/mL. In preparation for experiments, the cells were centrifuged at 400 × g for 5 minutes, washed with sterile PBS (pH 7.4), and suspended in culture medium without antibiotics. To induce differentiation, THP-1 cells were plated at 1×10⁶ cells/mL/well in 24-well plates (Costar, Corning Incorporated, Corning NY), in serum- and antibiotic-RPMI 1640 medium. Cells were then treated with 20 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) at 37°C in 5% CO₂ for 12 hours, then washed and re-suspended in the same medium. After 48 hours of further incubation, the cells were washed twice with serum- and antibiotic-free medium and used for experiments.

The H292 human muco-epidermoid bronchiolar carcinoma cell line (ATCC) was used at the passage numbers of 10-25. These cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS without antibiotics. Cells were grown at 37°C with 5% CO₂ and seeded every 3-4 days when confluency approached 80%. For viability testing, the cells were detached using 0.5% Trypsin-EDTA (Gibco, Eugene, OR), centrifuged at 400 × g for 5 minutes, washed with sterile PBS (pH 7.4), and suspended in culture medium. Cell viability was determined by the trypan blue exclusion method using a ViCell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA).

Pseudomonas aeruginosa strains and in vitro infectious model

Pseudomonas aeruginosa strain K wild type (PAK WT, provided by Dr. RJ Irvin, University of Alberta, Edmonton, AB) and the isogenic *P. aeruginosa* mutant PAK fliC (flagella deficient, provided by Dr. AS Prince, Columbia University, New York), as well as *P. aeruginosa* clinical isolates from sputum samples of CF patients were used (Table). One clinical isolate from an intermittently colonized and another from a chronically infected patient (the latter obtained during longitudinal observation at the Danish CF Center) were kindly provided by Dr. N Høiby (University Hospital Rigshospitalet, Copenhagen, Denmark). The characteristics of the isolates are described in our previous study (Hawdon et al. 2010).

The bacteria were maintained on Luria Burtani (LB) medium (Fischer Scientific, Fair Lawn, NJ) with 1% agar (LBA). A single colony of *P. aeruginosa* was grown overnight in sterile LB medium on a shaking platform at 150 rpm and diluted by a factor of 20 into fresh sterile LB medium. Cultures were allowed to grow for approximately 1 hour, until mid-log phase when optical density at 600 nm (OD_{600}) reached 0.30. The culture was then centrifuged at $3,500 \times g$ for 20 minutes at 4°C and washed twice in PBS. Following the final re-suspension, bacteria were diluted to an OD_{600} of 0.30 in RPMI 1640 that corresponded to approximately 2×10^8 CFU/mL, as determined by serial dilutions and drop plating on LBA. From this stock, bacteria were added to either H292 cells to obtain a multiplicity of infection (MOI) of 50, as was optimized in our previous experiments (Aval et al. 2013), or THP-1 cells at a MOI of 5. The latter conditions were optimized using THP-1 cells infected with PAK during 1, 2, 6, 12, or 18 hours at MOI of 1, 5, or 10.

Stimulation of THP-1 cells via Fcγ-receptor cross-linking

The 96-well plates (Falcon, Corning Incorporated) were coated with human IgG (Sigma-Aldrich) at concentrations of 10 and 100 µg/mL and incubated for 3 hours at 37°C, followed by overnight incubation at 4°C, then the plates were washed twice with sterile PBS. THP-1 cells at

concentration of 0.4×10^6 cells/mL in 200 μ L were added to the coated wells and incubated for 18 hours at 37°C with 5% CO₂.

Pretreatment with R406

THP-1 or H292 cells were grown for 24 hours to 0.4×10^6 cells/mL, or until they reached approximately 80% confluence, respectively, and R406 (AstraZeneca) dissolved in DMSO was added to the medium to achieve a final concentration of 10 μ M. The cells were incubated in the presence of R406 for 1 hour, then washed once with PBS and used for experiments. These conditions were developed based on published literature describing R406 pretreatments (Braselmann et al. 2006; Spalton et al. 2009; Chen et al. 2008; Quiroga et al. 2009) and our cellular viability testing using R406 concentrations of 1, 5, 10, 15, and 20 μ M. No noticeable effect of R406 concentrations up to 10 μ M on cell viability tested during one hour was detected (97-99% viable cells), nor significant decrease in either THP-1 or H292 cell viability following 18-hour-long incubation with 10 μ M of R406 occurred. Viability of THP-1 following 18 hour-long incubation with R406 or without R406 was 82% and 75% ($P > 0.05$), for H292 cells, it was 84% and 81%, correspondingly ($P > 0.05$).

Flow cytometry analysis of ICAM-1 expression

THP-1 cells (0.4×10^6) were infected with PAK WT for 6 hours at 37°C, 5% CO₂, then washed and re-suspended in 100 μ L of 0.1% BSA-PBS containing PE-conjugated mAb against ICAM-1 (Mouse anti-human CD54, BD Pharmigen, Mississauga, ON) at a dilution of 1:50 and incubated for 1 hour at 4°C. Following incubation, cells were washed twice with PBS and analyzed by flow cytometry on the FACSCalibur (BD Bioscience, Mississauga, ON, Canada). The data were analyzed using CellQuest Pro software and expressed as mean fluorescence intensity (MFI).

ELISA for cytokine detection

To measure the release of cytokines, PMA-differentiated THP-1 or H292 cells were infected with *P. aeruginosa* (MOI of 10, or 50, correspondingly), for 1 hour at 37°C, with 5% CO₂, and then 100 µg/mL gentamicin was added, followed by incubation for a further 17 hours at 37°C with 5% CO₂. Following stimulation, cell culture supernatants were collected and stored at -80°C until analysis. The levels of TNFα and IL-1β were measured using eBioscience Ready-Set-Go ELISA kits (San Diego, CA) according to the manufacturer's protocol. The lower detection limits of the assays were 2 pg/mL for IL-1β and 4 pg/mL for TNFα. Samples from three independent experiments were run in triplicate.

Immunoprecipitation and Western blot

THP-1 cells (2×10⁶) were infected with PAK WT at an MOI of 5 for 2 hours at 37°C, 5% CO₂. Following stimulation, the cells were centrifuged, washed, re-suspended in 100 µL of ice-cold RIPA lysis buffer, and incubated for 30 minutes at 4 °C. Following incubation, the cells were centrifuged at 8,000 × g for 10 minutes and protein lysate was collected. Isolated proteins were immunoprecipitated with polyclonal anti-Syk antibody (N-19) (Santa Cruz Biotechnology, CA) using magnetic Protein A beads (Bio-Rad, Hercules CA), according to the manufacturer's protocol. Samples were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, probed with primary antibody, i.e. anti-phospho-tyrosine (P-Tyr-100) (Cell Signaling Technology), or monoclonal anti-syk antibodies (4D10) (Santa Cruz Biotechnology, CA) followed by HRP-conjugated secondary antibody (7074S) (Cell Signaling Technology), and developed using enhanced chemiluminescence. Bands were scanned and images analyzed using ChemiDoc XRS (Bio-Rad). For analysis of total and phosphorylated intracellular signaling proteins, THP-1 cells were stimulated with PAK (MOI of 5), for 15, 30, or 60 minutes at 37°C. Protein lysates were collected and stored at -80 °C. For analysis of protein expression by Western blot we used: monoclonal anti-JNK (D-2), anti-phospho JNK (G-7), anti-ERK 2 (12A4), anti-

phospho ERK 2 (E-4), anti-p38 α (9F12), anti-phospho p38 α (E-1), anti-I κ B- α (H-4), anti-phospho I κ B- α (B-9), anti- β -actin (C4) and mouse IgG κ BP-HRP (Santa Cruz Biotechnology). In some cases, the blots were stripped and re-probed with other antibodies.

Statistical analysis

All the experiments were repeated at least 3 times. Data were expressed as mean $\pm$ SEM for n independent experiments. For comparison of two sample means, Student's t test was applied. GraphPad Prism 7.0 (La Jolla, CA, USA) was used for the analysis. P-values <0.05 were considered significant.

Draft

Results:*R406 down-regulates ICAM-1 expression induced by P. aeruginosa infection*

As the intercellular adhesion molecule 1 (ICAM-1) typically becomes up-regulated during inflammatory responses, particularly in cells infected with *P. aeruginosa* (Roebuck and Finnegan 1999; Sadikot et al. 2005) we tested the effect of R406 on the cell-surface expression of ICAM-1 in THP-1 cells exposed to a virulent *P. aeruginosa* strain K (PAK) at an MOI of 5. As shown on Fig.1A, 6-hour long infection resulted in >15-fold increase in ICAM-1 mean fluorescence intensity (MFI) compared to uninfected cells. One hour-long pre-incubation of THP-1 cells with R406 used in concentrations between 0.1 and 20 μ M down-regulated ICAM-1 expression in a dose-dependent manner, with a statistically significant effect for all the R406 concentrations >0.5 μ M (Fig. 1A). There was no noticeable effect of R406 concentrations up to 10 μ M on cell viability; the percentage of viable cells after one hour of incubation with R406 was 97-99% (data not shown). Likewise, one hour-long incubation of THP-1 cells with 1, 5, or 10 μ M of R406 did not have any visible effect on the baseline ICAM-1 expression (data not shown).

To confirm the effect of R406 on Syk in our model, we stimulated THP-1 cells via Fc γ -receptor (Fc γ R) cross-linking, which is known to induce Syk-dependent signaling (Darby et al. 1994). While stimulation of THP-1 cells with immobilized human IgG resulted in a significant increase in ICAM-1 surface expression in a dose-dependent manner, pre-treatment with 10 μ M R406 caused an attenuation of ICAM-1 expression at both 10 and 100 μ g/mL IgG concentrations ($P < 0.05$) (Fig. 1B). Moreover, tyrosine phosphorylation of Syk induced by two hour-long exposure of THP-1 cells to live bacteria was significantly down-regulated in cells, pre-treated with 10 μ M R406 (Fig. 1C).

These experiments imply that down-regulation of ICAM-1 in *P. aeruginosa* infected cells by R406 could be mediated by inhibition of Syk-mediated signaling.

R406 down-regulates the release of pro-inflammatory cytokines TNF α and IL-1 β induced by P. aeruginosa infection

To further test the effect of R406 in our model, we studied the release of cytokines TNF α and IL-1 β , which are the hallmarks of inflammatory responses caused by *P. aeruginosa* infection, by using a virulent *P. aeruginosa* strain K (PAK WT), the isogenic *P. aeruginosa* mutant PAK fliC (flagella-deficient), and clinical isolates from two CF patients (intermittently colonized and chronically infected). One hour-long infection of differentiated THP-1 cells, or H292 cells with PAK WT, followed by adding gentamicin with further 17 hours of incubation resulted in a large TNF α release by both cell types, with THP-1 cells producing over 35-fold greater amount of this cytokine as compared to H292 cells (Fig. 2B-C).

When flagella-deficient mutant (PAK fliC) was used for stimulation, TNF α release by both cell lines was lower compared to stimulation with PAK WT ($P < 0.01$). Interestingly, although stimulation of THP-1 cells with either clinical *P. aeruginosa* isolate resulted in lower TNF α release ($P < 0.0001$), in case of H292 cells, an isolate from a chronically infected CF patient induced higher TNF α release compared to PAK WT ($P < 0.001$), PAK fliC ($P < 0.0001$), and isolate from a CF patient with intermittent *P. aeruginosa* infection ($P < 0.001$). Nevertheless, TNF α release was significantly decreased in all infected cell cultures pretreated with R406, except for THP-1 cells stimulated with an isolate from a chronically infected CF patient (Fig. 2B-C).

While unstimulated differentiated THP-1 cells only produced a low amount of IL-1 β (78 ± 9 pg/mL), infection with PAK WT resulted in a large increase in IL-1 β release (3993 ± 245 pg/mL, $P < 0.0001$). Stimulation with PAK fliC, or either isolate from a CF patient with intermittent or chronically *P. aeruginosa* infected also significantly upregulated IL-1 β release, although to a

lesser degree compared to PAK WT ($P<0.0001$, $P<0.01$, and $P<0.05$ respectively). The lowest amount of IL-1 β (1310 ± 281 pg/mL) was released by THP-1 cells stimulated with an isolate from a chronically infected CF patient (significant lower than following stimulation with PAK WT, $P<0.0001$). Despite of different degrees of IL-1 β release induced by *P. aeruginosa* strains, pretreatment of differentiated THP-1 cells with R406 down-regulated this response (Fig. 2A).

R406 down-regulates the expression of phosphorylated ERK2, JNK, p-38, and I κ B α in P. aeruginosa infected THP-1 cells

As intracellular signaling molecules ERK2, JNK, p-38, and I κ B α have been recognized as important regulators of inflammatory responses induced by *P. aeruginosa* (Li et al. 1998; Ratner et al. 2001; Esen et al. 2001), we investigated the effect of R406 on the expression of these total and phosphorylated proteins in our model. Stimulation of THP-1 cells with PAK induced significant up-regulation of phosphorylated ERK2 at 30 min ($P<0.01$) and 60 min ($P<0.01$), JNK at 15 min ($P<0.001$), 30 min ($P<0.001$), and 60 min ($P<0.001$), p38 at 15 min ($P<0.001$), 30 min ($P<0.0001$), and 60 min ($P<0.0001$), and I κ B α at 30 min ($P<0.01$) and 60 min ($P<0.001$). Pretreatment of infected cells with R406 led to a decreased expression of all phosphorylated signaling molecules that was statistically significant for JNK and p-38 at 15, 30, and 60 minutes of stimulation, and for I κ B α and ERK2 at 30 and 60 minutes of stimulation (Fig. 3A-D).

Discussion

This study shows that a small molecule inhibitor of Syk down-regulates inflammatory responses of human cells infected with *P. aeruginosa*. Specifically, in monocytic cell line THP-1, R406 caused a significant decrease in cell surface expression of ICAM-1, an adhesion molecule, which mediates leukocyte migration to inflammatory sites, in a dose-dependent manner, as well as down-regulated the release of pro-inflammatory cytokines TNF α and IL-1 β . The transcriptional regulation of all these three molecules is largely dependent on the activation of transcription factor NF- κ B, which is known to be a downstream target of Syk-mediated signaling along with the MAPK cascade (Costello et al. 1996; Darby et al. 1994). Indeed, R406 caused a decrease in the expression of phosphorylated ERK2, JNK and p-38, as well as of I κ B α ; the latter, when phosphorylated, facilitates nuclear translocation of NF- κ B, which is required for its activation and resulting production of inflammatory mediators (Akira and Kishimoto 1997). In our previous study, inhibition of Syk using small interfering RNA caused down-regulation of the MAPK cascade phosphorylation and nuclear translocation of p65 NF- κ B induced by TNF α stimulation of lung epithelial cells (Ulanova et al. 2006). The data of the present study extend our earlier observations to monocytic cells and indicate that Syk is involved in the regulation of pro-inflammatory responses to *P. aeruginosa* infection via activation of downstream signalling pathways, including MAPK-mediated one. In support of this idea, we found an increase in the expression of tyrosine-phosphorylated Syk, an indicator of Syk activation, following two hour-long *P. aeruginosa* infection, and a decrease in the expression of phospho-Syk following pre-treatment with R406 (Fig. 1C). As release of mature IL-1 β requires inflammasome activation, in addition to IL-1 β gene transcription, the effect of R406 on IL-1 β release suggests Syk involvement in the regulation of inflammasome activation in our model (Dinarello 2009). This is not surprising as previous studies identified Syk as a key mediator of NLRP3 inflammasome activation and IL-1 β secretion in innate immune cells stimulated with fungi and crystals (Gross et al. 2009; del Fresno et al. 2013; Mao et al. 2014; Lin et al. 2015; Lima-Junior et al. 2017).

279

280 There are potentially multiple pathways of Syk activation during *P. aeruginosa* infection of
281 monocytic cells. This non-receptor protein tyrosine kinase is best known as a critical component
282 of immunoreceptor tyrosine-based activation motifs (ITAM)-dependent signaling in
283 hematopoietic cells involving Fc receptors, T-, B-, and NK cell receptors (Turner et al. 2000).
284 Congruently, in our experiments, we observed a strong inhibitory effect of R406 on ICAM-1
285 expression induced by a classical mechanism of Syk activation, i.e. via Fc γ receptor cross-linking,
286 with ICAM-1 expression level decreased to the baseline while using a 10 μ g/mL concentration of
287 human IgG for receptor activation (Fig. 1B). However, none of the cellular responses to *P.*
288 *aeruginosa* have been completely inhibited by R406, although we could achieve their significant
289 down-regulation using a concentration of 10 μ M, which was commonly used in studies by others
290 (Brasemann et al. 2006); in case of ICAM-1, lower concentrations of 0.5 to 5 μ M were also
291 effective (Fig 1A). The data suggest that although Syk is certainly involved in the regulation of
292 inflammatory responses to *P. aeruginosa* infection, it does not represent the major pathway
293 among multiple mechanisms operating in cellular responses to this highly virulent
294 microorganism, which is capable to interact with many pathogen-recognition receptors, including
295 Toll-like receptors, Nod-like receptors, integrins, C-type lectins, asialoGM1, *etc* (Sadikot et al.
296 2005; DiMango et al. 1995; Skerrett et al. 2007). Ability of *P. aeruginosa* to stimulate TNF α and
297 IL-1 β synthesis and release from human monocytes, and activation of transcription factors NF- κ B
298 and AP in infected cells have been established by previous studies (Li et al. 1998; Kube et al.
299 2001; Lagoumintzis et al. 2003; Wehkamp et al. 2006). Syk involvement in the regulation of
300 signals generated by the engagement of TLR-4 complex by its ligand LPS in human neutrophils
301 and macrophages has also been previously demonstrated (Arndt et al. 2004; Ulanova et al. 2007;
302 Miller et al. 2012), and this mechanism likely operates in our model. Recent studies expanded our
303 understanding of the role of Syk in fine-tuning of cellular responses stimulated by the
304 engagement of innate immune receptors (Aouar et al. 2016; Yin et al. 2016). For example, it was

demonstrated that in macrophages and dendritic cells, Syk regulates TNF α exocytosis induced by stimulation of TLR9 by bacterial CpG DNA (Rao et al. 2013); such mechanism may potentially be involved in responses of differentiated THP-1 cells to *P. aeruginosa*. In addition, innate immune responses activated by *P. aeruginosa* result in the amplification of inflammatory responses, as for example, TNF α further activates the inflammatory cascade via its own receptor associated signaling (Newton and Dixit 2012). The complexity of cellular responses to *P. aeruginosa* is further augmented by cross talk among multiple signalling pathways, including both pro- and anti-inflammatory (Lee and Kim 2007).

Syk may become activated following *P. aeruginosa* infection via several potential mechanisms. It is well recognized that Syk is significantly involved in several ITAM-independent signalling pathways, which are mediated by its interaction with G-protein coupled receptors, pattern recognition, and cytokine receptors (Ulanova et al. 2005a; Mocsai et al. 2010). In particular, Syk can be activated via interaction with integrin receptor cytoplasmic domains that is especially significant in lung epithelial cells, which do not express the plethora of innate immune receptors typical for leukocytes (Ulanova et al. 2005b). Our previous research demonstrated the involvement of integrin receptors in *P. aeruginosa* internalization and recognition by A549 alveolar epithelial cells; moreover, the data suggested an important role of integrin-mediated signaling in inflammation induced by this infection (Gravelle et al. 2010). In the present study, the release of TNF α by infected bronchiolar epithelial cells was significantly down-regulated by R406 implicating the involvement of Syk-dependent signaling in inflammatory responses to *P. aeruginosa* by lung epithelial cells, in addition to monocytes (Fig 1C). Indeed, we have previously demonstrated that H292 cells express Syk (Aval et al. 2013); however, it is uncertain whether or not Syk is exclusively engaged via integrin receptors in this particular cell line, or some other mechanisms, for example, those mediated by TNF-receptor signaling are involved (Takada and Aggarwal 2004).

Because Syk combines both kinase and adaptor protein properties, this molecule is capable to interact with multiple protein targets, and this explains why its inhibition leads to numerous biological effects. Indeed, Syk has been considered as a target for therapy of such diverse conditions as allergic diseases, rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, and B-cell lymphoma, with several pharmacological compounds undergoing clinical trials (Riccaboni et al. 2010). One potential application could be the use of Syk inhibitors to dampen severe pro-inflammatory responses associated with pulmonary *P. aeruginosa* infection, which affects CF patients, as well as occurs in ventilator-associated pneumonia, aggravates the course of chronic obstructive pulmonary disease (COPD), and causes severe complications in cancer patients with neutropenia, caused by chemotherapy that predisposes to *P. aeruginosa* pneumonia (Saiman and Siegel 2004; Hakki et al. 2007; Crouch Brewer et al. 1996; Lieberman and Lieberman 2003). In our previous study, we found that a natural Syk inhibitor piceatannol significantly suppressed inflammation, oxidative stress, apoptosis, and bacterial internalization in a model of *P. aeruginosa* infected pulmonary epithelial cells, although not all of these outcomes could be attributed to Syk-specific effect (Aval et al. 2013). Results of the current study corroborate our previous observations using this time both a model of infected THP-1 cells, which represent innate immune cells, and a bronchiolar epithelial cell line H292 (Carney et al. 1985)

As bronchiolar epithelial cells represent the major component of the airway lining, have receptors for *P. aeruginosa*, are the site of infection, generate inflammatory responses to this infectious agent, and express Syk, they could be the major targets for potential therapeutic intervention using Syk inhibitors. Importantly, the response of H292 cells to stimulation with various strains of *P. aeruginosa* was noticeably different from the response by differentiated monocytic THP-1 cells (Fig. 2B-C). Although the release of TNF α by H292 cells infected with PAK WT or PAK

fliC was approximately 50-times lower than the one by THP-1 cells, infection with clinical *P. aeruginosa* isolates caused relatively higher TNF α production in H292 cells. In particular, H292 cells infected with *P. aeruginosa* of a CF patient with long-term chronic infection released the largest amount of TNF α in comparison to other *P. aeruginosa* strains, i.e. 202 ± 5 pg/mL, although THP-1 cells produced significantly less TNF α when stimulated with either clinical isolate (1545 ± 237 pg/mL and 714 ± 102 pg/mL) as compared to both wild-type (4795 ± 463 pg/mL) and flagella-deficient (3691 ± 255 pg/mL) laboratory strains PAK. These data corroborate our previous observations that *P. aeruginosa* isolates from chronically infected CF patients have increased abilities of causing inflammatory responses of A549 alveolar epithelial cells in comparison to bacteria from patients with intermittent *P. aeruginosa* colonization, owing to the adaptation process in the CF lung during long-term infectious process (Hawdon et al. 2010). The isolate #19731A/92 was obtained from a CF patient with 18-year long chronic *P. aeruginosa* infection (Hawdon et al. 2010). As a flagella-deficient strain (PAK fliC) induced significantly lower release of cytokines TNF α and IL-1 β compared to the wild-type bacteria (Fig 2A-C) these data emphasize importance of flagella in stimulating potent pro-inflammatory responses to *P. aeruginosa* infection via the activation of pattern-recognition receptors such as TLR5 and NLRC4 inflammasome (Blohmke et al. 2010; Zhao et al. 2011). Importantly, in bronchiolar epithelial cells, R406 was able to significantly down-regulate TNF α release caused by *P. aeruginosa* isolates from both chronically infected and intermittently colonized CF patients, although to a lesser degree than when the inhibitor was applied to cells, stimulated with PAK WT or PAK fliC (Fig 2C) suggesting potential clinical application of this inhibitor. However, as recent studies found that Syk is essential for flagellin-specific T cell responses, it is important to consider complexity of the regulatory role of this signaling molecule in immune responses (Atif et al. 2017).

Compared to an early used inhibitor piceatannol, R406 has been demonstrated to be much more selective for Syk. However, R406 is not entirely specific to Syk, and able to inhibit JAK2 in addition to Syk of similar potency (Rolf et al. 2015). Although the present findings suggest Syk involvement in the regulation of *P. aeruginosa* triggered inflammatory responses in both human monocytic and bronchiolar epithelial cells, it will be highly desired to test more specific Syk inhibitors. However, creating a truly selective Syk inhibitor apparently represents a challenge; indeed, all of the existing compounds with Syk-inhibitory capacities, including the most recent ones express certain off-target specificity (Ferguson et al. 2016; Yaron et al. 2016). When Fostamatinib, of which R406 is the active metabolite, was tested in phase II-III clinical trials for rheumatoid arthritis, adverse events related to its off-target effect have been noticed (Kunwar et al. 2016). As it was demonstrated that inhibition of JAK2 down-regulated inflammatory responses in an animal model of polymicrobial sepsis, certain off-target effects of R406 may potentially be beneficial in case of *P. aeruginosa* infection (Pena et al. 2010). Conducting clinical trials to ascertain capacity of this Syk inhibitor to alleviate exaggerated inflammatory responses, which significantly contribute to the pathogenesis of *P. aeruginosa* pulmonary infections may represent a sensible approach.

Acknowledgments

This work was supported by a National Science and Engineering Research Council Discovery Grant and Ontario Lung Association Grant to M.U. We thank Dr. Niels Høiby (University Hospital Rigshospitalet, Copenhagen, Denmark), Dr. Randal Irvin (University of Alberta, Edmonton, Canada), and Dr. Alice Prince, Columbia University, New York) for kindly providing *P. aeruginosa* clinical isolates and strains, AstraZeneca AB (Södertälje, Sweden) for providing R406), and Jessica Rosengren (Lakehead University, Thunder Bay, Canada) for help with optimizing experimental conditions.

References

- Akira, S. and Kishimoto, T. 1997. NF-IL6 and NF-kappa B in cytokine gene regulation. *Adv Immunol*, 65, 1-46.
- Aouar, B., Kovarova, D., Letard, S., Font-Haro, A., Florentin, J., Weber, J., Durantel, D., Chaperot, L., Plumas, J., Trejbalova, K., Hejnar, J., Nunes, J. A., Olive, D., Dubreuil, P., Hirsch, I. and Stranska, R. 2016. Dual Role of the Tyrosine Kinase Syk in Regulation of Toll-Like Receptor Signaling in Plasmacytoid Dendritic Cells. *PLoS One*, 11(6), e0156063. doi: 10.1371/journal.pone.0156063. PMID: 27258042.
- Arndt, P. G., Suzuki, N., Avdi, N. J., Malcolm, K. C. and Worthen, G. S. 2004. Lipopolysaccharide-induced c-Jun NH2-terminal kinase activation in human neutrophils: role of phosphatidylinositol 3-Kinase and Syk-mediated pathways. *J Biol Chem*, 279(12), 10883-91. doi: 10.1074/jbc.M309901200. PMID: 14699155.
- Atif, S. M., Lee, S. J., Li, L. X., Uematsu, S., Akira, S., Gorjestani, S., Lin, X., Schweighoffer, E., Tybulewicz, V. L. and McSorley, S. J. 2015. Rapid CD4+ T-cell responses to bacterial flagellin require dendritic cell expression of Syk and CARD9. *Eur J Immunol*, 45(2), 513-24. doi: 10.1002/eji.201444744. PMID: 25430631.
- Aval, P. S., Werner, J., Cerqueira, A., Balfour-Boehm, J. and Ulanova, M. 2013. Piceatannol modulates lung epithelial cellular responses to *Pseudomonas aeruginosa*. *Inflamm Allergy Drug Targets*, 12(5), 297-307.
- Blohmke, C. J., Park, J., Hirschfeld, A. F., Victor, R. E., Schneiderman, J., Stefanowicz, D., Chilvers, M. A., Durie, P. R., Corey, M., Zielenski, J., Dorfman, R., Sandford, A. J., Daley, D. and Turvey, S. E. 2010. TLR5 as an anti-inflammatory target and modifier gene in cystic fibrosis. *J Immunol*, 185(12), 7731-8. doi: 10.4049/jimmunol.1001513. PMID: 21068401.
- Brasemann, S., Taylor, V., Zhao, H., Wang, S., Sylvain, C., Baluom, M., Qu, K., Herlaar, E., Lau, A., Young, C., Wong, B. R., Lovell, S., Sun, T., Park, G., Argade, A., Jurcevic, S., Pine, P., Singh, R., Grossbard, E. B., Payan, D. G. and Masuda, E. S. 2006. R406, an orally

- 434 available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune
435 complex-mediated inflammation. *J Pharmacol Exp Ther*, 319(3), 998-1008. doi:
436 10.1124/jpet.106.109058. PMID: 16946104.
- 437 Carney, D. N., Gazdar, A. F., Bepler, G., Guccion, J. G., Marangos, P. J., Moody, T. W., Zweig,
438 M. H. and Minna, J. D. 1985. Establishment and identification of small cell lung cancer cell
439 lines having classic and variant features. *Cancer Res*, 45(6), 2913-23. 2985257.
- 440 Cha, H. S., Boyle, D. L., Inoue, T., Schoot, R., Tak, P. P., Pine, P. and Firestein, G. S. 2006. A
441 novel spleen tyrosine kinase inhibitor blocks c-Jun N-terminal kinase-mediated gene
442 expression in synoviocytes. *J Pharmacol Exp Ther*, 317(2), 571-8. doi:
443 10.1124/jpet.105.097436. PMID: 16452391.
- 444 Chen, L., Monti, S., Juszczynski, P., Daley, J., Chen, W., Witzig, T. E., Habermann, T. M.,
445 Kutok, J. L. and Shipp, M. A. 2008. SYK-dependent tonic B-cell receptor signaling is a
446 rational treatment target in diffuse large B-cell lymphoma. *Blood*, 111(4), 2230-7. doi:
447 10.1182/blood-2007-07-100115. PMID: 18006696.
- 448 Cheng, K., Ashby, D. and Smyth, R. L. 2013. Oral steroids for long-term use in cystic fibrosis.
449 *Cochrane Database Syst Rev*(6), CD000407. doi: 10.1002/14651858.CD000407.pub3.
450 PMID: 23794322.
- 451 Costello, P. S., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P. H., Downward, J. and
452 Tybulewicz, V. L. 1996. Critical role for the tyrosine kinase Syk in signalling through the
453 high affinity IgE receptor of mast cells. *Oncogene*, 13(12), 2595-605.
- 454 Crouch Brewer, S., Wunderink, R. G., Jones, C. B. and Leeper, K. V., Jr. 1996. Ventilator-
455 associated pneumonia due to *Pseudomonas aeruginosa*. *Chest*, 109(4), 1019-29.
- 456 Darby, C., Geahlen, R. L. and Schreiber, A. D. 1994. Stimulation of macrophage Fc gamma
457 RIIIA activates the receptor-associated protein tyrosine kinase Syk and induces
458 phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. *J*
459 *Immunol*, 152(11), 5429-37.

- del Fresno, C., Soulat, D., Roth, S., Blazek, K., Udalova, I., Sancho, D., Ruland, J. and Ardavin, C. 2013. Interferon-beta production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to *C. albicans*. *Immunity*, 38(6), 1176-86. doi: 10.1016/j.immuni.2013.05.010. PMID: 23770228.
- DiMango, E., Zar, H. J., Bryan, R. and Prince, A. 1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J Clin Invest*, 96(5), 2204-10. doi: 10.1172/JCI118275. PMID: 7593606.
- Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol*, 27, 519-50. doi: 10.1146/annurev.immunol.021908.132612. PMID: 19302047.
- Esen, M., Grassme, H., Riethmuller, J., Riehle, A., Fassbender, K. and Gulbins, E. 2001. Invasion of human epithelial cells by *Pseudomonas aeruginosa* involves src-like tyrosine kinases p60Src and p59Fyn. *Infect Immun*, 69(1), 281-7. doi: 10.1128/IAI.69.1.281-287.2001. PMID: 11119516.
- Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H. and Prince, A. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun*, 66(1), 43-51. 9423837.
- Ferguson, G. D., Delgado, M., Plantevin-Krenitsky, V., Jensen-Pergakes, K., Bates, R. J., Torres, S., Celeridad, M., Brown, H., Burnett, K., Nadolny, L., Tehrani, L., Packard, G., Pagarigan, B., Haelewyn, J., Nguyen, T., Xu, L., Tang, Y., Hickman, M., Baculi, F., Pierce, S., Miyazawa, K., Jackson, P., Chamberlain, P., LeBrun, L., Xie, W., Bennett, B. and Blease, K. 2016. A Novel Triazolopyridine-Based Spleen Tyrosine Kinase Inhibitor That Arrests Joint Inflammation. *PLoS One*, 11(1), e0145705. doi: 10.1371/journal.pone.0145705. PMID: 26756335.
- Gravelle, S., Barnes, R., Hawdon, N., Shewchuk, L., Eibl, J., Lam, J. S. and Ulanova, M. 2010. Up-regulation of integrin expression in lung adenocarcinoma cells caused by bacterial

- infection: in vitro study. *Innate Immun*, 16(1), 14-26. doi: 10.1177/1753425909106170. PMID: 19710103.
- Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hanneschlager, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., Mocsai, A., Tschopp, J. and Ruland, J. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*, 459(7245), 433-6. doi: 10.1038/nature07965. PMID: 19339971.
- Hakki, M., Limaye, A. P., Kim, H. W., Kirby, K. A., Corey, L. and Boeckh, M. 2007. Invasive *Pseudomonas aeruginosa* infections: high rate of recurrence and mortality after hematopoietic cell transplantation. *Bone Marrow Transplant*, 39(11), 687-93. doi: 10.1038/sj.bmt.1705653. PMID: 17401395.
- Hawdon, N. A., Aval, P. S., Barnes, R. J., Gravelle, S. K., Rosengren, J., Khan, S., Ciofu, O., Johansen, H. K., Hoiby, N. and Ulanova, M. 2010. Cellular responses of A549 alveolar epithelial cells to serially collected *Pseudomonas aeruginosa* from cystic fibrosis patients at different stages of pulmonary infection. *FEMS Immunol Med Microbiol*, 59(2), 207-20. doi: 10.1111/j.1574-695X.2010.00693.x. PMID: 20528926.
- Kipnis, E., Sawa, T. and Wiener-Kronish, J. 2006. Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. *Med Mal Infect*, 36(2), 78-91. doi: 10.1016/j.medmal.2005.10.007. PMID: 16427231.
- Kube, D., Sontich, U., Fletcher, D. and Davis, P. B. 2001. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *Am J Physiol Lung Cell Mol Physiol*, 280(3), L493-502.
- Kunwar, S., Devkota, A. R. and Ghimire, D. K. 2016. Fostamatinib, an oral spleen tyrosine kinase inhibitor, in the treatment of rheumatoid arthritis: a meta-analysis of randomized controlled trials. *Rheumatol Int*, 36(8), 1077-87. doi: 10.1007/s00296-016-3482-7. PMID: 27113955.
- Lagoumintzis, G., Christofidou, M., Dimitracopoulos, G. and Paliogianni, F. 2003. *Pseudomonas*

aeruginosa slime glycolipoprotein is a potent stimulant of tumor necrosis factor alpha gene expression and activation of transcription activators nuclear factor kappa B and activator protein 1 in human monocytes. *Infect Immun*, 71(8), 4614-22.

Lee, M. S. and Kim, Y. J. 2007. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu Rev Biochem*, 76, 447-80. doi: 10.1146/annurev.biochem.76.060605.122847. PMID: 17328678.

Li, J. D., Feng, W., Gallup, M., Kim, J. H., Gum, J., Kim, Y. and Basbaum, C. 1998. Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. *Proc Natl Acad Sci U S A*, 95(10), 5718-23.

Lieberman, D. and Lieberman, D. 2003. Pseudomonal infections in patients with COPD: epidemiology and management. *Am J Respir Med*, 2(6), 459-68.

Lima-Junior, D. S., Mineo, T. W. P., Calich, V. L. G. and Zamboni, D. S. 2017. Dectin-1 Activation during *Leishmania amazonensis* Phagocytosis Prompts Syk-Dependent Reactive Oxygen Species Production To Trigger Inflammasome Assembly and Restriction of Parasite Replication. *J Immunol*, 199(6), 2055-2068. doi: 10.4049/jimmunol.1700258. PMID: 28784846.

Lin, Y. C., Huang, D. Y., Wang, J. S., Lin, Y. L., Hsieh, S. L., Huang, K. C. and Lin, W. W. 2015. Syk is involved in NLRP3 inflammasome-mediated caspase-1 activation through adaptor ASC phosphorylation and enhanced oligomerization. *J Leukoc Biol*, 97(5), 825-835. doi: 10.1189/jlb.3HI0814-371RR. PMID: 25605870.

Mao, L., Zhang, L., Li, H., Chen, W., Wang, H., Wu, S., Guo, C., Lu, A., Yang, G., An, L., Abliz, P. and Meng, G. 2014. Pathogenic fungus *Microsporum canis* activates the NLRP3 inflammasome. *Infect Immun*, 82(2), 882-92. doi: 10.1128/IAI.01097-13. PMID: 24478101.

McAdoo, S. P. and Tam, F. W. 2011. Fostamatinib Disodium. *Drugs Future*, 36(4), 273.

Miller, Y. I., Choi, S. H., Wiesner, P. and Bae, Y. S. 2012. The SYK side of TLR4: signalling

- 538 mechanisms in response to LPS and minimally oxidized LDL. *Br J Pharmacol*, 167(5), 990-
539 9. doi: 10.1111/j.1476-5381.2012.02097.x. PMID: 22776094.
- 540 Mocsai, A., Ruland, J. and Tybulewicz, V. L. 2010. The SYK tyrosine kinase: a crucial player in
541 diverse biological functions. *Nat Rev Immunol*, 10(6), 387-402. doi: 10.1038/nri2765.
542 PMID: 20467426.
- 543 Newton, K. and Dixit, V. M. 2012. Signaling in innate immunity and inflammation. *Cold Spring*
544 *Harb Perspect Biol*, 4(3). doi: 10.1101/cshperspect.a006049. PMID: 22296764.
- 545 Pasloske, B. L., Finlay, B. B. and Paranchych, W. 1985. Cloning and sequencing of the
546 *Pseudomonas aeruginosa* PAK pilin gene. *FEBS Lett*, 183(2), 408-12. 2985436.
- 547 Pena, G., Cai, B., Deitch, E. A. and Ulloa, L. 2010. JAK2 inhibition prevents innate immune
548 responses and rescues animals from sepsis. *J Mol Med (Berl)*, 88(8), 851-9. doi:
549 10.1007/s00109-010-0628-z. PMID: 20393690.
- 550 Quiroga, M. P., Balakrishnan, K., Kurtova, A. V., Sivina, M., Keating, M. J., Wierda, W. G.,
551 Gandhi, V. and Burger, J. A. 2009. B-cell antigen receptor signaling enhances chronic
552 lymphocytic leukemia cell migration and survival: specific targeting with a novel spleen
553 tyrosine kinase inhibitor, R406. *Blood*, 114(5), 1029-37. doi: 10.1182/blood-2009-03-
554 212837. PMID: 19491390.
- 555 Rao, S., Liu, X., Freedman, B. D. and Behrens, E. M. 2013. Spleen tyrosine kinase (Syk)-
556 dependent calcium signals mediate efficient CpG-induced exocytosis of tumor necrosis
557 factor alpha (TNFalpha) in innate immune cells. *J Biol Chem*, 288(18), 12448-58. doi:
558 10.1074/jbc.M113.454405. PMID: 23515313.
- 559 Ratner, A. J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S., Cheung, A. and
560 Prince, A. 2001. Cystic fibrosis pathogens activate Ca²⁺-dependent mitogen-activated
561 protein kinase signaling pathways in airway epithelial cells. *J Biol Chem*, 276(22), 19267-
562 75. doi: 10.1074/jbc.M007703200. PMID: 11278360.
- 563 Riccaboni, M., Bianchi, I. and Petrillo, P. 2010. Spleen tyrosine kinases: biology, therapeutic

- 564 targets and drugs. *Drug Discov Today*, 15(13-14), 517-30. doi:
565 10.1016/j.drudis.2010.05.001. PMID: 20553955.
- 566 Roebuck, K. A. and Finnegan, A. 1999. Regulation of intercellular adhesion molecule-1 (CD54)
567 gene expression. *J Leukoc Biol*, 66(6), 876-88.
- 568 Rolf, M. G., Curwen, J. O., Veldman-Jones, M., Eberlein, C., Wang, J., Harmer, A., Hellowell, C.
569 J. and Braddock, M. 2015. In vitro pharmacological profiling of R406 identifies molecular
570 targets underlying the clinical effects of fostamatinib. *Pharmacol Res Perspect*, 3(5), e00175.
571 doi: 10.1002/prp2.175. PMID: 26516587.
- 572 Sadikot, R. T., Blackwell, T. S., Christman, J. W. and Prince, A. S. 2005. Pathogen-host
573 interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med*, 171(11),
574 1209-23. doi: 10.1164/rccm.200408-1044SO. PMID: 15695491.
- 575 Saiman, L. and Siegel, J. 2004. Infection control in cystic fibrosis. *Clin Microbiol Rev*, 17(1), 57-
576 71.
- 577 Skerrett, S. J., Wilson, C. B., Liggitt, H. D. and Hajjar, A. M. 2007. Redundant Toll-like receptor
578 signaling in the pulmonary host response to *Pseudomonas aeruginosa*. *Am J Physiol Lung*
579 *Cell Mol Physiol*, 292(1), L312-22. doi: 10.1152/ajplung.00250.2006. PMID: 16936244.
- 580 Spalton, J. C., Mori, J., Pollitt, A. Y., Hughes, C. E., Eble, J. A. and Watson, S. P. 2009. The
581 novel Syk inhibitor R406 reveals mechanistic differences in the initiation of GPVI and
582 CLEC-2 signaling in platelets. *J Thromb Haemost*, 7(7), 1192-9. doi: 10.1111/j.1538-
583 7836.2009.03451.x. PMID: 19422460.
- 584 Takada, Y. and Aggarwal, B. B. 2004. TNF activates Syk protein tyrosine kinase leading to TNF-
585 induced MAPK activation, NF-kappaB activation, and apoptosis. *J Immunol*, 173(2), 1066-
586 77. PMID: 15240695.
- 587 Turner, M., Schweighoffer, E., Colucci, F., Di Santo, J. P. and Tybulewicz, V. L. 2000. Tyrosine
588 kinase SYK: essential functions for immunoreceptor signalling. *Immunol Today*, 21(3), 148-
589 54.

- 590 Ulanova, M., Asfaha, S., Stenton, G., Lint, A., Gilbertson, D., Schreiber, A. and Befus, D. 2007.
 591 Involvement of Syk protein tyrosine kinase in LPS-induced responses in macrophages. *J*
 592 *Endotoxin Res*, 13(2), 117-25. doi: 10.1177/0968051907079125. PMID: 17621553.
- 593 Ulanova, M., Duta, F., Puttagunta, L., Schreiber, A. D. and Befus, A. D. 2005*a*. Spleen tyrosine
 594 kinase (Syk) as a novel target for allergic asthma and rhinitis. *Expert Opin Ther Targets*,
 595 9(5), 901-21. doi: 10.1517/14728222.9.5.901. PMID: 16185147.
- 596 Ulanova, M., Marcet-Palacios, M., Munoz, S., Asfaha, S., Kim, M. K., Schreiber, A. D. and
 597 Befus, A. D. 2006. Involvement of Syk kinase in TNF-induced nitric oxide production by
 598 airway epithelial cells. *Biochem Biophys Res Commun*, 351(2), 431-7. doi:
 599 10.1016/j.bbrc.2006.10.073. PMID: 17070777.
- 600 Ulanova, M., Puttagunta, L., Marcet-Palacios, M., Duszyk, M., Steinhoff, U., Duta, F., Kim, M.
 601 K., Indik, Z. K., Schreiber, A. D. and Befus, A. D. 2005*b*. Syk tyrosine kinase participates in
 602 beta1-integrin signaling and inflammatory responses in airway epithelial cells. *Am J Physiol*
 603 *Lung Cell Mol Physiol*, 288(3), L497-507. doi: 10.1152/ajplung.00246.2004. PMID:
 604 15557085.
- 605 Wehkamp, K., Schwichtenberg, L., Schroder, J. M. and Harder, J. 2006. *Pseudomonas*
 606 *aeruginosa*- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is
 607 controlled by NF-kappaB and AP-1. *J Invest Dermatol*, 126(1), 121-7. doi:
 608 10.1038/sj.jid.5700020. PMID: 16417227.
- 609 Yaron, J. R., Rao, M. Y., Gangaraju, S., Zhang, L., Kong, X., Su, F., Tian, Y., Glenn, H. L. and
 610 Meldrum, D. R. 2016. The oxindole Syk inhibitor OXSI-2 blocks nigericin-induced
 611 inflammasome signaling and pyroptosis independent of potassium efflux. *Biochem Biophys*
 612 *Res Commun*, 472(3), 545-50. doi: 10.1016/j.bbrc.2016.03.021. PMID: 26970308.
- 613 Yin, H., Zhou, H., Kang, Y., Zhang, X., Duan, X., Alnabhan, R., Liang, S., Scott, D. A., Lamont,
 614 R. J., Shang, J. and Wang, H. 2016. Syk negatively regulates TLR4-mediated IFNbeta and
 615 IL-10 production and promotes inflammatory responses in dendritic cells. *Biochim Biophys*

- 616 Acta, 1860(3), 588-98. doi: 10.1016/j.bbagen.2015.12.012.PubMed PMID: 26708990.
- 617 Zhao, Y., Yang, J., Shi, J., Gong, Y. N., Lu, Q., Xu, H., Liu, L. and Shao, F. 2011. The NLRC4
- 618 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature,
- 619 477(7366), 596-600. doi: 10.1038/nature10510. PMID: 21918512.
- 620

Draft

Figure Legends

Fig. 1 The effect of R406 on ICAM-1 expression induced by *Pseudomonas aeruginosa* strain K (PAK WT) infection, or Fcγ receptor (FcγR) cross-linking. **a)** One hour-long pre-treatment of THP-1 cells with various concentrations of R406 decreased ICAM-1 expression in THP-1 cells infected with PAK at an MOI of 5 for 6 hours in a dose-dependent manner. **b)** Pre-treatment of THP-1 cells with 10 μM R406 for 1 hour prior to their stimulation with immobilized human IgG at concentrations of 10 and 100 μg/mL decreased up-regulation of ICAM-1. THP-1 cells were infected with PAK or stimulated via FcγR cross-linking as described in Materials & Methods, and ICAM-1 surface expression determined using immunostaining and flow cytometry analysis. Data are expressed as mean fluorescence intensity (MFI). Results represent the mean ± SEM of 3 independent experiments; ###P<0.001, difference between un-stimulated and stimulated cells; *P<0.05, **P<0.01, ***P<0.001, difference between stimulated R406 treated vs. un-treated cells. **c)** Ratios of Western blotting band intensity of Syk phosphorylated on tyrosine to total Syk. The lanes from left to right: un-stimulated THP-1 cells, THP-1 cells infected with PAK for 2 hours, THP-1 cells pretreated with 10 μM R406 followed by infection with PAK WT. The bands of Syk and phosphotyrosine on immunoprecipitated Syk were detected at 72 kDa. Results represent 3 independent experiments. *** P < 0.001, difference between R406-treated and un-treated infected THP-1 cells.

Fig. 2 The effect of R406 pre-treatment on cytokine expression induced by *Pseudomonas aeruginosa*.

Differentiated THP-1 cells were infected with *P. aeruginosa* for 1 hours at an MOI of 10 per 1×10^6 cells, and cultured for another 17 h in the presence of 100 μg/mL gentamicin. Unstimulated differentiated THP-1 cells (1×10^6) in complete culture medium served as a negative control. The supernatant was collected and IL-1β (A) and TNFα (B) concentrations (pg/mL) in culture

supernatants were examined using ELISA. For TNF α expression by H292 cells (C), the cells were infected at an MOI of 50 for 1 h and further cultured as described above. In samples involving R406, cells were pre-treated with 10 μ M R406 for 1 hour prior to infection. Results represent the mean $\pm$ SEM of 3 independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, difference between R406-treated and un-treated infected cells.

Fig. 3 The effect of R406 on expression of phosphorylated and total intracellular signaling proteins.

THP-1 cells were stimulated with *P. aeruginosa* strain K (PAK WT) at an MOI of 5 for 15, 30, or 60 minutes. Following stimulation, the levels of total and phosphorylated ERK2 (42 kDa), JNK (46 kDa), I κ B α (41 kDa), and p-38 (38 kDa) were determined in cellular lysates by Western blot. Results are expressed as ratios of phosphorylated/total protein band intensity. In samples involving R406, cells were pre-treated with 10 μ M R406 for 1 hour prior to infection. β -actin served as a loading control. Results represent the mean $\pm$ SEM of 2 independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, difference between R406-treated and un-treated infected cells.

1 **Table 1: Strains and Clinical Isolates of *P. aeruginosa* used in this study.**
2

Strain/Isolate	Source/Reference
Wild-type PAK (PAK WT)	R.J. Irvin/Pasloske et al. (1985)
Flagella-deficient PAK (PAK fliC)	A.S. Prince/Feldman et al. (1998)
Isolate from intermittently colonized CF patient (9793/92)	Danish CF Centre/Hawdon et al. (2010)
Isolate from chronically infected CF patient (19731A/92)	Danish CF Centre/Hawdon et al. (2010)

3

Draft

Fig. 1 The effect of R406 on ICAM-1 expression induced by *Pseudomonas aeruginosa* strain K (PAK WT) infection, or Fc γ R cross-linking. a) One hour-long pre-treatment of THP-1 cells with various concentrations of R406 decreased ICAM-1 expression in THP-1 cells infected with PAK at an MOI of 5 for 6 hours in a dose-dependent manner. b) Pre-treatment of THP-1 cells with 10 μM R406 for 1 hour prior to their stimulation with immobilized human IgG at concentrations of 10 and 100 $\mu\text{g/mL}$ decreased up-regulation of ICAM-1. THP-1 cells were infected with PAK or stimulated via Fc γ R cross-linking as described in Materials & Methods, and ICAM-1 surface expression determined using immunostaining and flow cytometry analysis.

Data are expressed as mean fluorescence intensity (MFI). Results represent the mean $\pm$ SEM of 3 independent experiments; ### P <0.001, difference between un-stimulated and stimulated cells; * P < 0.05, ** P <0.01, *** P <0.001, difference between stimulated R406 treated vs. un-treated cells. c) Ratios of Western blotting band intensity of Syk phosphorylated on tyrosine to total Syk. The lanes from left to right: un-stimulated THP-1 cells, THP-1 cells infected with PAK for 2 hours, THP-1 cells pretreated with 10 μM R406 followed by infection with PAK WT. The bands of Syk and phosphotyrosine on immunoprecipitated Syk were

detected at 72 kDa. Results represent 3 independent experiments. *** $P < 0.001$, difference between R406-treated and un-treated infected THP-1 cells.

232x305mm (300 x 300 DPI)

Draft

Fig. 2 The effect of R406 pre-treatment on cytokine expression induced by *Pseudomonas aeruginosa*. Differentiated THP-1 cells were infected with *P. aeruginosa* for 1 hours at an MOI of 10 per 1×10^6 cells, and cultured for another 17 h in the presence of 100 $\mu\text{g/mL}$ gentamicin. Unstimulated differentiated THP-1 cells (1×10^6) in complete culture medium served as a negative control. The supernatant was collected and IL-1 β (A) and TNF α (B) concentrations (pg/mL) in culture supernatants were examined using ELISA. For TNF α expression by H292 cells (C), the cells were infected at an MOI of 50 for 1 h and further cultured as described above. In samples involving R406, cells were pre-treated with 10 μM R406 for 1 hour prior to infection. Results represent the mean $\pm$ SEM of 3 independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, difference between R406-treated and un-treated infected cells.

231x351mm (300 x 300 DPI)

Draft