‘’ Investigation of Rho signalling in therapeutic targeting of neutrophil recruitment during lung inflammation’’

Research Project

for

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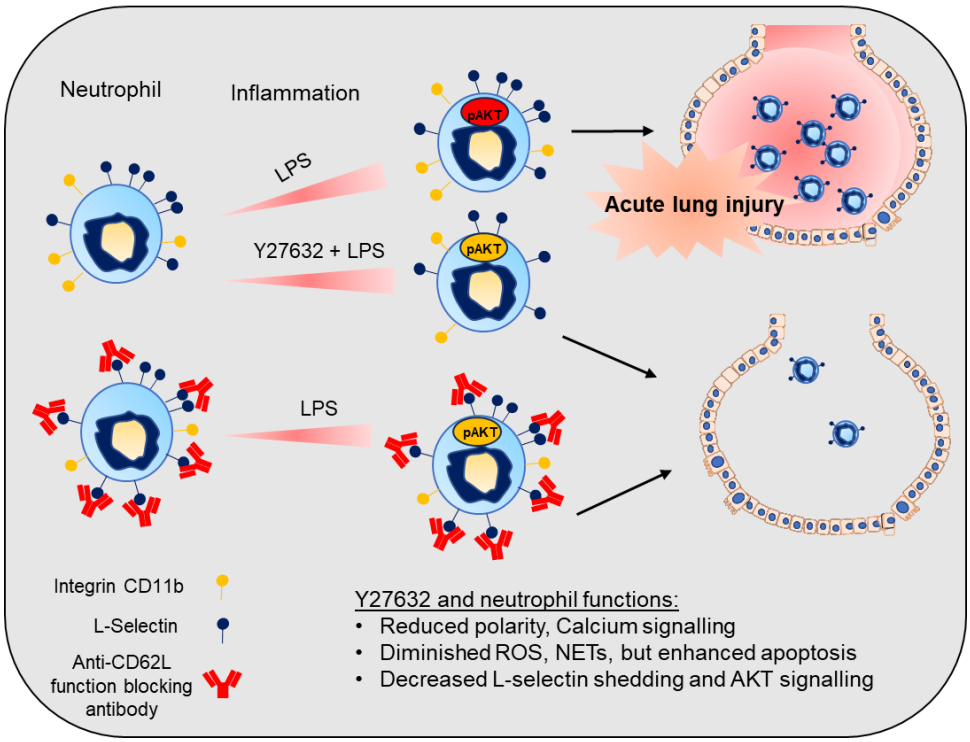
Highlights of the study:

Neutrophils are the key immune cells to infiltrate in large number to eliminate pathogens during infection. Excessive release of their intracellular toxic granular proteases, reactive oxygen species (ROS), Neutrophil extracellular traps (NETs) can lead to exuberated inflammation and tissue damage. Thus, targeting neutrophil recruitment remains an attractive strategy for treatment of multiple inflammatory diseases including acute lung injury, acute respiratory distress syndrome (ARDS) etc. Here, we identified:

- *Inhibition of Rho signalling via Y27632 reduces LPS induced lung inflammation via targeting neutrophil infiltration.*
- *Y27632 treated neutrophils exhibits reduced adhesion, migration, ROS production and NETosis to apoptotic switch under invitro condition.*
- *L-selectin/Akt signalling regulates neutrophil infiltration during LPS induced lung inflammation.*



1. Introduction:

Targeting of Neutrophils in pathological conditions including lung injury:

Neutrophils are the most abundant leukocytes that are first to be recruited at the site of the infection to eliminate the pathogenic insult. Infiltration of neutrophils at the site of infection is essential to promote pathogen elimination (Nemeth, Sperandio et al. 2020). Neutrophils performs multiple functions including *phagocytosis, degranulation, NETs release* in order to combat against the infection (Nathan 2006, Amulic, Cazalet et al. 2012). However, their overactivation and release of their intracellular toxic granular proteases, NETs, oxidants contribute to the *exuberated inflammation and tissue damage* during multiple inflammatory diseases including Cystic Obstructive Pulmonary Disease (COPD), sepsis, SARS-CoV2, ARDS. High levels of Neutrophil extracellular traps, citrullinated H3 histones, caprolectin are observed in COVID-19 patients and represent severity of COVID-19. Further, defective clearance and accumulation of NETs components including chromatin, Histones H3 and H4 could induce further activation of Coagulation factor XII leading to COVID-19-associated pulmonary thrombo-inflammation. ***Recombinant DNases or Pulmozyme*** degrades extracellular DNA reduces viscosity of sputum and improves lung function in patients with COPD. 7589382. Other strategies including inhibition of neutrophil recruitment via **CXCR2-blockade** using AZD5069 and neutrophil elastase inhibitor AZD9668 show positive outcomes in patients with asthma and lung diseases.
"Thus, neutrophil targeted strategies either by reducing neutrophil recruitment or their functional overactivation can be helpful in treatment of multiple inflammatory diseases"

Neutrophil migration is coordinated process and prerequisite for neutrophil recruitment at site of infection or inflammation. *Members of the Rho-GTPase family including Rho, Rac and Cdc42 are critical for migration mechanisms*. Deficiency or loss of function of Cdc42, Rac 1, Rac 2 leads to defects in the neutrophil migration (Filippi, Szczur et al. 2007, Kumar, Xu et al. 2012, Record, Malinova et al. 2015). *Rho A localizes towards uropod /back in migrating neutrophils and regulates myosin II regulatory light chain (p-MLC) mediated contraction at back* (Xu, Wang et al. 2003). Deficiency of its downstream effector myosin light-chain kinase (MYLK) leads to defective neutrophil adhesion and recruitment to inflamed tissue (Xu, Gao et al. 2008). While, Rho effector Rho-kinase (ROCK) deletion induces recruitment and chemotaxis of neutrophils to the site of infection (Vemula, Shi et al. 2010). Mitigation of Rho-kinase was also observed to protect lungs from acute injury in the Cecal ligation and puncture (CLP) model of sepsis (Cinel, Ark et al. 2012). In contrast, Jennings et al., revealed that Rho A suppresses premature neutrophil priming (Jennings, Strengert et al. 2014), as Rho-deficient neutrophils exhibited hyperactivity with enhanced random migration and exacerbated LPS-mediated lung injury (Jennings, Strengert et al. 2014) ***These studies suggest a context dependent and complex Rho signaling in neutrophil migration and functions.***

In this study, ***we explored cell-intrinsic functions of neutrophils and their modulation by mitigation of Rho signaling using LPS induced acute lung injury model and other approaches.*** We found Rho signaling inhibitor in animals to rescue intra-tracheal LPS induced alveolar wall thickening, hemorrhage and infiltration of neutrophils into inflamed tissue.

Adoptive transfer of neutrophils identified a specific defect in neutrophil migration capacity in cell-intrinsic manner. Further *in vitro* studies revealed altered actin dynamics, L-selectin levels and MAPK signaling in neutrophils upon Rho signaling mitigation. Induced selectin shedding caused defect in neutrophil polarization and infiltration of adoptively transferred neutrophils into inflamed lungs. Detailed single cell level analyses identified MAPK signaling as downstream effector of Rho signaling and selectin mediated effects.

2. Objectives of the study:

To examine the role of Rho signalling in neutrophil during LPS induced lung inflammation, following objectives were undertaken:

- *To establish role of Rho in neutrophils during LPS induced lung inflammation.*
- *To access effect of Rho signalling inhibition via Y27632 on neutrophil functions including chemotaxis, ROS production, apoptosis, NETs production.*
- *To identify molecular mechanism involved in regulating Rho signaling inhibition mediated mitigation of lung injury.*

3. Materials and methods:

Mice strain: C57BL/6 male mice, age 8-16 weeks, were utilized in the present study. All experimental procedures and animal protocols were approved and as per the guideline of the Institution animal ethical committee (IAEC).

LPS induced Lung inflammation: The mice were challenged with 40 µg LPS from *Escherichia coli O111*:*B4* intra-tracheally after ketamine and xylazine anesthesia. Y-27632 treatment (15mg/kg) was given intra-peritoneally 1 hour before LPS administration. Bronchoalveolar lavages (BALs) and lung histology were performed as previously described after 4h of LPS administration (Kumar, Xu et al. 2012).

Neutrophil isolation and adoptive transfer: Neutrophils were isolated from mouse bone marrow using Percoll density centrifugation as described previously (Kumar, Xu et al. 2012). Neutrophils were labelled with CellTracker Green; 2.5 µM; Invitrogen) and CellTracker orange; 5 µM; Invitrogen) or vice-versa, at 37°C for 10 min. These cells were then treated with vehicle or Y-27632 or 10 µg/ml CD62L blocking antibody (obtained from BioLegend) mixed in equal ratio and transferred to C57Bl/6 mice recipients through i.v. injection at the time of LPS lung challenge. Relative neutrophil migration to lungs was calculated as the ratio of Y-27632 or anti-CD62L treated relative to vehicle-treated neutrophils (Kumar, Xu et al. 2012).

Immunofluorescence: Neutrophils were stimulated with fMLP in HBSS containing 0.1% BSA, 1mM Ca2+, 1mM Mg2+, on fibrinogen-coated slides for 0–10 minutes at 37°C. The cells were fixed with 2% PFA, permeabilized with 0.1% Triton X-100 and stained for the intracellular protein or antibodies as previously described. Fluorescence images were captured using a Leica DMI6000 fluorescence microscope at 40×/1.3 NA objective using Leica software (Kumar, Xu et al. 2014) .

Adhesion assay: Neutrophils were treated with vehicle or Y-27632 and added to the coverslips coated with diverse ligands followed by stimulation with fMLP for 5 min at 37°C. The assay was stopped by immersing the coverslip in 4% PFA for 20 min. The non-adherent cells were removed by gently replacing the formaldehyde solution with HBSS. Adherent cells were counted using a Nikon Eclipse TS2 microscope with a 40× objective.

Superoxide generation: Neutrophils (2×10^5) were incubated with 10 µM L012 in HBSS buffer for 10 min and treated with inhibitors including Y-27632 (10µM), or CCG1243 (10µM). Cells were stimulated with fMLP 10 µM or PMA (20 nM). Chemiluminescence was measured at every 5 s for 20 min with fMLP and at every 20 s for 45 min with PMA using BMG LABTECH luminometer (Kumar, Xu et al. 2014).

NETs: Cell-free DNA in BAL supernatant obtained from mice challenged with LPS and Y-27632 was quantified using cell-impermeable dye Sytox green (Invitrogen). 100µl of sample was incubated with 2.5µM of SYTOX-Green for 10 minutes and fluorescence intensity was measured using BMG LABTECH luminometer.

F-Actin quantitation by flow cytometry: Total amount of F-actin per neutrophil was quantified using flow cytometry as previously described (Kumar, Xu et al. 2014). Neutrophils were stimulated with fMLP in HBSS containing 0.1% BSA, 1mM $CaCl_2$, 1mM $MgCl_2$ at 37°C and fixed, permeabilized with 0.1% Triton X-100, and stained with rhodamine-phalloidin (Invitrogen). 10,000 cells were acquired using a flow cytometer. Data are reported as arbitrary unit of mean cellular fluorescence (AU) with unstimulated cells arbitrarily assigned a value of 100%.

Preparation of whole cell lysates, DRM fraction and Western blotting: 2 million neutrophils treated with 10µM of Y-27632 or 10ug/ml anti-CD62L blocking antibody (BioLegend). After removal of non-adherent cells, adherent cells were lysed with lysis buffer for whole cell lysate, cytosolic and detergent resistant membrane (DRM) fraction to estimate expression of protein in various cellular domains. Cell lysates containing equal amounts of protein were separated by SDS-PAGE and probed for p-AKT, p-ERK, and pMLC (Cell Signaling Technology, Boston, MA) and actin (DSHB).

Measurement of intracellular pAKT, pERK and IL-1β levels by flow cytometry: Neutrophils (1×10^6) in suspension were stimulated with fMLP (10µM) in HBSS containing 0.1% BSA, 1mM Ca2+, 1mM Mg2+ for 5min and fixed with 2% PFA for 20 minutes at 37°C. Cells were pre-treated with 10µM of Y-27632 for 30 minutes and stimulated with fMLP. The cells were fixed and permeabilized (Thermo Fischer Scientific) for 10 minutes at room temperature and stained for intracellular proteins pAKT and pERK (from Cell Signalling Technology, Boston, MA). 10,000 cells were analyzed using flow cytometer.

Annexin V/PI staining: Neutrophils were cultured for 16h in RPMI media containing 2% FBS with or without inhibitors Y-27632 (10µM) in the presence of 10µg/ml LPS. Cells were stained with annexin V (BD) and PI for 30 min in annexin V binding buffer containing (0.01M HEPES, 0.14M NaCl and 2.5mM $CaCl_2$). 20,000 cells were acquired for analysis using flow cytometry.

Results:

- ***Objective 1:*** *Rho signaling in neutrophils during LPS induced lung inflammation.*

Diminished neutrophil infiltration in inflamed lungs targeted for Rho signaling

To examine the impact of Rho signaling inhibitor, Y-27632 on lung inflammation, we established LPS-induced acute lung injury model (Kumar, Xu et al. 2012). LPS treatment led to the increased lung inflammation and infiltration of immune cells **(Fig. 1A, B)** compared to the vehicle treated mice, as described previously (Kumar, Xu et al. 2012). Further this effect was mitigated by Y-27632 indicated by ***reduced damage in the architecture of the airway and infiltration of immune cells,*** **majorly neutrophils** in bronchoalveolar lavage (BAL) of LPS challenged mice **(Fig. 1A, B, C)**. These findings were further validated using flow cytometric analyses of neutrophils in blood and BALF **(Fig. 1D, E)**. Interestingly, neutrophils in the blood of mice treated with vehicle as well as Y27632 were increased to the similar levels suggesting no effect of Y-27632 treatment on LPS mediated systemic increase of neutrophils **(Fig. 1D)**. In contrast, the percent of neutrophils in BALF were significantly reduced in Y-27632 treated mice than the vehicle group challenged with LPS **(Fig. 1E)**. Together, these data reveal ***Rho signaling inhibitor mediated mitigation of neutrophil recruitment protective effect on the LPS induced lung injury***.

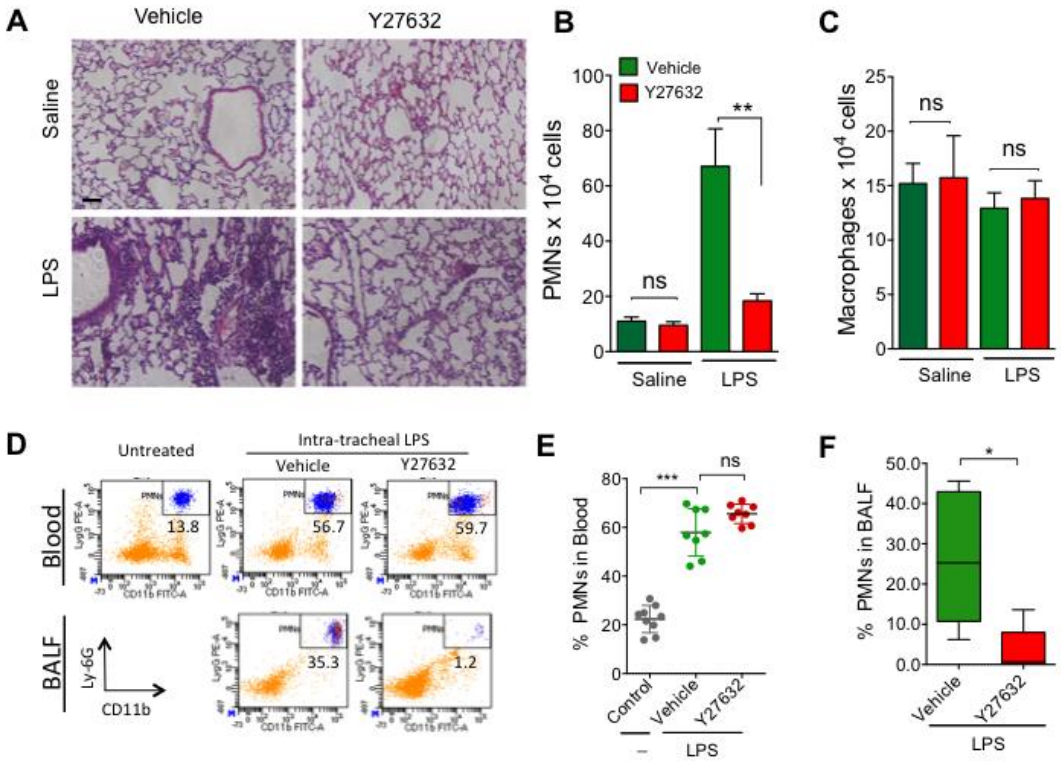


Figure 1. Effect of Rho signaling mitigation on LPS induced acute lung injury. (A) Lung histology images in vehicle or Y-27632 treated mice after LPS challenge in the lung. **(B) (C)** % Neutrophils and macrophages in the LPS

challenged BALF of vehicle and Y27632 treated mice **(D)** Flow cytometric analysis of neutrophils in the blood and BALF of LPS challenged mice. **(E)** Quantification of circulating neutrophils in blood in untreated control and LPS treated animals with vehicle and Y27632. **(F)** Percentage of neutrophils in BALF of vehicle and Y27632 treated mice post LPS challenge. **(*Singhal et al.*, BBA-MCR, 2021)**

Role of cell intrinsic Rho signaling inhibition in neutrophil infiltration to inflamed lungs

The above findings suggest a possible role of neutrophil infiltration in exuberating lung inflammation and injury. Further to identify any difference in localized lung-specific signals for diminished recruitment of neutrophils in mice treated with vehicle or Y-27632 we performed the adoptive transfer of vehicle or Y-27632 treated neutrophils, tagged with different dyes, into the same mice challenged with LPS intra-tracheally and analysed using flow cytometry **(Fig. 2A, B)**. Flow cytometry analyses of labeled neutrophils in the circulating blood confirmed the similar input of labeled neutrophils **(Fig. 2C, E,** left panels**)**. While labeled cells in the BALF (right panels) confirmed the reduced infiltration of Y-27632 treated neutrophils in BALF 4 hours after LPS challenge **(Fig. 2C, E)**. Futher, we found this effect to be independent of dye used for the labeling of vehicle or inhibitor-treated neutrophils **(Fig. 2C, E)** indicating ***a defective in vivo infiltrating ability of neutrophils targeted for Rho signaling inhibition.***

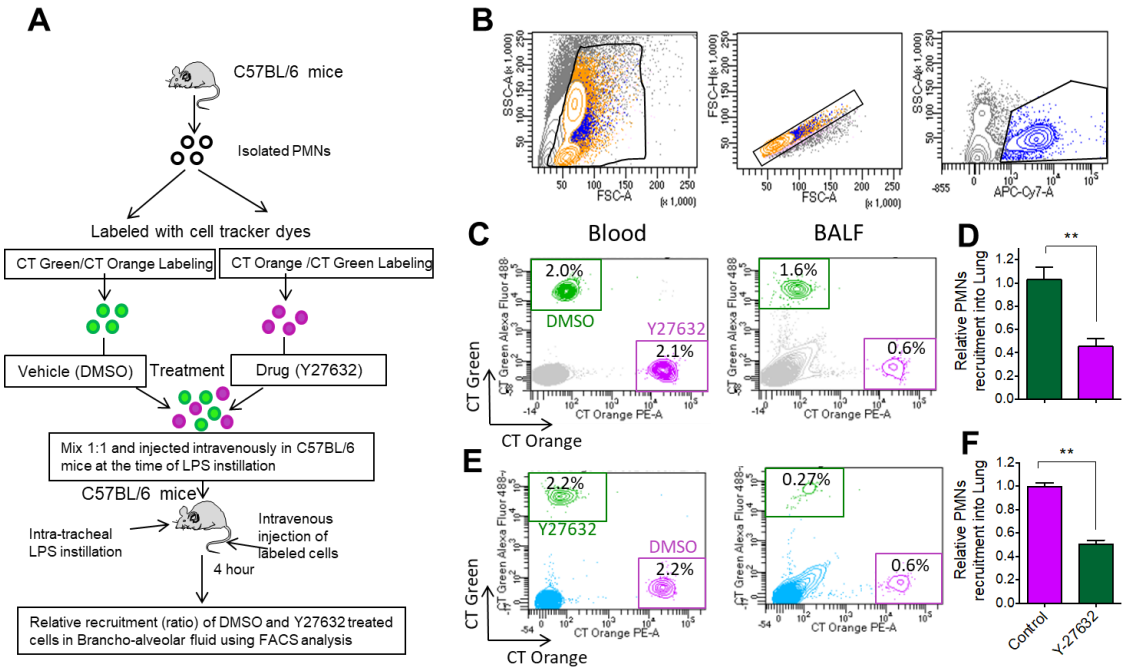


Figure 2. Adoptive transfer reveals specific effect of Y-27632 on neutrophil infiltration to inflamed lungs. **(A)** Experimental design of recruitment of adoptively transferred neutrophils in the lung. **(B)** Representative flow cytometry gating strategy to identify neutrophils in blood and BALF using Ly-6G antibody. **(C)** Flowcytometry density plot of labeled neutrophils in the blood (left panel), in the BALF (right panel). **(D)** Relative recruitment of neutrophils calculated as ratio of Y-27632 treated neutrophils (labeled with CMRA) relative to vehicle treated cells (labeled with CMFDA). **(E)** Density plot profile of labeled neutrophils vehicle-CMRA: Y-27632-CMFDA in the blood (left panel), in the BALF (right panel). **(F)** Relative recruitment of neutrophils calculated as ratio of Y-27632 treated neutrophils relative to vehicle (vehicle-CMRA: Y-27632-CMFDA). **(*Singhal et al.*, BBA-MCR, 2021)**

- ***Objective 2:*** *To access effect of Rho signalling inhibition via Y27632 on neutrophil functions including chemotaxis, ROS production, apoptosis, NETs production.*

Rho signaling at neutrophil uropod is essential for polarity

Neutrophils use chemotaxis towards diverse intruders and DAMPs to enable their recruitment to infected tissue. During this process, neutrophil undergoes cytoskeletal reorganization and acquire a polarized shape that is important for persistent migration towards chemo-attractants, and its failure leads in overall defect in neutrophil migration (Kumar, Xu et al. 2012). Based on *in-vivo* results, we investigated the effect of Rho signaling inhibition on neutrophil polarity. Western blot and immunofluorescence (IF) analyses confirmed the inhibition of pMLC, an effector of Rho signaling (Amano, Ito et al. 1996), by Y-27632 in fMLP stimulated neutrophils **(Fig. 3A)**. Further analysis of neutrophil polarity using F-actin distribution revealed multiple protrusions at the front and a long-elongated tail in Y-27632 treated cells **(Fig. 3B-D),** suggesting a loss of polarity after Rho signaling inhibition. Consistently we observed the similar results with another Rho signaling inhibitor, CCG-1423 **(Fig. 3C)**. In polarized cells, myosin contractile fibers get localized towards the uropod to facilitate neutrophil migration, in contrast, we observed defective p-MLC localization in Y-27632 and CCG-1423 treated cells **(Fig. 3E, F)**. Altogether, these data confirm the ***loss of neutrophil polarity after Rho signaling inhibition***.

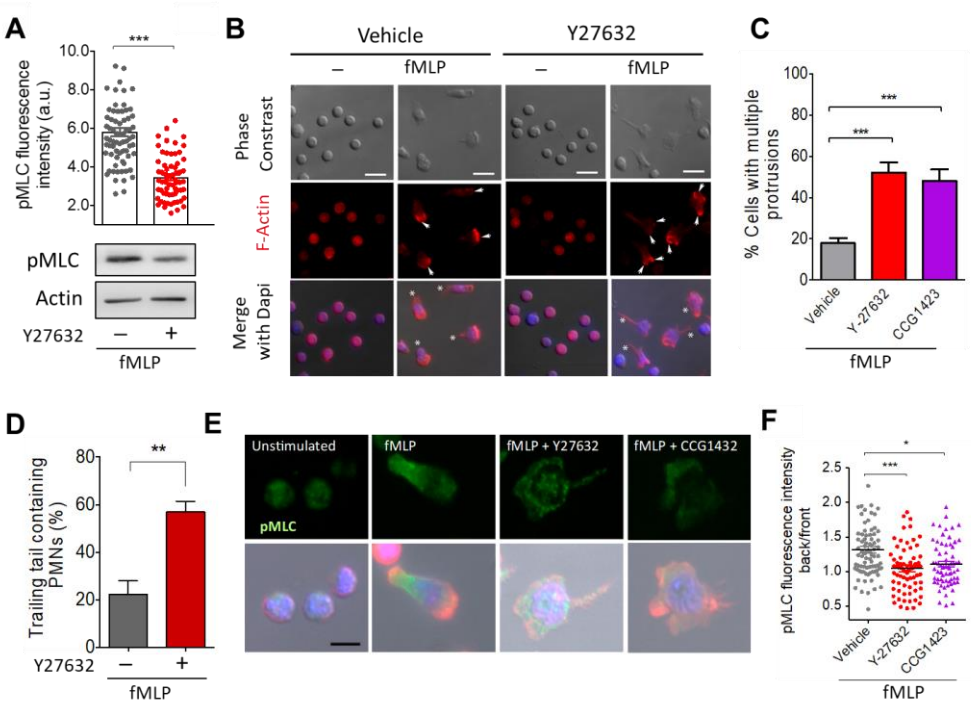


Figure 3. Effect of Rho signaling inhibition on neutrophil polarization and cytoskeletal reorganization (A) Immunofluorescence and Western blot analysis of myosin light chain phosphorylation in presence or absence of Y-27632 in neutrophils stimulated with fMLP. **(B)** Representative pictures depicting effect of Y-27632 treatment on morphology of neutrophil stimulated with fMLP on adherent condition. **(C)** % Neutrophils of fMLP stimulated showing multiple protrusions in presence of Y-27632 and CCG-1423. **(D)** % Neutrophils exhibiting >5µm long trailing tail in polarized conditions **(E)** Immunofluorescence analysis of p-MLC localization in fMLP stimulated neutrophils treated

with Y-27632 and CCG-1423. **(F)** Ratiometric analysis of pMLC intensity observed at the back vs front side of neutrophils, cells were divided into front and back half as region of interest. **(*Singhal et al.*, BBA-MCR, 2021)**

Diminished ROS and NETs generation, while increased apoptotic program in Rho targeted neutrophils

Neutrophils use diverse anti-microbial mechanisms, including ROS generation and NETs to eliminate pathogenic insults, however their uncontrolled activation can lead to tissue damage. We observed mitigation of Rho signaling led to significant inhibition of fMLP as well PMA induced ROS generation and formed fewer NETs in response to PMA and A23187 than vehicle-treated neutrophils **(Fig. 4 A, B)**. These *in vitro* results were further corroborated by a reduced cell-free DNA in the BALF of Y-27632 treated challenged with LPS *in vivo* **(Fig. 4C)**. In contrast to diminished NETosis, we observed an increase in spontaneous as well as LPS induced apoptosis in Y-27632 treated neutrophils **(Fig. 4D, E)**. NETs have been shown to exuberate tissue injuries, while apoptosis is a silent death program without inducing inflammatory responses (Savill and Fadok 2000). Together, these results suggest a possible ***switching of the cell death pathway from NETosis to apoptosis after targeting Rho signaling*****,** and may be contributing to decreased lung damage in Y-27632 treated mice.

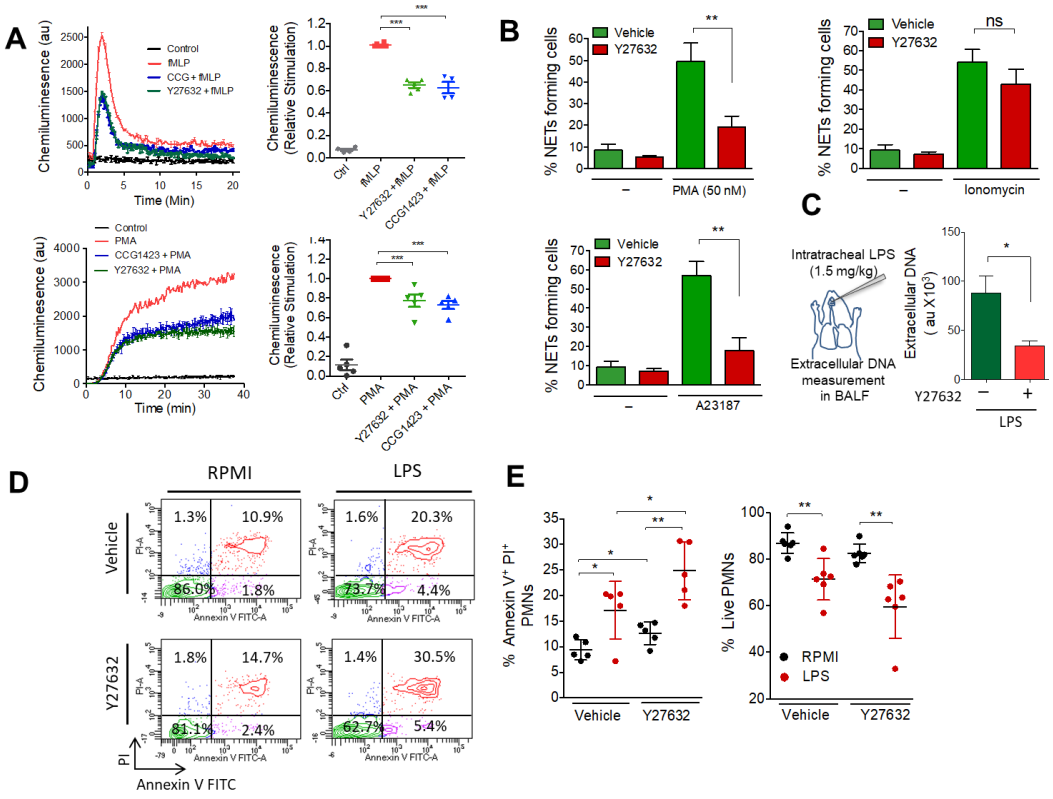


Figure 4. Abrogation of Rho signaling switches NETosis and apoptosis in neutrophils. (A) time kinetics of ROS generation in untreated or Y27632 and CCG1423 treated neutrophils with fMLP or PMA **(B)** Effect of Y-27632 on NETs forming neutrophils in response to PMA, Ionomycin and A23187 **(C)** Extracellular DNA was measured in BAL fluid of LPS challenged mice that were treated with vehicle or Y-27632. **(D)** Flow cytometric analysis of neutrophils apoptosis in the presence Rho signaling inhibitor. **(E)** Percentage of dead neutrophils identified as PI^{+}Annexin V^{+} was increased

while live cells (PI$^-$Annexin V$^-$ cells) were decreased in presence of Y-27632. **(*Singhal et al.*, BBA-MCR, 2021)**

- ***Objective 3:*** *To identify molecular mechanism involved in regulating Rho signaling inhibition mediated mitigation of lung injury.*

Rho signaling inhibition blocks neutrophil L-selection shedding

To further understand the Rho signaling inhibition mediated protective mechanism of neutrophil centric inflammation, we investigated the effect of Y27632 on neutrophils adhesion a phenomenon intricately linked to selectins and integrins in naïve and activated neutrophils. We observed decreased neutrophils adhesion after Y-27632 treatment with and without fMLP stimulation **(Fig. 5A)**. Further, we found modest change in integrin-CD11b, following Rho inhibition in fMLP stimulated PMNs **(Fig. 5B)**. Invivo, BALF neutrophil exhibited increased CD11b expression compared to circulating neutrophils but was not affected in vehicle and Y27632 treated mice after LPS challenge. **(Fig. 5C)**.

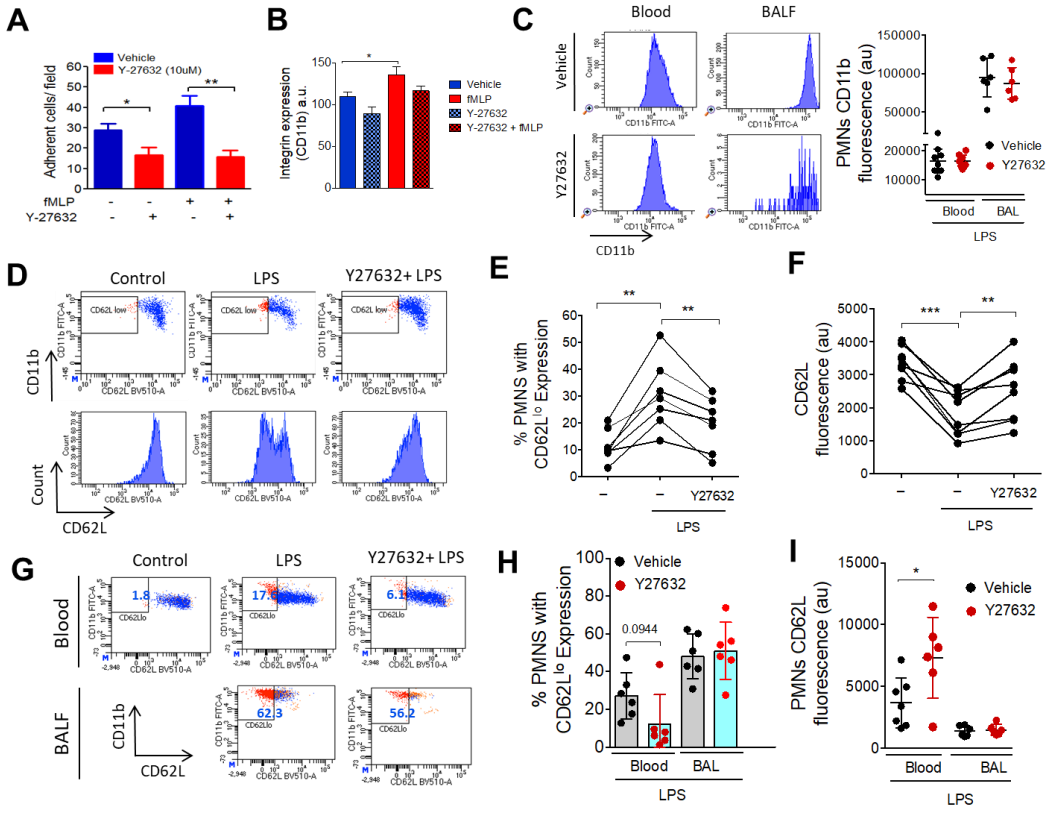


Figure 5. Stimuli dependent L-selectin shedding is blocked by Rho signaling inhibitor. (A) Effect of Y-27632 on fMLP induced adhesion of neutrophils **(B)** Expression of integrin CD11b using flow cytometry in unstimulated and fMLP stimulated neutrophils with or without Y27632 **(C)** Flow cytometric analyses of CD11b expression on blood and neutrophil infiltrated into BALF of LPS challenged animals treated with vehicle or Y-27632 **(D) (E)** Dot plot and histogram depicting percentage of CD62L^{lo} cells and fluorescence intensity of L-selectin on neutrophils with or without Y27632. **(G)** In vivo effect of Y27632 on % CD62L^{lo}CD11b^{hi} cells in blood and BALF **(H) %** Neutrophils exhibiting low level of CD62L in blood and BALF of mice challenged with LPS. **(I)** CD62L fluorescence intensity in LPS and Y-27632 treated mice blood and BALF. **(*Singhal et al.*, BBA-MCR, 2021)**

Next, we investigated the effect of Y-27632 on L-selectin (CD62L) levels present on the neutrophils. LPS, a known shedding agent, led to significant L-selectin shedding on neutrophils upon ex-vivo treatment to whole blood (**Fig. 5D** upper panel), which was blocked with Y-27632 treatment (**Fig. 5D, E)**. Consistently, ***LPS dependent decrease in CD62L intensity was also rescued by Rho signaling blocker*** **(Fig. 5D** lower panel**, F)**. These results were further confirmed *in-vivo*, indeed LPS challenged mice treated with Y-27632 showed less percentage of blood neutrophils with low CD62L expression in contrast to the vehicle group (**Fig. 5G-I)**.

L-Selectin signaling regulates neutrophil infiltration and polarization via AKT signaling

To understand the role of L-selectin signaling in neutrophil polarization and infiltration, we treated neutrophils with a functional CD62L blocking antibody and evaluated neutrophil migration to inflamed lung tissue. Surprisingly, functional blocking of L-selectin led to a significant decreased neutrophils infiltration in BALF, while no difference was observed in circulation, ruling out the possibility of differential survival of neutrophils after blocking of L-selectin signaling (**Fig. 6A, B)**. To further understand the differential responses of Rho inhibition and functional blocking of selectin, we looked into MAPK signaling under these settings.

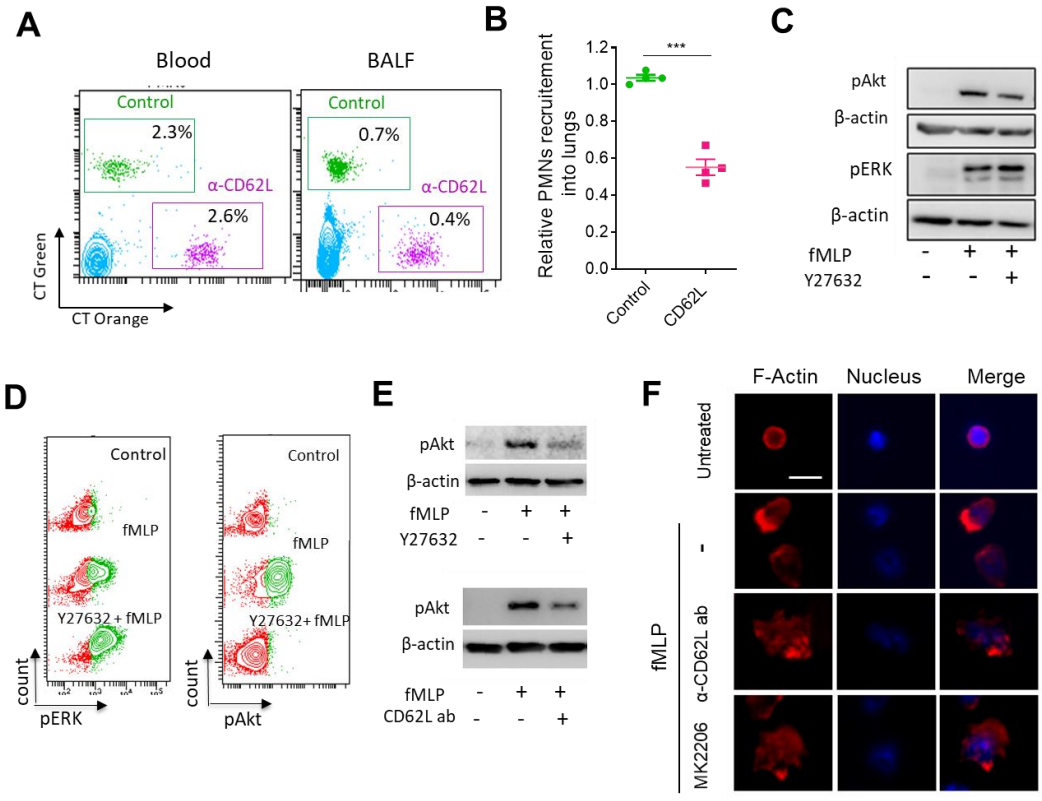


Figure 6: L-Selectin signaling and neutrophil infiltration into the lungs, effect on AKT activation and neutrophil polarization (A, B) Flow cytometric analysis and quantification of relative recruitment of adoptive transferred labelled neutrophils treated with anti-CD62L blocking antibody in blood and lungs. **(C)** Western analysis for AKT and ERK phosphorylation in fMLP stimulated and unstimulated neutrophils in the presence or absence of Y-27632 **(D)** Density plot showing % PMNs expressing high levels of pAKT and pERK in the presence or absence of Y-27632. **(E)** Western analysis for AKT phosphorylation in DRMs of fMLP stimulated and unstimulated neutrophils in Y-27632 and anti-

CD62L antibody **(F)** Immunofluorescence analysis of f-actin polarization in fMLP stimulated and unstimulated neutrophils after functional blocking of L-selectin and pAKT inhibitor, MK2206. **(*Singhal et al.,* BBA-MCR, 2021)**

Importantly, AKT signaling plays a crucial role in directed migration and cellular invasion activity including during neutrophil trans-endothelial migration (Chin and Toker 2009, Kumar, Xu et al. 2014). While extracellular signal-regulated kinase (ERK) activity regulates the protrusive activity and overall motility (Szczur, Xu et al. 2006). Interestingly, ERK activity was induced post-stimulation that was further increased significantly in Rho inhibited cells (**Fig. 6C, D)**. While, fMLP induced AKT activity was significantly ameliorated in neutrophils post Y-27632 treatment (**Fig. 6C, D)**. Based on the role of AKT signaling on neutrophils invasion activity, we next investigated the effect of anti-CD62L antibody on AKT activity in detergent-resistant membrane domains (DRMs), that functions as signalosome underneath the plasma membrane. We observed decreased pAKT levels in DRM of αCD62L antibody and Y-27632 treated neutrophils stimulated with fMLP (**Fig. 6E).** Altogether, these studies suggested the role of ***AKT signaling as a decisive mechanism in neutrophil activation and tissue infiltration in vivo*****.** These premises were further confirmed with defective polarization of neutrophils in presence of AKT inhibitor, MK-2206 as well as αCD6L antibody treatment (**Fig. 6F)**.

5. Statistics

All the experiments were performed at least three times. An unpaired Student's *t* test (normally distributed) was performed as statistics using Prism 5 software (GraphPad) for comparison of experimental groups unless specified. For survival curve statistics analysis, Log-rank test was used. Data are mean ± SD. The p-value of *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ were considered as significant.

6. Discussion

In this study, we demonstrated neutrophil-centric mechanistic regulation of Rho signaling mitigation during lung injury and inflammation. Using diverse *in-vitro* and *in-vivo* approaches, we observed a protective effect of Rho signaling inhibitor**,** Y-27632 during LPS induced lung inflammation. Adoptive transfer of labeled neutrophils identified a ***specific defect in migration capacity of Y-27632 treated neutrophil in a cell-intrinsic manner***. Rho family members are shown to regulate cytoskeleton, adhesion and detachment dynamics during neutrophil and macrophage migration (Alblas, Ulfman et al. 2001, Konigs, Jennings et al. 2014), Rho signaling inhibitor modulated actin dynamics, cytoskeletal reorganization leading to the loss of polarity and defective neutrophil migration as previously witnessed in the case of RhoA/B double KO neutrophils (Jennings, Strengert et al. 2014).

We observed ***mitigation of ROS, NETosis to apoptotic switching in Rho inhibited neutrophils***. A recent study advocated Y-27632 induced apoptosis as a major cause for reduced accumulation of neutrophils at the site of inflammation (Galvao, Athayde et al. 2019). In addition, mitigation of Rho signaling using Y-27632 has been suggested to enhance efferocytosis of apoptotic cells (Tosello-Trampont, Nakada-Tsukui et al. 2003)**.** ***Mechanistically, Y27632***

mediated protection from LPS induced lung inflammation was observed to be regulated via L-selectin/MAPK signalling.

7. Impact of the research in the advancement of knowledge or benefit to mankind

As Increased neutrophil infiltration and NETs formation has been related to multiple inflammatory diseases including- *Systemic lupus Erythromatosus, gout, COVID 19, sepsis, atherosclerosis, rheumatoid arthritis, COPD, ARDS, pneumonia, Cystic fibrosis, asthma, Transfusion-related acute lung injury* etc. (Porto and Stein 2016). Thus, targeting neutrophil functions and recruitment seems rationale in multiple pathophysiological disease (Hidalgo, Libby et al. 2022).

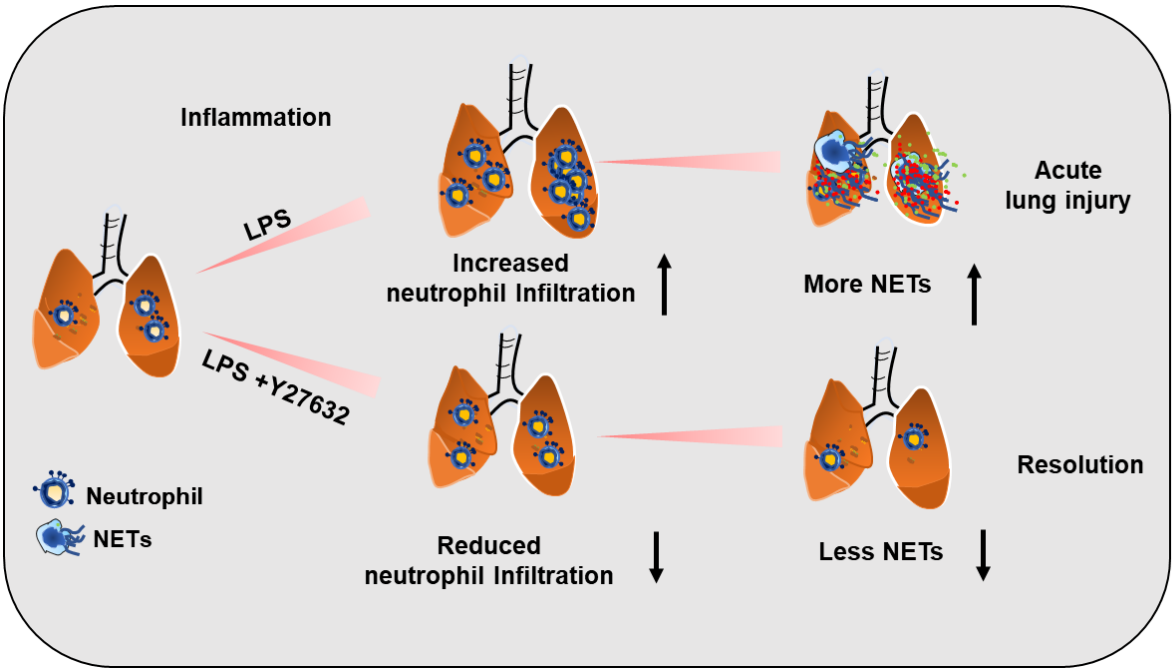
Acute lung injury (ALI) is still a major clinical challenge and new pharmacological therapies are required. Acute lung injury can progress in to acute respiratory distress syndrome which is more severe associated with massive pulmonary edema, diffuse alveolar damage (DAD) and respiratory failure. Therefore, targeting at early stage before progression to more severe form – ARDS might be an appropriate strategy. In our study *we* identified Rho A inhibition to significantly *reduce neutrophil infiltration and associated tissue damage in LPS induced acute lung injury model*. (Early stage) We observed reduced ROS generation, NETs formation in *Rho inhibited* neutrophils that might be helpful in prevention of NETs associated pathologies.

Though Rho small GTPases are well described to regulate cytoskeletal dependent functions. Pharmacological inhibitors of these lack nM range efficacy in cellular systems and this highlights the need of novel molecules or derivatives with higher sensitivity. *This work further establishes the therapeutic efficacy for targeting Rho signaling to mitigate neutrophil infiltration during lung injury, in future synthesis and derivatization of new chemical entities/ molecules to better target Rho signaling may provide better therapeutic options for various neutrophil mediated inflammatory diseases.*

Moreover, targeting downstream pathways may also provide better druggable targets. Here, we demonstrated that Rho signaling inhibitor reduces the AKT activation thus possibly altering neutrophil migratory phenotype through AKT signaling causing less neutrophil infiltration into inflamed tissue. Interestingly, ARQ 092, an orally-active AKT inhibitor, a phase Ib clinical trial anti-cancer drug inhibits neutrophil adhesion and interaction with platelets in sickle cell disease patients (Kim, Li et al. 2017). This suggests that targeting of Rho or downstream signaling may provide therapeutic approaches in diverse diseases associated with exuberated infiltration of activated neutrophils.

For more details, please see-

Singhal A, Dhankani P, Mazumder J, Adithya R, Dikshit M, **Kumar S.** Rho signaling inhibition mitigates lung injury via targeting neutrophil recruitment and selectin-AKT signaling. **Biochimica et Biophysica Acta (BBA)-Molecular Cell Research** 2021 Nov;1868(12):119122.



Rho inhibition mediated mitigation of neutrophil mediated Lung inflammation

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