Elsevier Editorial System(tm) for The International Journal of Biochemistry & Cell Biology
Manuscript Draft

Manuscript Number: BC-D-16-00018R2

Title: TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

Article Type: Research paper

Keywords: corticosteroid insensitivity, TLR2, MKP-1, PP2A

Corresponding Author: Prof. Alaina Ammit,

Corresponding Author's Institution: University of Sydney

First Author: Alaina Ammit

Order of Authors: Alaina Ammit; Md. Mostafizur Rahman; Pavan Prabhala; Nowshin Rumzhum; Brijeshkumar Patel; Thomas Wickop; Philip Hansbro; Nicole Verrills

Manuscript Region of Origin: AUSTRALIA

Covering Letter to Editor

UNIVERSITY OF TECHNOLOGY SYDNEY

21st July 2016

Professor of Respiratory Pharmacology
Faculty of Science
School of Life Sciences
City Campus
Building 4, Level 7, Room 322
PO Box 123 Broadway
NSW 2007 Australia
T: +61 2 9514 8403
M: +61 0400 351307
Alaina.Ammit @uts.edu.au
www.uts.edu.au

UTS CRICOS PROVIDER CODE 00099F

Professor Geoff Laurent, Editor-in-Chief
International Journal of Biochemistry and Cell Biology

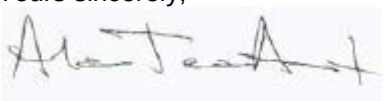
Dear Professor Laurent,

RE: BC-D-16-00018R1

Thank you for the detailed review of our manuscript. We are grateful for your clear guidance and we are glad that Reviewers' #1-#3 thought that we had made significant improvements in our revised manuscript. We also pleased that we have addressed the main concerns of Reviewer #4 and confirm that the Western blot data shown in Figures 6 and 7 are derived from the same blot. We have addressed the final request raised by Reviewer #4 in the attached *Response to Reviewers' Comments* and provided original blots.

We are hopeful that our manuscript is now acceptable for publication in the *International Journal of Biochemistry and Cell Biology.* Please do not hesitate to contact me if you require further information or clarification for our manuscript.

Yours sincerely,



Alaina J. Ammit Ph.D.

MANUSCRIPT NO. BC-D-16-00018R1
RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #1
No comment

RESPONSE TO REVIEWER #2
Comment #1: The authors have corrected the mistakes and performed additional experiments to answer the questions, so my suggestion is that the manuscript can be considered for publication now.
Response #1: Thank you.

RESPONSE TO REVIEWER #3
Comment #1: The authors have made significant improvement in scientific and technical content of the manuscript. The manuscript is now in acceptable format.
Response #1: Thank you.

RESPONSE TO REVIEWER #4
Comment #1: The authors have addressed most of my main concerns and the additional experiments have improved the manuscript. I would like to see, however, whether the Western blot data from Figs 6 and 7 are derived from the same blot and would thus like to ask for the original blot data of the three replicates. Quantification of expression levels is only possible when samples are run on the same gel and have been processed and exposed in a similar manner. As such, depicting quantitative Western blots as in Figs 6 and 7 is irritating and not recommended.
Response #1: I can confirm that the Western blot data from the new n=3 experiments performed for the Figures 6 and 7 in the revised manuscript are derived from the same blot. We agree with the reviewer that quantification of expression levels is only possible when samples are run on the same gel and have been processed and exposed in a similar manner (our standard practice). In accordance with the reviewer's request I have attached the original blot data – see attached.

No. BC-D-16-00018R1

TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

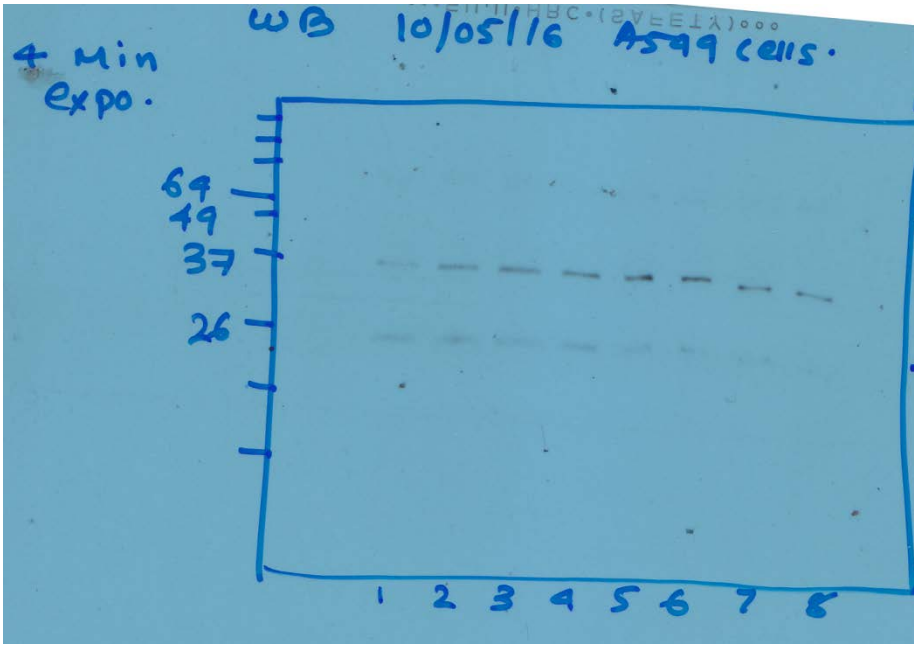
The International Journal of Biochemistry & Cell Biology

Original blots used for the three experimental replicates used for Figure 6

Representative blots are from Experiment 1

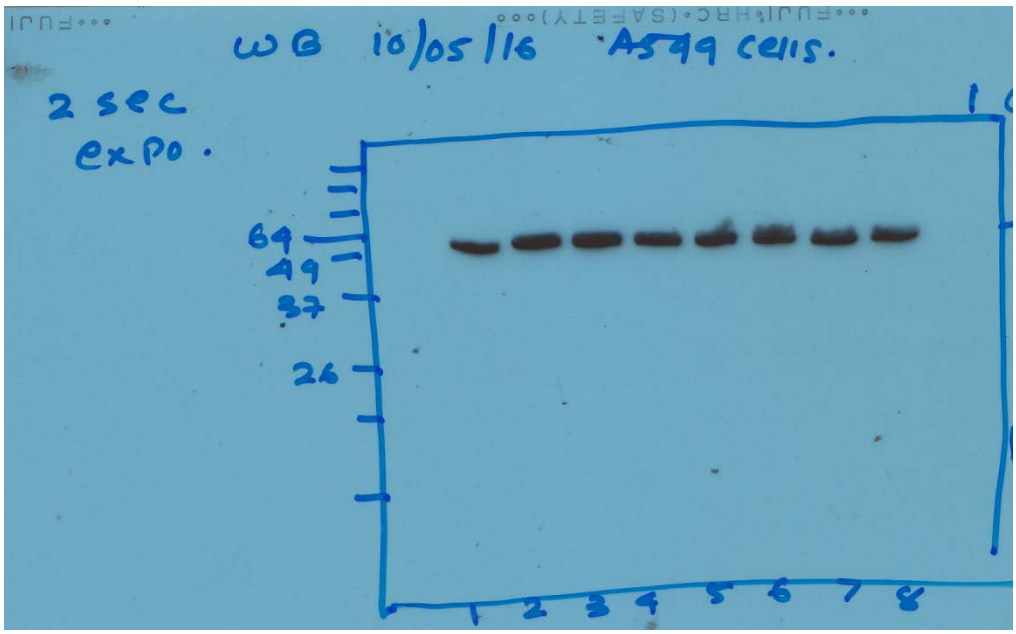
Data Used: Lanes 1 (vehicle), 4 (Pam3CSK4) and 7 (H_20_2)

HO-1



←HO-1

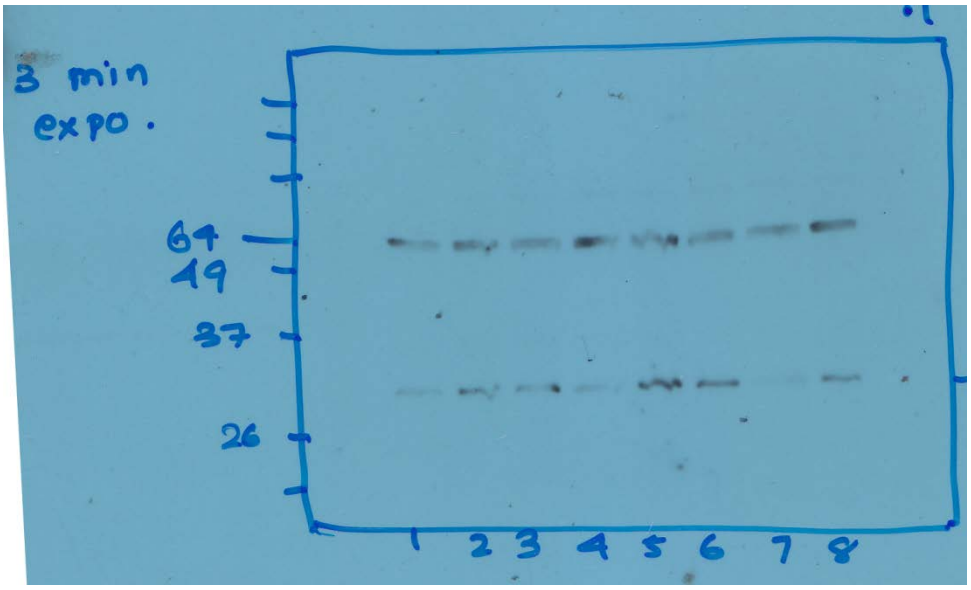
Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

Experiment 1

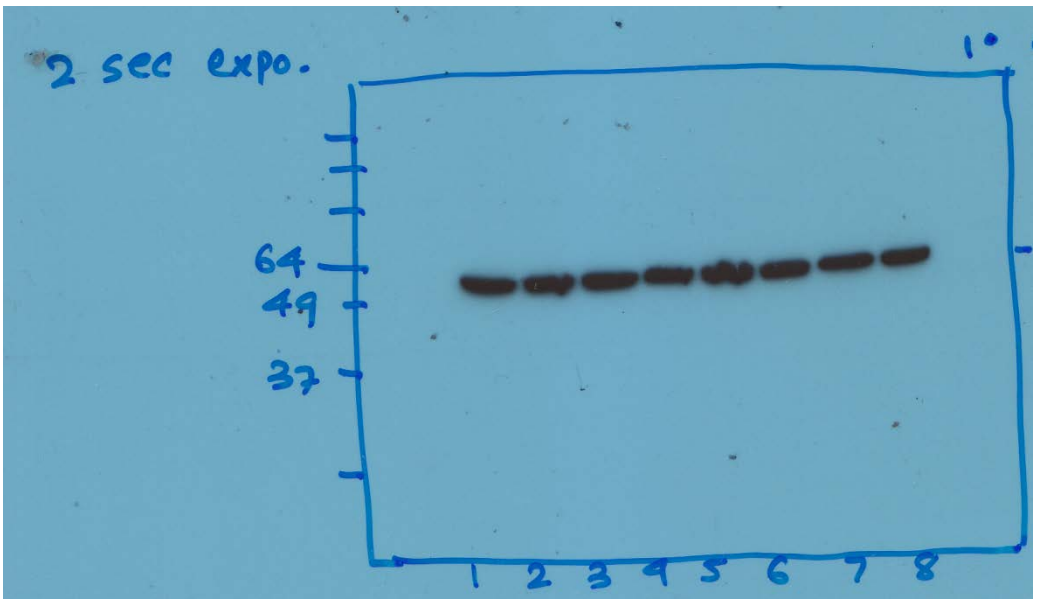
HO-1



←HO-1

Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

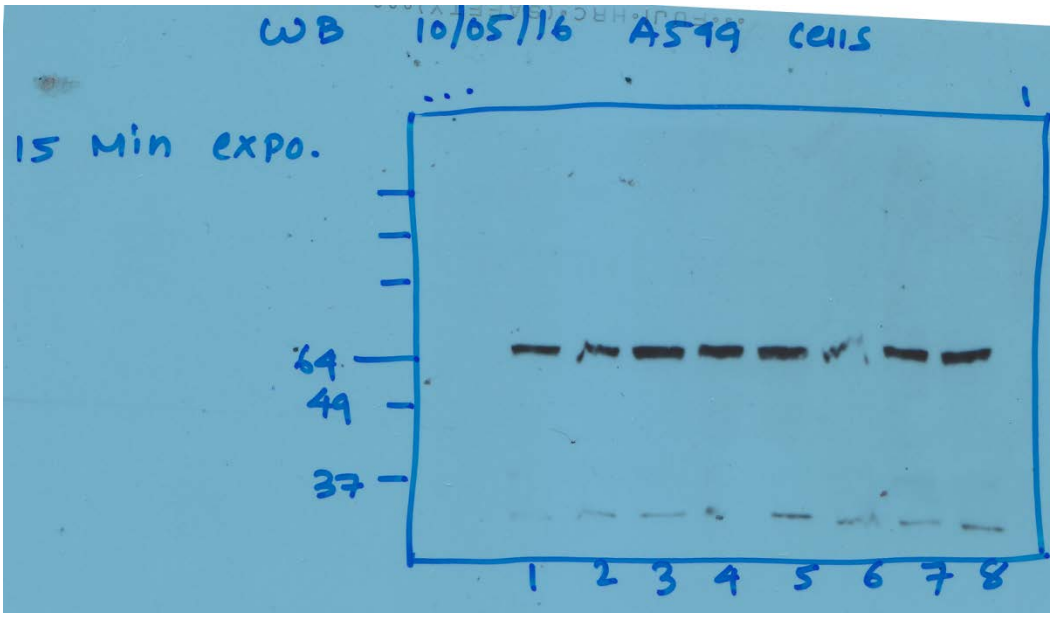
tubulin



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

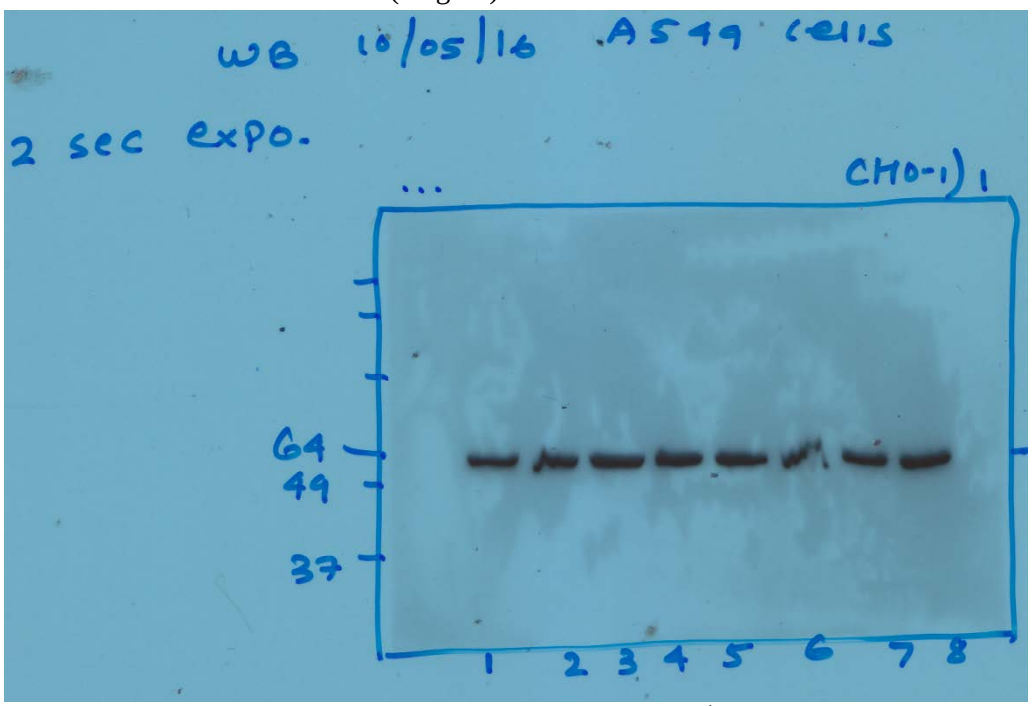
Experiment 2

HO-1



←HO-1

Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

Experiment 3

No. BC-D-16-00018R1

TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

The International Journal of Biochemistry & Cell Biology

Original blots used for the three experimental replicates used for Figure 7C

Representative blots are from…

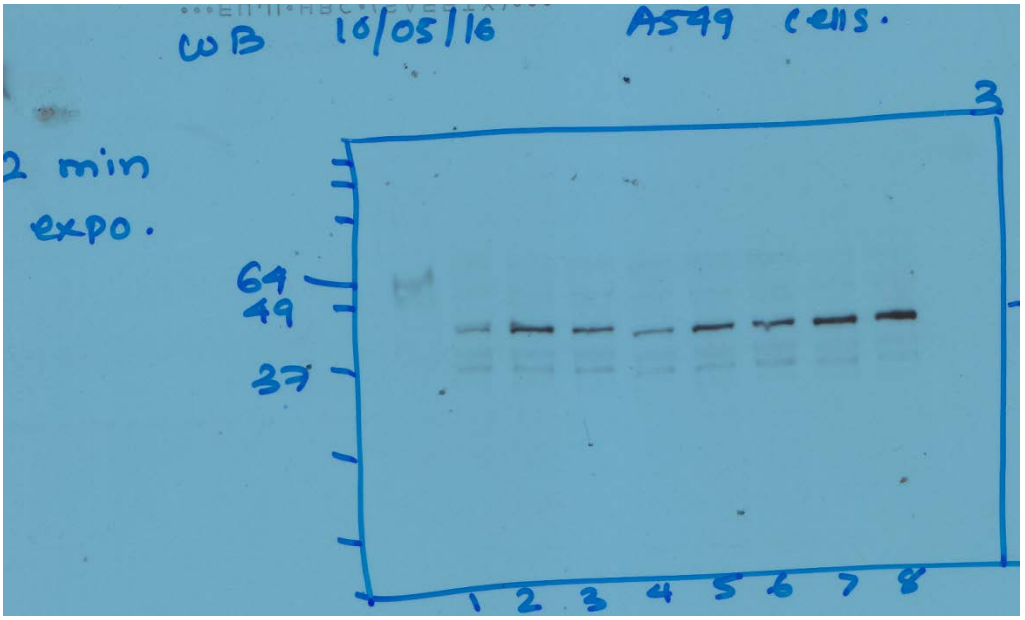
phospho-p38 MAPK/total p38 MAPK - Experiment 1

Phospho-ERK/total ERK – Experiment 2

phospho-JNK/total JNK – Experiment 1

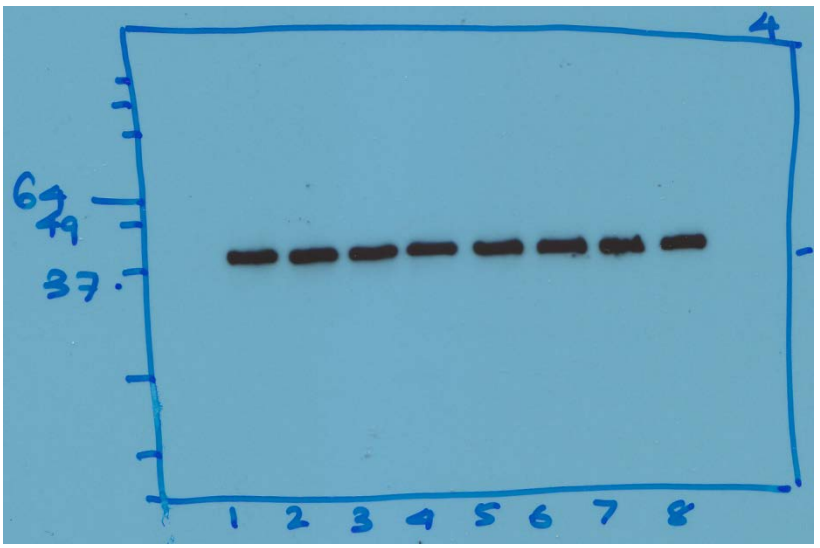
Data Used: Lanes 3 (Dex + TNFα) and 6 (Pam3CSK4 + Dex + TNFα)

phospho-p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

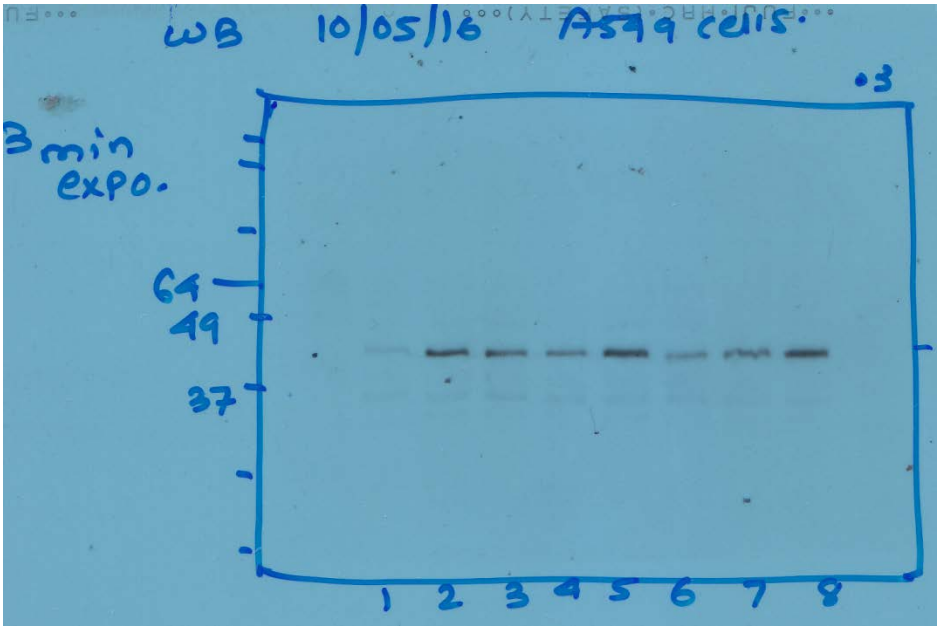
total p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

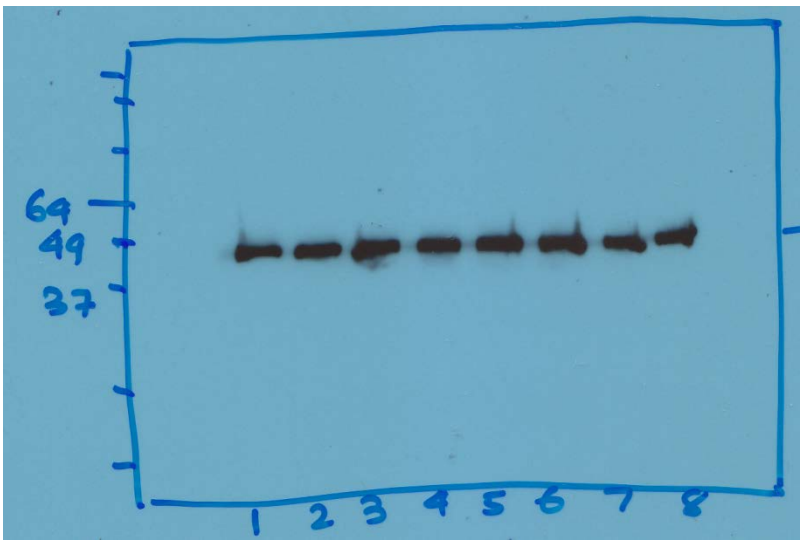
Experiment 1

phospho-p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

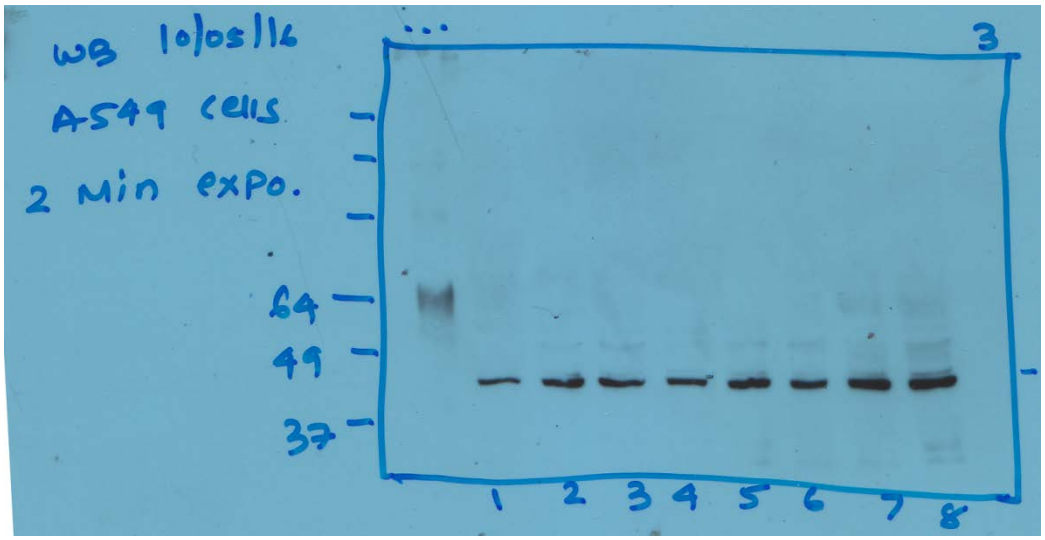
total p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

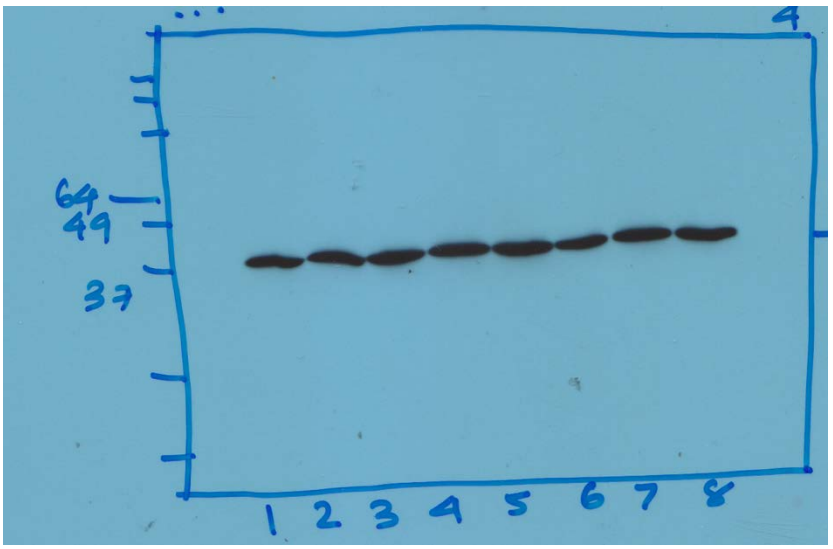
Experiment 2

phospho-p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

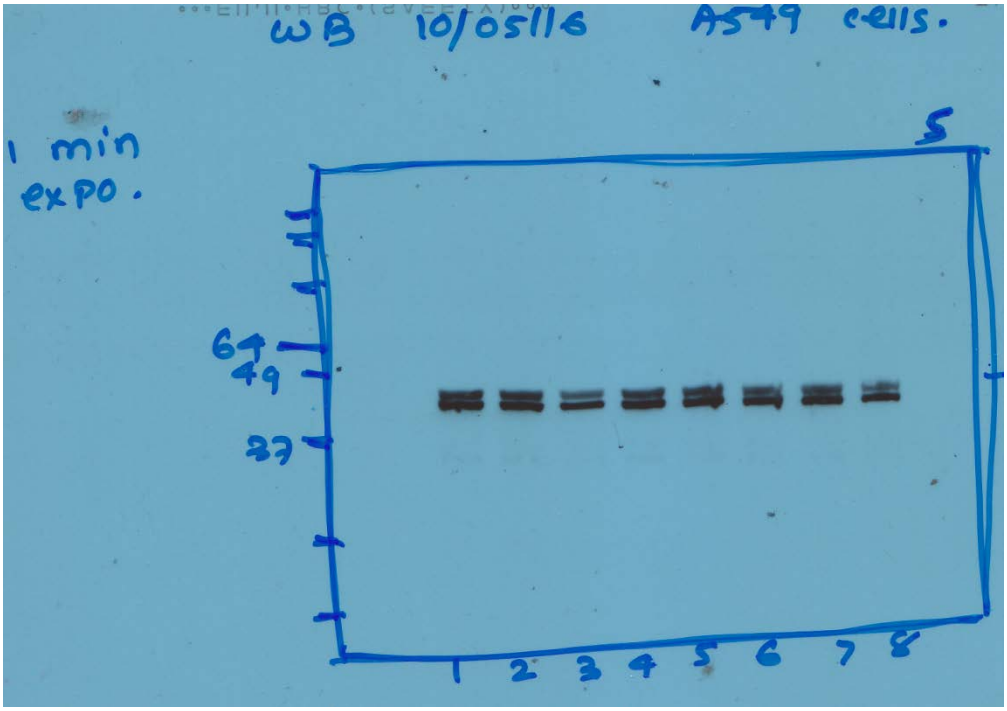
total p38 MAPK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

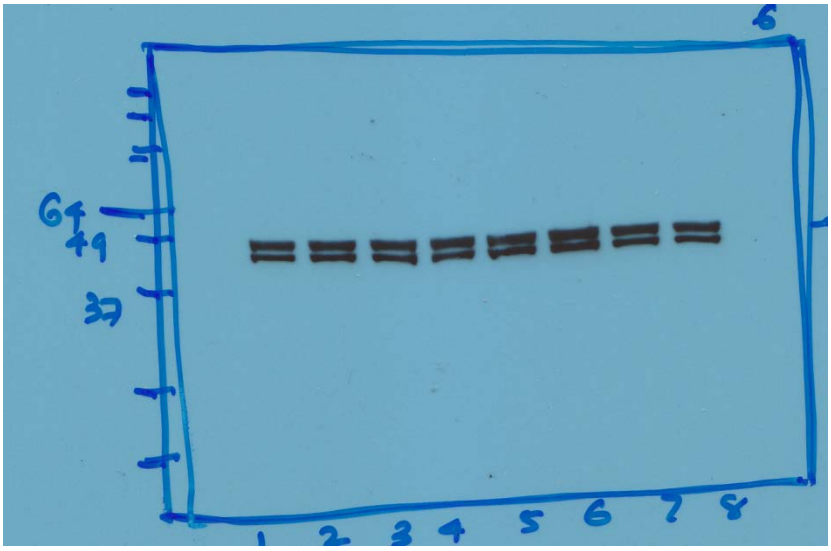
Experiment 3

phospho-ERK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

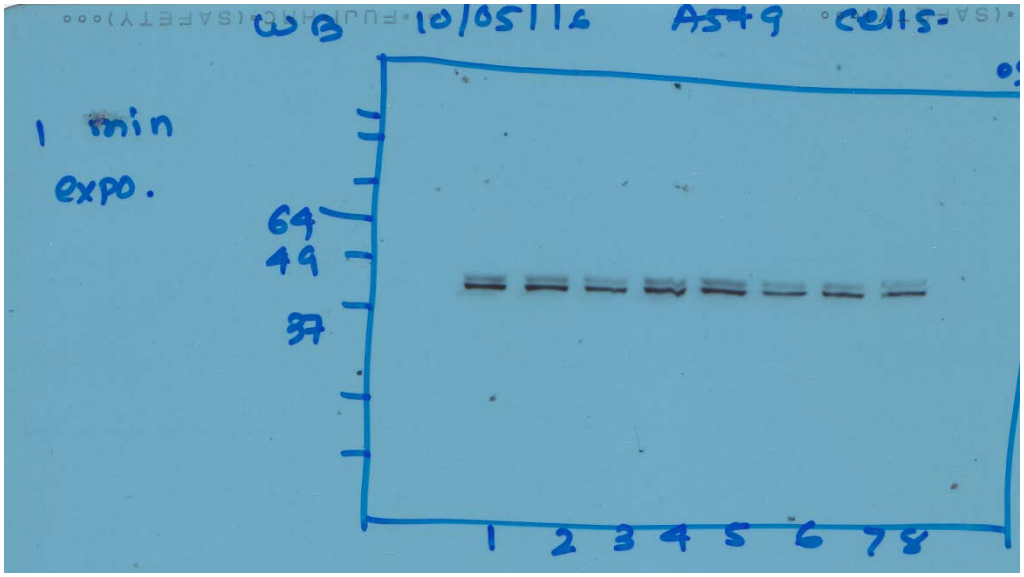
total ERK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

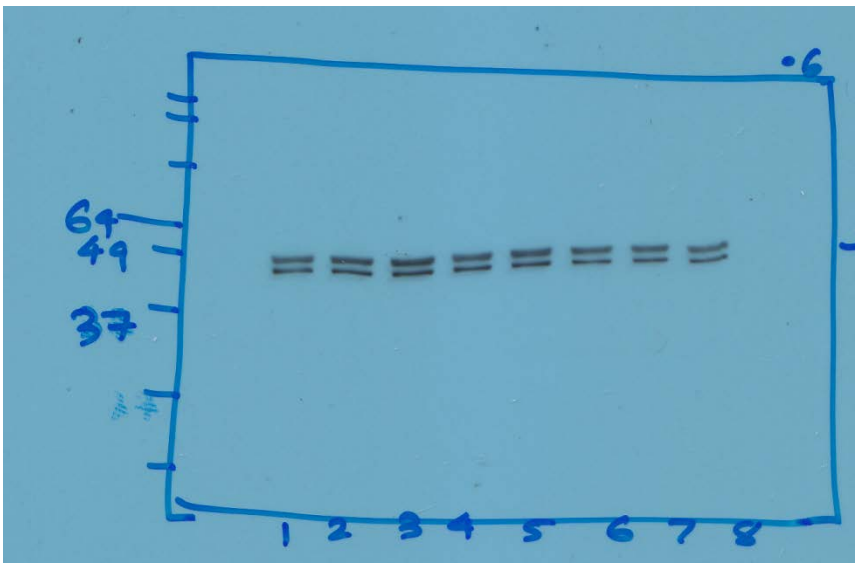
Experiment 1

phospho-ERK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

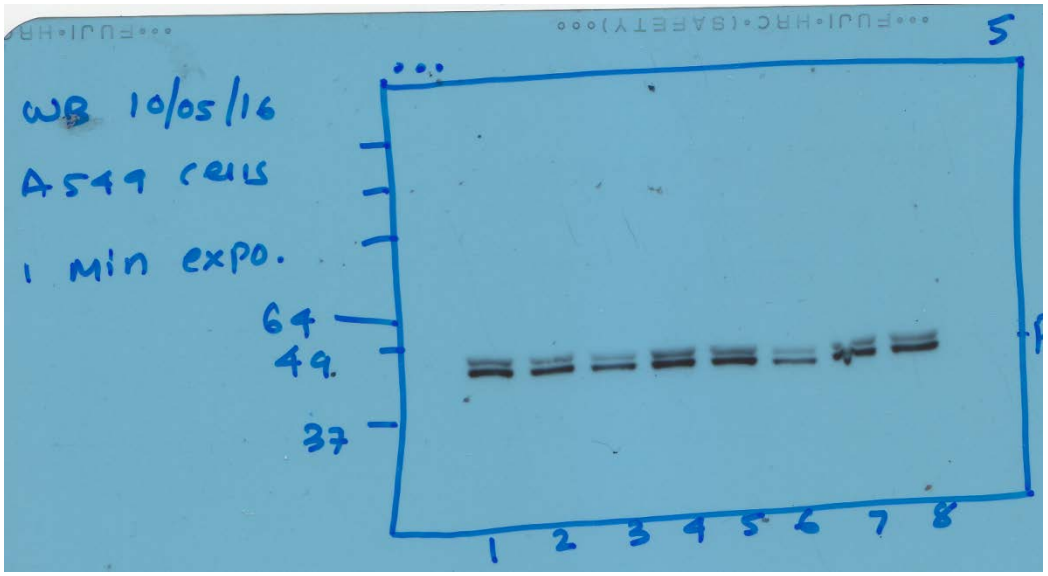
total ERK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

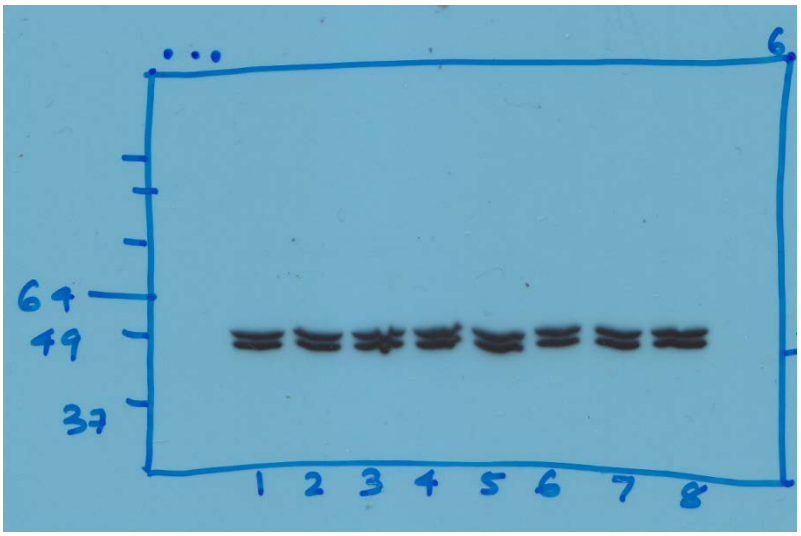
Experiment 2

phospho-ERK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

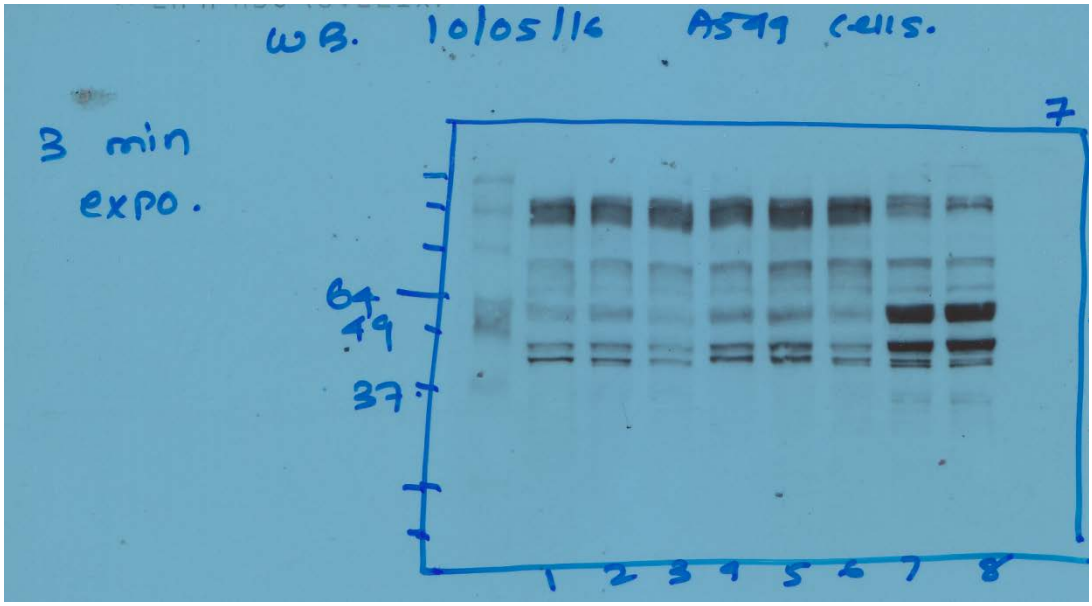
total ERK



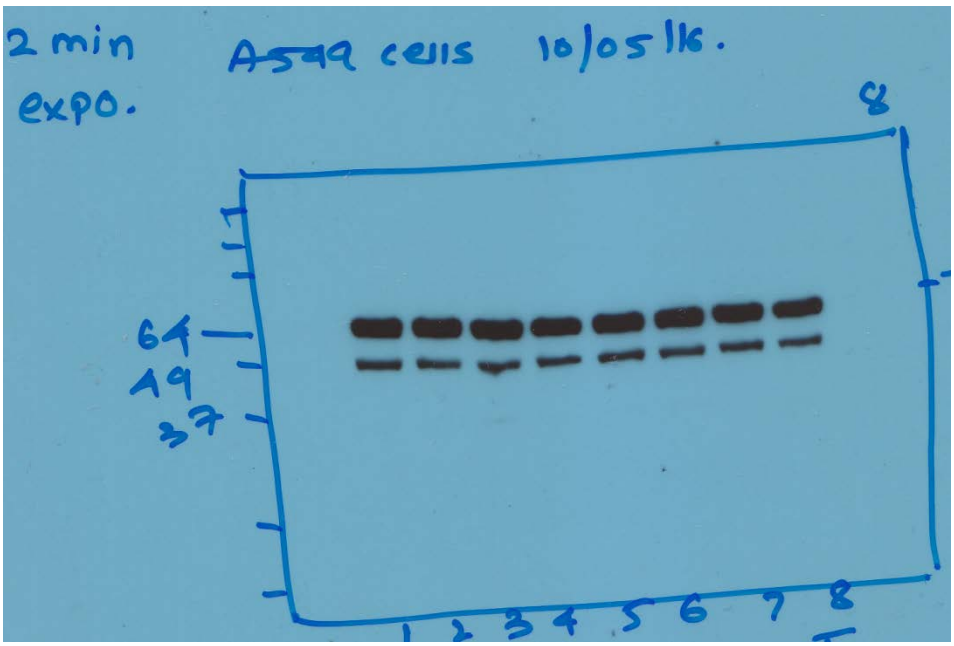
Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

Experiment 3

phospho-JNK



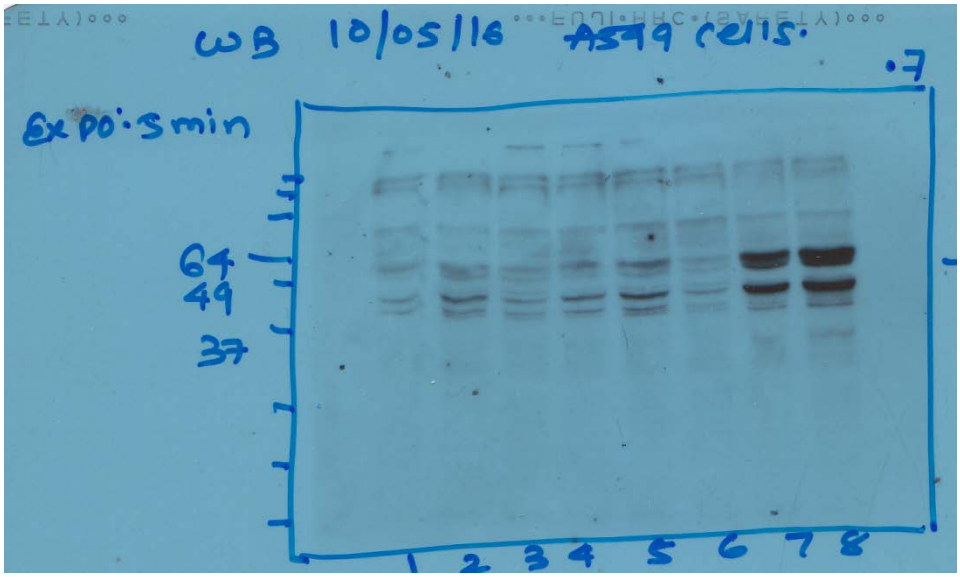
Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

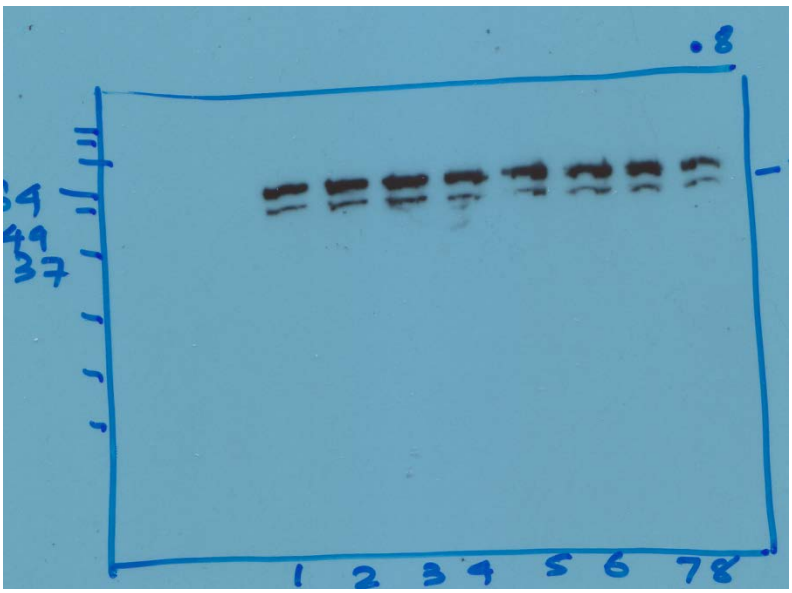
Experiment 1

phospho-JNK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

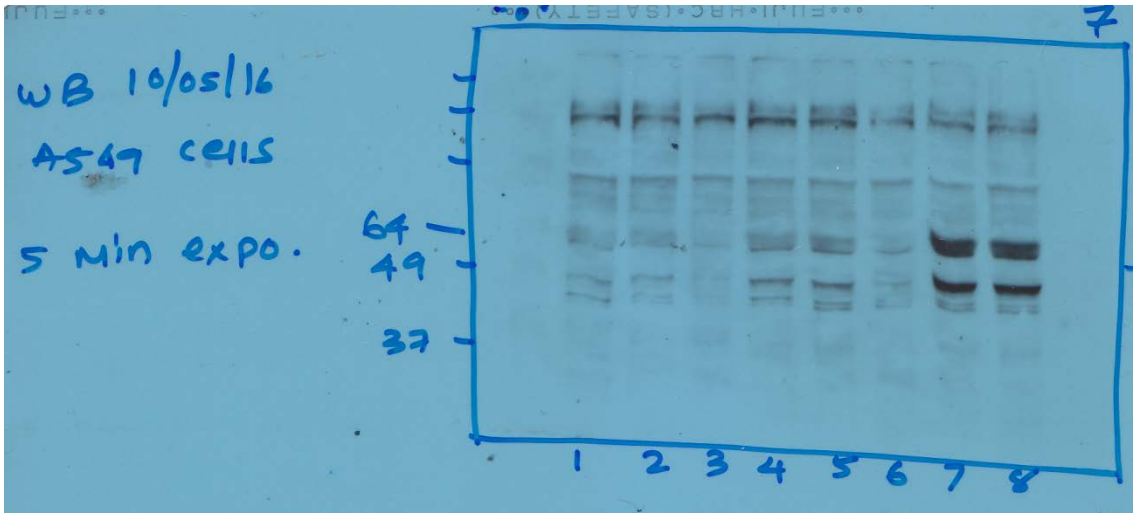
total JNK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

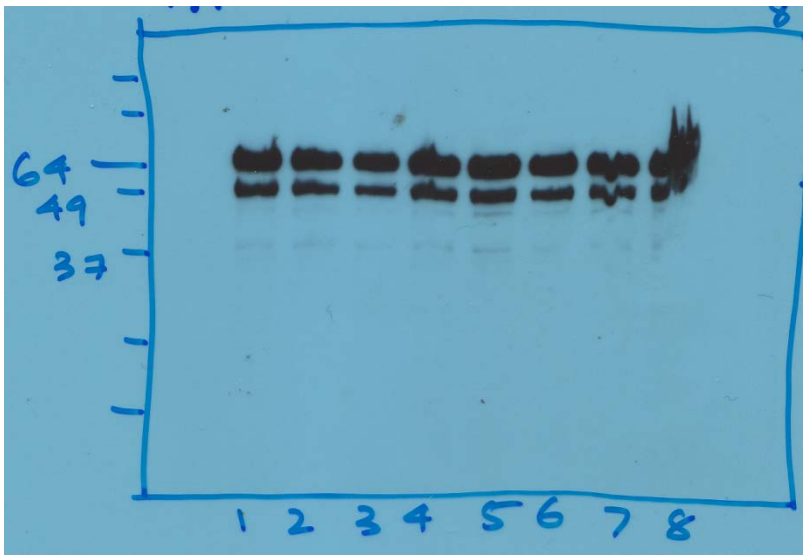
Experiment 2

phospho-JNK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

total JNK



Pam3CSK4 (1 µg/ml)	-	-	-	+	+	+	-	-
Dex (0.01 µM)	-	-	+	-	-	+	-	-
H_20_2 (100 µM)	-	-	-	-	-	-	+	-
TNFα (4 ng/ml)	-	+	+	-	+	+	-	+

Experiment 3

TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

Md. Mostafizur Rahman[a], Pavan Prabhala[a], Nowshin N. Rumzhum[a], Brijeshkumar S. Patel[a], Thomas Wickop[a], Philip M. Hansbro[b], Nicole M. Verrills[c] and Alaina J. Ammit[d,e]

[a]Faculty of Pharmacy
University of Sydney
NSW Australia

[b]Priority Research Centre for Respiratory Diseases
Hunter Medical Research Institute and The University of Newcastle
Newcastle, Australia.

[c]School of Biomedical Sciences and Pharmacy
Faculty of Health and Medicine
University of Newcastle
NSW Australia

[d]Woolcock Emphysema Centre
Woolcock Institute of Medical Research
University of Sydney
NSW Australia

[e]School of Life Sciences
Faculty of Science
University of Technology Sydney
NSW Australia

Corresponding author: Alaina J. Ammit Ph.D.
Phone: +61 2 91140368
E-mail: Alaina.Ammit@uts.edu.au

Abstract

Corticosteroids are effective anti-inflammatory therapies widely utilized in chronic respiratory diseases. But these medicines can lose their efficacy during respiratory infection resulting in disease exacerbation. Further *in vitro* research is required to understand how infection worsens lung function control in order to advance therapeutic options to treat infectious exacerbation in the future. In this study, we utilize a cellular model of bacterial exacerbation where we pretreat A549 lung epithelial cells with the synthetic bacterial lipoprotein Pam3CSK4 (a TLR2 ligand) to mimic bacterial infection and tumor necrosis factor α (TNFα) to simulate inflammation. Under these conditions, Pam3CSK4 induces corticosteroid insensitivity; demonstrated by substantially reduced ability of the corticosteroid dexamethasone to repress TNFα-induced interleukin 6 secretion. We then explored the molecular mechanism responsible and found that corticosteroid insensitivity induced by bacterial mimics was not due to altered translocation of the glucocorticoid receptor into the nucleus, nor an impact on the NF-κB pathway. Moreover, Pam3CSK4 did not affect corticosteroid-induced upregulation of anti-inflammatory MAPK deactivating phosphatase - MKP-1. However, Pam3CSK4 can induce oxidative stress and we show that a proportion of the MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was rendered inactive by oxidation. Thus to combat inflammation in the context of bacterial exacerbation we sought to discover effective strategies that bypassed this road-block. We show for the first time that known (FTY720) and novel (theophylline) activators of the phosphatase PP2A can serve as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

Keywords: corticosteroid insensitivity, TLR2, MKP-1, PP2A

1. Introduction

Corticosteroids are front-line anti-inflammatory therapies that are widely used to treat people with chronic lung disease. In asthma, although corticosteroids have proven clinical efficacy, it is increasingly recognized that their anti-inflammatory effectiveness can vary widely depending on disease context (such as when people with asthma are experiencing respiratory infections). This is known as corticosteroid insensitivity. In severe asthma, the degree of insensitivity increases to become corticosteroid resistance; a steroid-refractory condition experienced by 10% of people with asthma. Thus, the impact of corticosteroid insensitivity is increasingly recognized as a major problem limiting the efficacy of anti-inflammatory therapy in chronic respiratory disease (reviewed in (Ammit, 2013; Chung, 2013)). Moreover, corticosteroids are much less effective in chronic obstructive pulmonary disease (COPD) than in asthma, and this is considered due to relative corticosteroid insensitivity that exists in COPD (Marwick and Chung, 2010). To combat inflammation, steroid dose can be increased, but this is not ideal. Thus, alternative anti-inflammatory strategies that could effectively treat inflammation when corticosteroid insensitivity exists are urgently required.

Cellular models of corticosteroid insensitivity are invaluable *in vitro* tools in our quest to develop novel and efficacious pharmacotherapeutic strategies to treat respiratory disease in the future. They are an essential first step towards elucidating the mechanisms responsible for respiratory infections and the exacerbation of chronic lung diseases. This was highlighted in a recent review by Saturni *et al*. (Saturni et al., 2015), and demonstrated by Papi *et al.* (Papi et al., 2013), where A549 lung epithelial cells exposed *in vitro* to rhinoviral infection resulted in corticosteroid insensitivity. The molecular mechanisms responsible were explored and shown to be due to reduced nuclear translocation of the receptor for corticosteroids – the glucocorticoid-receptor (GR) (Papi et al., 2013). Ligand-activated GR translocation is a critical step in corticosteroid function; without this, GRs are unable to interact in a *cis*- or

trans-manner to exert transcriptional control. Notably, upregulation of a critical, corticosteroid-induced, anti-inflammatory protein – mitogen-activated protein kinase phosphatase 1 (MKP-1) - was attenuated by rhinoviral infection and contributed to corticosteroid insensitivity. While the impact of respiratory viruses on corticosteroid insensitivity have begun to be uncovered (reviewed in (Saturni et al., 2015)), the influence of bacterial infection warrants further investigation.

Accordingly, we have established an *in vitro* cellular model of bacterial exacerbation utilizing the synthetic bacterial lipoprotein Pam3CSK4 (Alkhouri et al., 2014; Hirota et al., 2013; Manetsch et al., 2012c). The impact of this TLR2 ligand on corticosteroid insensitivity and the role played by GR translocation and MKP-1 upregulation was previously unknown. We address this herein and show that Pam3CSK4 induces corticosteroid insensitivity in A549 lung epithelial cells. Interestingly, the mechanism is not via retardation of GR translocation into the nucleus, the mechanism responsible for rhinoviral-induced corticosteroid insensitivity (Papi et al., 2013). Instead we reveal that while the total amount of anti-inflammatory MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was unaffected, a proportion of MKP-1 was rendered inactive by oxidation of the catalytic cysteine. MKP-1 is one of the major ways in which corticosteroids achieve anti-inflammatory action (Che et al., 2014; Issa et al., 2007; King et al., 2009; Manetsch et al., 2012a; Quante et al., 2008; Rahman et al., 2014). Given that this anti-inflammatory pathway is less effective under *in vitro* conditions mimicking bacterial exacerbation, we were compelled to uncover alternative, non-steroidal anti-inflammatory strategies. We focused on activators of ubiquitous serine/threonine phosphatase protein phosphatase 2A (PP2A). PP2A dephosphorylates a number of proteins that control inflammatory cell signalling (Junttila et al., 2008; Miskolci et al., 2003; Shanley et al., 2001) and our recent research has revealed that known and novel PP2A activators can also have substantial anti-inflammatory effect in the

context of respiratory inflammation (Patel et al., 2015; Rahman et al., 2016; Rahman et al., 2015). In this study we are the first to examine the impact of PP2A activators in an *in vitro* model of bacterial exacerbation, and notably, we show that PP2A activators can repress inflammation when corticosteroid insensitivity exists.

2. Material and Methods

2.1 Chemicals

Pam3CSK4 was purchased from InVivoGen (San Diego, CA), tumor necrosis factor α (TNFα) was from R&D Systems (Minneapolis, MN) and FTY720 was from Cayman Chemical Company (Ann Arbor, MI). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell culture

The human alveolar epithelial cell line (A549) was cultured in Ham's F-12K (Kaighn's) medium (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). All experiments were performed after an overnight serum-starvation period (14-16 h) in Ham's F-12K supplemented with sterile BSA (0.1%), and cells were stimulated with 4 ng/ml TNFα in accordance with previous publications (Cornell et al., 2009; Rahman et al., 2015). A minimum of three experimental replicates performed on separate days were used for each experiment.

2.3 ELISA

IL-6 ELISA was performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

2.4 Western blotting

To examine GR and p65 NF-κB translocation, cytoplasmic and nuclear protein extraction was performed using NE-PER nuclear and cytosolic extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). Western blotting was performed using rabbit polyclonal IgG antibodies raised against GR (E-20: Santa Cruz

Biotechnology, Santa Cruz, CA) or an XP® rabbit monoclonal antibody against p65 NF-κB (D14E12: Cell Signaling Technologies, Danvers, MA). Mouse monoclonal antibodies to α-tubulin (IgG_1, DM1A: Santa Cruz Biotechnology) and rabbit polyclonal antibodies to lamin A/C (Cell Signaling Technology) were used as loading controls for the cytosolic and nuclear fractions, respectively. To measure MKP-1upregulation in whole cell lysates, MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology). IκB-α degradation and heme oxygenase 1 (HO-1) upregulation were also detected using rabbit polyclonal antibodies from Santa Cruz (C19) or Cell Signaling Technologies (P249), respectively. For all experiments in whole cell lysates, α-tubulin was utilized as the loading control. Antibodies to detect phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total ERK, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK were from Cell Signaling Technology. Primary antibodies were detected with goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.5 MKP-1 oxidation

The method used to measure oxidized MKP-1 was an adaptation of the PROP (purification of reversibly oxidized proteins) technique developed by Templeton *et al.* (Templeton et al., 2010; Victor et al., 2012). A549 cells were subjected to oxidative stress conditions (see experimental text) before commencing the PROP procedure. Briefly, the non-oxidized thiols were blocked using N-ethylmaleimide, then the oxidized thiols were reduced with dithiothrietol and eluted with thiol affinity chromatography (thiopropyl sepharose 6B: GE Healthcare, Little Chlafont, UK). Oxidized MKP-1 was then measured using Western blot

and the degree of oxidation compared to native MKP-1 measured in whole cell lysates run in parallel.

2.6 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way ANOVA with Fisher's post-hoc multiple comparison test or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean+SEM of $n \geq 3$ independent replicates.

3. Results

3.1 TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells

Herein we adapt a clinically-relevant model of corticosteroid insensitivity (Papi et al., 2013) to mimic bacterial exacerbation *in vitro* and demonstrate that exposure to a synthetic bacterial lipoprotein (TLR2/TLR1 ligand; Pam3CSK4) induces relative corticosteroid insensitivity. We chose to use 1 μg/ml Pam3CSK4 based on our earlier studies with this bacterial mimic (Hirota et al., 2013; Manetsch et al., 2012c). When A549 cells were pretreated with Pam3CSK4, the dose-dependent repression of TNFα-induced IL-6 secretion achieved with dexamethasone was significantly altered (Figure 1: $P<0.05$). This is particularly notable at 0.01-1 μM dexamethasone, where the inhibitory impact of dexamethasone on cytokine secretion from A549 cells is significantly attenuated by Pam3CSK4 (Figure 1: $P<0.05$).

3.2 Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus

We then wished to explore the molecular mechanism responsible for corticosteroid insensitivity exerted by the TLR2 ligand. Corticosteroids mediate their actions via interaction with the cytosolic GR that then undergoes translocation into the nucleus. As this is an essential step necessary for corticosteroid action, we examined whether the molecular basis of corticosteroid insensitivity established by Pam3CSK4 is due to a retardation of GR translocation. We address this in Figure 2, where the effect of Pam3CSK4 pretreatment on GR translocation induced by dexamethasone (0.0001-1 μM) was assessed under two experimental conditions: i.e. in the absence (Figure 2A) or presence of stimulation with TNFα (Figure 2B). Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR determined by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. As shown in Figure 2A, GR

resides in the cytosol and is undetectable in the nucleus under unstimulated conditions. After treatment with dexamethasone however, GR undergoes translocation in a concentration-dependent manner. Notably, dexamethasone-induced GR translocation is unaffected by Pam3CSK4 (Figure 2A). These results were independent of stimulation with TNFα; as confirmatory experiments (performed in Figure 2B) show a similar pattern of GR translocation in cells that have been treated with TNFα. Taken together these experiments demonstrate that corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus.

3.3 Corticosteroids have no effect on TNFα-induced IκB-α degradation and p65 NF-κB nuclear entry

Corticosteroids also have the potential to form inhibitory interactions with the pro-inflammatory DNA-binding transcription factors, such as NF-κB (reviewed in (Ammit, 2013)). To exclude the possible inhibition of NF-κB activity as a mechanism for corticosteroid insensitivity we took two experimental approaches. Firstly, we showed that corticosteroids had no effect on TNFα-induced IκB-α degradation (Figure 3). IκB-α is a constitutively expressed protein that forms an inhibitory complex with NF-κB. TNFα can induce the degradation of IκB-α, freeing NF-κB from its inhibitory control and allowing it to exert transcriptional impact. This is shown in Figure 3, where the basal level of inhibitory IκB-α is significantly degraded by TNFα. Notably, corticosteroids do not affect TNFα-induced IκB-α degradation in A549 cells. We examined this after pretreating cells with a range of dexamethasone concentrations (0.0001-1 μM) and, as shown in Figure 3, there was no significant effect on the extent of IκB-α degradation achieved with TNFα in the presence of dexamethasone (P<0.05). Secondly, we examined whether dexamethasone repressed the translocation of the p65 NF-κB subunit into the nucleus. Cells were pretreated with a range of

dexamethasone concentrations (0.0001-1 μM) and the effect on TNFα-induced nuclear translocation assessed. As shown in Figure 4, corticosteroids have no effect on the nuclear entry of p65 NF-κB induced by TNFα. Taken together, the results presented in Figures 3 and 4 confirm that corticosteroids do not act to repress the TNFα-induced NF-κB pathway.

3.4 Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1

Thus far we have shown that Pam3CSK4 reduces the anti-inflammatory efficacy of corticosteroids on TNFα-induced cytokine production without affecting GR nor p65 NF-κB translocation. We (Che et al., 2014; Manetsch et al., 2012b; Quante et al., 2008; Rahman et al., 2014) and others (Issa et al., 2007; King et al., 2009) have shown that a major mechanism responsible for the anti-inflammatory action of corticosteroids is via the upregulation of MAPK-deactivating phosphatase – MKP-1; thus, we investigated whether impaired MKP-1 upregulation was responsible for corticosteroid insensitivity induced by bacterial ligand Pam3CSK4. This is explored in Figure 5, where MKP-1 upregulation in whole cell lysates of A549 cells was compared by Western blotting (Figure 5A) and analyzed by densitometry (Figure 5B). We demonstrate that TNFα induces significant upregulation of MKP-1 protein (3.2±0.5-fold compared to vehicle-treated cells (designated as 1) (P<0.05)); in alignment with previous studies in A549 cells (King et al., 2009). MKP-1 is corticosteroid-inducible (King et al., 2009) and accordingly, TNFα-induced MKP-1 in A549 cells pretreated with dexamethasone is significantly enhanced at 0.01-1 μM (Figure 5B: P<0.05). However, when we examine the influence of Pam3CSK4 pretreatment on MKP-1 upregulation there was no significant effect; as demonstrated by Western blotting (Figure 5A) and confirmed by densitometric analysis (Figure 5B). Thus, our hypothesis that the bacterial ligand Pam3CSK4 attenuated upregulation of MKP-1 protein was not supported.

3.5 Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1

However, MKP-1 protein is subject to redox regulation (Hou et al., 2008; Kamata et al., 2005) as the catalytic cysteine responsible for phosphatase action can be oxidized and thus reduce anti-inflammatory function (Bonham and Vacratsis, 2009). Notably, Pam3CSK4 has been shown to increase oxidative stress in a TLR2-mediated manner (Singh et al., 2015). To test this in A549 cells we measured the expression of heme oxygenase 1 as a surrogate measure of oxidative stress. HO-1 is an inducible enzyme that can be detected by Western blotting and a recognised marked of oxidative stress (Kamalvand et al., 2003). We examined the effect of Pam3CSK4, alone and in combination with TNF, on HO-1 protein upregulation, and as shown in Figure 6, Pam3CSK4 significantly increases HO-1 protein upregulation as detected by Western blotting (Figure 6A) and assessed densitometrically (Figure 6B)($P<0.05$). The increases in HO-1 upregulation were similar to those achieved with 100 μM H_2O_2 (Figure 6), a positive control for oxidative stress

We were now intrigued to examine whether MKP-1 expressed under these conditions may be oxidized. To do this, we utilized a technique referred to as PROP (purification of reversibly oxidized proteins) (Templeton et al., 2010; Victor et al., 2012). A549 cells were treated under conditions established above to induce corticosteroid insensitivity (dexamethasone (at 10 nM) + TNFα without/with Pam3CSK4) in order to ask the question - is the MKP-1 produced in cells that have been pretreated with Pam3CSK4 oxidized? In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1. As shown in Figures 7A and 7B, and in confirmation with our earlier data (Figure 5), Pam3CSK4 had no significant effect on the dexamethasone + TNFα-induced

upregulation of MKP-1 detected in whole cell lysates (see native MKP-1 (WCL)). In contrast, there was a significant (P<0.05) increase in oxidized MKP-1 detected by the PROP technique (Figure 7B). To examine whether this oxidized MKP-1 resulted in altered MKP-1 activity and an impact on downstream effectors, we examined the phosphorylation of MAPK superfamily members (p38 MAPK, ERK and JNK) under identical conditions. These data are shown by Western blot in Figure 7C and densitometrically in Figure 7D, where we see a small but significant increase in p38 MAPK phosphorylation in the presence of Pam3CSK4 (P<0.05). ERK phosphorylation was unaffected, while there was some variability in the JNK response. In cells pretreated with the widely-used positive control for oxidative stress – H_2O_2 (Kamata et al., 2005; Templeton et al., 2010; Victor et al., 2012) before TNFα, we can confirm that the PROP technique detects increases in oxidized MKP-1 (Figures 7E and 7F). Taken together these results suggest that MKP-1 oxidation induced by Pam3CSK4 may be a contributing factor in corticosteroid insensitivity.

3.6 Alternative anti-inflammatory approaches to overcome corticosteroid insensitivity: known and novel PP2A activators

By modeling bacterial exacerbation *in vitro* we have demonstrated that exposure to a bacterial ligand induces corticosteroid insensitivity. We revealed, in part, a contributing molecular mechanism (oxidation of MKP-1) and show that translocation of GR and p65 NF-κB is unaffected. We now turn to exploring alternative anti-inflammatory approaches and hypothesize that PP2A activators, being non-steroidal, may prove to inhibit cytokine production when corticosteroid insensitivity exists. To address this we examine the impact of two compounds: FTY720 and theophylline. FTY720 is a well-established activator of PP2A (Perrotti and Neviani, 2013; Rahman et al., 2015); while theophylline has only been recently reported by us (Patel et al., 2015) to function as a novel activator of PP2A. The

concentrations of FTY720 and theophylline utilized in this study were in accordance with previous publications (Patel et al., 2015; Rahman et al., 2015). We show that FTY720 (2.5 μM) in combination with dexamethasone (0.001-1 μM) achieves significant repression of IL-6 with lower concentrations of corticosteroids (Figure 8A), and inhibits IL-6 secretion in cells where corticosteroid insensitivity has been established by exposure to the bacterial ligand Pam3CSK4 (Alkhouri et al., 2014; Manetsch et al., 2012c)(Figure 8B) (P<0.05). At a concentration of 10 μM, theophylline is anti-inflammatory and can repress TNFα-induced IL-6 secretion (Figure 9A); in confirmation of our earlier publication (Patel et al., 2015). It also appeared to have an additive repressive effect together with corticosteroids, although this difference was not significant. However, theophylline can still significantly repress IL-6 secretion even when corticosteroid insensitivity exists (Figure 9B: P<0.05). Thus, PP2A activators are a non-steroidal anti-inflammatory alternative and/or corticosteroid-sparing approach in respiratory inflammation where corticosteroid insensitivity exists.

4. Discussion

Corticosteroid insensitivity is a major factor limiting treatment of chronic respiratory disease today. The relative responsiveness to corticosteroids can be influenced by a range of endogenous and exogenous factors, including respiratory infections. In this study we modeled bacterial infection *in vitro* and showed for the first time that ligation of the TLR2 receptor with a synthetic bacterial lipoprotein induces corticosteroid insensitivity. We then explored the cellular mechanisms responsible and demonstrate that, unlike rhinoviral-induced corticosteroid insensitivity, Pam3CSK4 did not perturb GR nuclear translocation. Notably, while the overall amount of native MKP-1 upregulation protein produced under these conditions was unaffected by Pam3CSK4 pretreatment, a significant proportion of MKP-1 was oxidized and consequently was rendered inactive. Finally, we explored alternative modalities to repress cytokine production and demonstrate the utility of PP2A activators as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

When a person with chronic lung disease (such as asthma and COPD) experiences an infection their lung function deteriorates and they require greater amounts of their anti-inflammatory medicines to treat their respiratory symptoms. This is because viral or bacterial infection changes the molecular pathways by which respiratory medicines act; making them less sensitive. These changes are due to interaction of viral and bacterial products with pattern recognition receptors, particularly TLRs expressed on airway cells (reviewed in (Saturni et al., 2015)). We have focused on mimicking bacterial infection *in vitro* with Pam3CSK4 and have previously shown that TLR2 ligation robustly increased TNFα-induced secretion of cytokines implicated as playing a role in exacerbation (Manetsch et al., 2012c). Moreover, TLR2 activation causes tachyphylaxis to bronchodilatory β_2-agonists *in vitro* and *ex vivo* (Alkhouri et al., 2014). In the current study, we reveal an additional detrimental

consequence of bacterial infection beyond amplification of inflammation and β_2-adrenergic desensitization; namely, we show that Pam3CSK4 induces corticosteroid insensitivity. This *in vitro* evidence is in support of a recent study examining clinical samples from people with COPD that linked increased TLR2 with this corticosteroid insensitive condition (Simpson et al., 2013).

However, the molecular mechanisms responsible differ from those reported for corticosteroid insensitivity induced in A549 cells by rhinoviral infection (Papi et al., 2013). Corticosteroids initiate their effects on gene expression by interacting with cytoplasmic GRs. Translocation of GR into the nucleus is a requisite step to allow GR to act as a ligand-dependent transcription factor. Once in the nucleus, ligand-activated GRs then repress inflammation by: (i) activation of glucocorticoid response elements and upregulation of anti-inflammatory genes, including MKP-1 and glucocorticoid-inducible leucine zipper 1; (ii) inhibitory interactions with proinflammatory DNA-binding transcription factors, such as NF-κB; and (iii) effects on recruitment of transcriptional co-activators, co-repressors and the chromatin machinery (reviewed in (Ammit, 2013)). Papi *et al.* (Papi et al., 2013) showed that rhinoviral infection reduced GR nuclear translocation, thus levels of MKP-1 upregulation was lower. Additionally, they demonstrated induction of MAPK signaling pathways (JNK in particular) and NF-κB activation. In contrast, we show that GR translocation was unaffected by Pam3CSK4 and that the upregulation of MKP-1 in whole cell lysates was unchanged. We also confirm that corticosteroids have no effect on TNFα-induced IκB-α degradation and p65 NF-κB nuclear entry, excluding the possibility that corticosteroid insensitivity is due to inhibitory interactions with the pro-inflammatory DNA-binding transcription factors, particularly NF-κB.

However, just examining the overall protein levels of MKP-1 may not reveal the entire story. It is increasingly recognized that the MAPK-deactivating ability of dual-specificity

phosphatases (with MKP-1, aka DUSP1, being a founding member of this family) can be regulated post-translationally by oxidation (Bonham and Vacratsis, 2009). The catalytic cysteine of MKP-1 can be modified by oxidation, thereby reducing its function as phosphatase. Redox regulation of MKP-1 has been demonstrated *in vitro* (Hou et al., 2008; Kamata et al., 2005) and highlighted as a potential mechanism responsible for corticosteroid insensitivity in our recent *in vivo* study exposing MKP-1 wild-type and knock-out mice to ozone (Pinart et al., 2014). Notably, Pam3CSK4 can increase oxidative stress in a TLR2-mediated manner (Singh et al., 2015) and herein we show that Pam3CSK4 increases production of a surrogate marker for oxidative stress, HO-1. Therefore we utilized the PROP technique to show that a proportion of MKP-1 expressed under these conditions is oxidized. This results in altered MKP-1 activity with impact on downstream effectors; notably p38MAPK phosphorylation is increased under these conditions. Taken together, these results suggest that Pam3CSK4-mediated oxidation of MKP-1 may contribute to corticosteroid insensitivity.

Finally, we explored alternative means of repressing cytokines when corticosteroid insensitivity exists. We focused on small molecules that are known and novel activators of the ubiquitous serine-threonine phosphatase – PP2A. PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling, including MAPKs and NF-κB (Junttila et al., 2008; Miskolci et al., 2003; Rahman et al., 2015; Shanley et al., 2001). A number of small molecules can activate PP2A (Perrotti and Neviani, 2013). The best known of these is FTY720, and we recently showed that it activates TNFα-induced PP2A enzymatic activity and represses cytokine production in A549 cells (Rahman et al., 2015). In the current study we examine its efficacy when used as an adjunct therapy with corticosteroids *in vitro* and show that FTY720 can act additively in a corticosteroid-sparing manner. Most importantly in the context of the bacterial exacerbation, FTY720 can significantly repress cytokine

production when corticosteroid insensitivity exists. Lastly, we explored the potential effects of theophylline. Theophylline has beneficial effects in asthma and COPD and acts via a number of different molecular pathways (reviewed in (Barnes, 2013)). We recently reported a new anti-inflammatory function for theophylline; it increases TNFα-induced PP2A enzymatic activity in A549 cells, as well as primary cultures of airway smooth muscle (Patel et al., 2015). Thus, we examined its impact on corticosteroid insensitivity and although the impact was not as great as FTY720, theophylline was able overcome corticosteroid insensitivity. It is important to note however, that we believe that these repressive effects on cytokine function exerted by FTY720 and theophylline are anti-inflammatory effects acting via alternative, PP2A-dependent pathways, rather than restoration of corticosteroid sensitivity, although further investigation is warranted.

Collectively, our study shows that, like rhinoviral infection modeled *in vitro,* bacterial ligands exert corticosteroid insensitivity. These models allow us to explore the molecular mechanisms responsible and highlight potential therapeutic strategies to combat inflammation in infectious exacerbation. We reveal that oxidation of anti-inflammatory MKP-1, rather than retardation of nuclear translation, occurs via TLR2-mediated pathways. Finally, we suggest that activating PP2A may prove to be efficacious, non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in the context of bacterial exacerbation.

Acknowledgements

This research was supported by National Health and Medical Research Council of Australia (APP1025637) and philanthropic funding from Mr Maurice Renshaw. Md. Mostafizur Rahman was supported by an Endeavour Postgraduate Award; Pavan Prabhala received scholarship support from Mr Renshaw; Nowshin Nowaz Rumzhum received an International Postgraduate Research Scholarship; Brijeshkumar S. Patel was supported by an Australian Postgraduate Award.

References

Alkhouri, H., Rumzhum, N.N., Rahman, M.M., FitzPatrick, M., de Pedro, M., Oliver, B.G., Bourke, J.E., Ammit, A.J., 2014. TLR2 activation causes tachyphylaxis to beta2 -agonists in vitro and ex vivo: modelling bacterial exacerbation. Allergy 69(9), 1215-1222.

Ammit, A.J., 2013. Glucocorticoid insensitivity as a source of drug targets for respiratory disease. Curr Opin Pharmacol 13(3), 370-376.

Barnes, P.J., 2013. Theophylline. Am. J. Respir. Crit. Care Med. 188(8), 901-906.

Bonham, C.A., Vacratsis, P.O., 2009. Redox Regulation of the Human Dual Specificity Phosphatase YVH1 through Disulfide Bond Formation. The Journal of Biological Chemistry 284(34), 22853-22864.

Che, W., Parmentier, J., Seidel, P., Manetsch, M., Ramsay, E.E., Alkhouri, H., Ge, Q., Armour, C.L., Ammit, A.J., 2014. Corticosteroids inhibit sphingosine 1-phosphate-induced interleukin-6 secretion from human airway smooth muscle via mitogen-activated protein kinase phosphatase 1-mediated repression of mitogen and stress-activated protein kinase 1. Am. J. Respir. Cell Mol. Biol. 50(2), 358-368.

Chung, K.F., 2013. New treatments for severe treatment-resistant asthma: targeting the right patient. The Lancet Respiratory Medicine 1(8), 639-652.

Cornell, T.T., Hinkovska-Galcheva, V., Sun, L., Cai, Q., Hershenson, M.B., Vanway, S., Shanley, T.P., 2009. Ceramide-dependent PP2A regulation of TNFalpha-induced IL-8 production in respiratory epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 296(5), L849-856.

Hirota, J.A., Im, H., Rahman, M.M., Rumzhum, N.N., Manetsch, M., Pascoe, C., Bunge, K., Alkhouri, H., Oliver, B.G., Ammit, A.J., 2013. The NLRP3 Inflammasome is not

Activated in Airway Smooth Muscle Upon TLR2 Ligation. Am. J. Respir. Cell Mol. Biol. 49(4), 517-524.

Hou, N., Torii, S., Saito, N., Hosaka, M., Takeuchi, T., 2008. Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. Endocrinology 149(4), 1654-1665.

Issa, R., Xie, S.P., Khorasani, N., Sukkar, M., Adcock, I.M., Lee, K.Y., Chung, K.F., 2007. Corticosteroid inhibition of growth-related oncogene protein-alpha via mitogen-activated kinase phosphatase-1 in airway smooth muscle cells. J. Immunol. 178(11), 7366-7375.

Junttila, M.R., Li, S.-P., Westermarck, J., 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 22(4), 954-965.

Kamalvand, G., Pinard, G., Ali-Khan, Z., 2003. Heme-oxygenase-1 response, a marker of oxidative stress, in a mouse model of AA amyloidosis. Amyloid 10(3), 151-159.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., Karin, M., 2005. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120(5), 649-661.

King, E.M., Holden, N.S., Gong, W., Rider, C.F., Newton, R., 2009. Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. J. Biol. Chem. 284(39), 26803-26815.

Manetsch, M., Che, W., Seidel, P., Chen, Y., Ammit, A.J., 2012a. MKP-1: a negative feedback effector that represses MAPK-mediated pro-inflammatory signaling pathways and cytokine secretion in human airway smooth muscle cells. Cell. Signal. 24(4), 907-913.

Manetsch, M., Ramsay, E., King, E., Seidel, P., Che, W., Ge, Q., Hibbs, D., Newton, R., Ammit, A., 2012b. Corticosteroids and beta(2) -agonists upregulate mitogen-activated protein kinase phosphatase 1: in vitro mechanisms. Br. J. Pharmacol. 166(7), 2049-2059.

Manetsch, M., Seidel, P., Heintz, U., Che, W., Hughes, J.M., Ge, Q., Sukkar, M.B., Ammit, A.J., 2012c. TLR2 ligand engagement upregulates airway smooth muscle TNFalpha-induced cytokine production. Am J Physiol Lung Cell Mol Physiol 302(9), L838-845.

Marwick, J.A., Chung, K.F., 2010. Glucocorticoid insensitivity as a future target of therapy for chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 5, 297-309.

Miskolci, V., Castro-Alcaraz, S., Nguyen, P., Vancura, A., Davidson, D., Vancurova, I., 2003. Okadaic acid induces sustained activation of NFκB and degradation of the nuclear IκBα in human neutrophils. Arch. Biochem. Biophys. 417(1), 44-52.

Papi, A., Contoli, M., Adcock, I.M., Bellettato, C., Padovani, A., Casolari, P., Stanciu, L.A., Barnes, P.J., Johnston, S.L., Ito, K., Caramori, G., 2013. Rhinovirus infection causes steroid resistance in airway epithelium through nuclear factor κB and c-Jun N-terminal kinase activation. J. Allergy Clin. Immunol. 132(5), 1075-1085.e1076.

Patel, B.S., Rahman, M.M., Rumzhum, N.N., Oliver, B.G., Verrills, N.M., Ammit, A.J., 2015. Theophylline Represses IL-8 Secretion From ASM Cells Independently of PDE Inhibition: Novel Role as a PP2A Activator. Am. J. Respir. Cell Mol. Biol.

Perrotti, D., Neviani, P., 2013. Protein phosphatase 2A: a target for anticancer therapy. Lancet Oncol 14(6), e229-e238.

Pinart, M., Hussain, F., Shirali, S., Li, F., Zhu, J., Clark, A.R., Ammit, A.J., Chung, K.F., 2014. Role of mitogen-activated protein kinase phosphatase-1 in corticosteroid insensitivity of chronic oxidant lung injury. Eur. J. Pharmacol. 744, 108-114.

Quante, T., Ng, Y.C., Ramsay, E.E., Henness, S., Allen, J.C., Parmentier, J., Ge, Q., Ammit, A.J., 2008. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. Am. J. Respir. Cell Mol. Biol. 39(2), 208-217.

Rahman, M.M., Alkhouri, H., Tang, F., Che, W., Ge, Q., Ammit, A.J., 2014. Sphingosine 1-phosphate induces neutrophil chemoattractant IL-8: repression by steroids. PLoS One 9(3), e92466.

Rahman, M.M., Rumzhum, N.N., Hansbro, P.M., Morris, J.C., Clark, A.R., Verrills, N.M., Ammit, A.J., 2016. Activating protein phosphatase 2A (PP2A) enhances tristetraprolin (TTP) anti-inflammatory function in A549 lung epithelial cells. Cell. Signal. 28(4), 325-334.

Rahman, M.M., Rumzhum, N.N., Morris, J.C., Clark, A.R., Verrills, N.M., Ammit, A.J., 2015. Basal protein phosphatase 2A activity restrains cytokine expression: role for MAPKs and tristetraprolin. Scientific reports 5, 10063.

Saturni, S., Contoli, M., Spanevello, A., Papi, A., 2015. Models of Respiratory Infections: Virus-Induced Asthma Exacerbations and Beyond. Allergy Asthma Immunol Res 7(6), 525-533.

Shanley, T.P., Vasi, N., Denenberg, A., Wong, H.R., 2001. The Serine/Threonine Phosphatase, PP2A: Endogenous Regulator of Inflammatory Cell Signaling. J. Immunol. 166(2), 966-972.

Simpson, J.L., McDonald, V.M., Baines, K.J., Oreo, K.M., Wang, F., Hansbro, P.M., Gibson, P.G., 2013. Influence of age, past smoking, and disease severity on TLR2, neutrophilic inflammation, and MMP-9 levels in COPD. Mediators Inflamm. 2013, 462934.

Singh, A., Singh, V., Tiwari, R.L., Chandra, T., Kumar, A., Dikshit, M., Barthwal, M.K., 2015. The IRAK-ERK-p67phox-Nox-2 axis mediates TLR4, 2-induced ROS

production for IL-1beta transcription and processing in monocytes. Cellular & molecular immunology.

Templeton, D.J., Aye, M.S., Rady, J., Xu, F., Cross, J.V., 2010. Purification of reversibly oxidized proteins (PROP) reveals a redox switch controlling p38 MAP kinase activity. PLoS One 5(11), e15012.

Victor, K.G., Rady, J.M., Cross, J.V., Templeton, D.J., 2012. Proteomic profile of reversible protein oxidation using PROP, purification of reversibly oxidized proteins. PLoS One 7(2), e32527.

Figure Legends

Figure 1. TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle or dexamethasone (0.0001-1 μM) for 30 min. After stimulation with TNFα (4 ng/ml) for 24 h, IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant reduction of corticosteroid-mediated repression by Pam3CSK4 (P<0.05)). Data are mean+SEM values from n=9 independent experiments.

Figure 2. Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle or dexamethasone (0.0001-1 μM) for 30 min. GR translocation was examined in the (A) absence or (B) or presence of stimulation with TNFα (4 ng/ml) for 1 h. Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR determined by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. Data are representative blots from n=3 independent experiments.

Figure 3. Corticosteroids have no effect on TNFα-induced IκB-α degradation. A549 cells were pretreated with vehicle or dexamethasone (0.0001-1 μM) for 30 min. Cells were then stimulated without and with TNFα (4 ng/ml) for 1 h and the impact on IκB-α degradation examined by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of IκB-α degradation (expressed as a percentage of IκB-α measured in vehicle-treated cells, normalized to α-tubulin). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple

comparison test (where * denotes significant degradation of IκB-α induced by TNFα (P<0.05); there was no significant effect of dexamethasone). Data are mean+SEM values from n=3 independent experiments.

Figure 4. Corticosteroids have no effect on TNFα-induced p65 NF-κB nuclear translocation. A549 cells were pretreated with vehicle or dexamethasone (0.0001-1 μM) for 30 min. Cells were then stimulated without and with TNFα (4 ng/ml) for 1 h, nuclear protein extracts prepared and entry of p65 NF-κB into the nucleus determined by Western blotting, compared with lamin A/C as a loading control. (A) Results are representative Western blots and (B) densitometric analysis of p65 NF-κB nuclear translocation (expressed as fold increase in p65 NF-κB in the nucleus of vehicle-treated cells, normalized to lamin A/C). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant p65 NF-κB nuclear translocation induced by TNFα (P<0.05); there was no significant effect of dexamethasone). Data are mean+SEM values from n=3 independent experiments.

Figure 5. Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle or dexamethasone (0.0001-1 μM) for 30 min. Cells were treated without and with TNFα (4 ng/ml) for 1 h and the impact on MKP-1 upregulation compared by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to vehicle-treated cells). Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant

increase in MKP-1 protein upregulation induced by TNFα, and § denotes significant potentiation of TNFα-induced MKP-1 by dexamethasone (P<0.05); there was no effect of Pam3CSK4). Data are mean+SEM values from n=3 independent experiments.

Figure 6. Pam3CSK4 increases heme oxygenase 1 (HO-1) protein upregulation: a marker of oxidative stress. A549 cells were treated for 2 h with either vehicle, Pam3CSK4 (1 μg/ml) or H_2O_2 (100 μM) as a positive control for oxidative stress. HO-1 upregulation was examined by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of HO-1 protein upregulation (expressed as a fold increase compared to vehicle-treated cells, normalized to α-tubulin). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant upregulation of HO-1 induced by both Pam3CSK4 and H_2O_2 (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 7. Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1. (A-D) A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by dexamethasone (0.01 μM) for 30 min then TNFα (4 ng/ml) for 1 h. (A, B) In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates (WCL) to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1 and Western blotting performed. (C, D) To demonstrate the impact of oxidized MKP-1 on downstream effectors, the phosphorylation of MAPK family members (p38 MAPK, ERK and JNK) in WCL were measured by Western blotting (normalized to total p38 MAPK, ERK and JNK, respectively). (E, F) As a positive control for oxidative stress, A549 cells were pretreated for 30 min

without and with 100 μM H_2O_2 before stimulation with TNFα (4 ng/ml) for 1 h and processed as above (A, B). (A, C, E) Results are representative Western blots and (B, D, F) densitometric analysis (results expressed as fold increase compared to cells treated without (A-D) Pam3CSK4 or (E-F) H_2O_2). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test or Student's unpaired *t* test (where * denotes significant increase (P<0.05). Data are mean+SEM values from n=3-4 independent experiments.

Figure 8. FTY720 is corticosteroid-sparing and overcomes corticosteroid insensitivity. A549 were pretreated for 6 h with vehicle or FTY720 (2.5 μM), before 30 min with (A) vehicle or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min. After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of FTY720 (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 9. Theophylline is anti-inflammatory and can overcome corticosteroid insensitivity. A549 were pretreated for 30 min with (A) vehicle or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min, without or with theophylline (10 μM). After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of theophylline (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

Md. Mostafizur Rahman[a], Pavan Prabhala[a], Nowshin N. Rumzhum[a], Brijeshkumar S. Patel[a], Thomas Wickop[a], Philip M. Hansbro[b], Nicole M. Verrills[c] and Alaina J. Ammit[d,e]

[a]Faculty of Pharmacy
University of Sydney
NSW Australia

[b]Priority Research Centre for Respiratory Diseases
Hunter Medical Research Institute and The University of Newcastle
Newcastle, Australia.

[c]School of Biomedical Sciences and Pharmacy
Faculty of Health and Medicine
University of Newcastle
NSW Australia

[d]Woolcock Emphysema Centre
Woolcock Institute of Medical Research
University of Sydney
NSW Australia

[e]School of Life Sciences
Faculty of Science
University of Technology Sydney
NSW Australia

Corresponding author: Alaina J. Ammit Ph.D.
Phone: +61 2 91140368
E-mail: Alaina.Ammit@uts.edu.au

Abstract

Corticosteroids are effective anti-inflammatory therapies widely utilized in chronic respiratory diseases. But these medicines can lose their efficacy during respiratory infection resulting in disease exacerbation. Further *in vitro* research is required to understand how infection worsens lung function control in order to advance therapeutic options to treat infectious exacerbation in the future. In this study, we utilize a cellular model of bacterial exacerbation where we pretreat A549 lung epithelial cells with the synthetic bacterial lipoprotein Pam3CSK4 (a TLR2 ligand) to mimic bacterial infection and tumor necrosis factor α (TNFα) to simulate inflammation. Under these conditions, Pam3CSK4 induces corticosteroid insensitivity; demonstrated by substantially reduced ability of the corticosteroid dexamethasone to repress TNFα-induced interleukin 6 secretion. We then explored the molecular mechanism responsible and found that corticosteroid insensitivity induced by bacterial mimics was not due to altered translocation of the glucocorticoid receptor into the nucleus, nor an impact on the NF-κB pathway. Moreover, Pam3CSK4 did not affect corticosteroid-induced upregulation of anti-inflammatory MAPK deactivating phosphatase - MKP-1. However, Pam3CSK4 can induce oxidative stress and we show that a proportion of the MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was rendered inactive by oxidation. Thus to combat inflammation in the context of bacterial exacerbation we sought to discover effective strategies that bypassed this road-block. We show for the first time that known (FTY720) and novel (theophylline) activators of the phosphatase PP2A can serve as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

Keywords: corticosteroid insensitivity, TLR2, MKP-1, PP2A

1. Introduction

Corticosteroids are front-line anti-inflammatory therapies that are widely used to treat people with chronic lung disease. In asthma, although corticosteroids have proven clinical efficacy, it is increasingly recognized that their anti-inflammatory effectiveness can vary widely depending on disease context (such as when people with asthma are experiencing respiratory infections). This is known as corticosteroid insensitivity. In severe asthma, the degree of insensitivity increases to become corticosteroid resistance; a steroid-refractory condition experienced by 10% of people with asthma. Thus, the impact of corticosteroid insensitivity is increasingly recognized as a major problem limiting the efficacy of anti-inflammatory therapy in chronic respiratory disease (reviewed in (Ammit, 2013; Chung, 2013)). Moreover, corticosteroids are much less effective in chronic obstructive pulmonary disease (COPD) than in asthma, and this is considered due to relative corticosteroid insensitivity that exists in COPD (Marwick and Chung, 2010). To combat inflammation, steroid dose can be increased, but this is not ideal. Thus, alternative anti-inflammatory strategies that could effectively treat inflammation when corticosteroid insensitivity exists are urgently required.

Cellular models of corticosteroid insensitivity are invaluable *in vitro* tools in our quest to develop novel and efficacious pharmacotherapeutic strategies to treat respiratory disease in the future. They are an essential first step towards elucidating the mechanisms responsible for respiratory infections and the exacerbation of chronic lung diseases. This was highlighted in a recent review by Saturni *et al*. (Saturni et al., 2015), and demonstrated by Papi *et al.* (Papi et al., 2013), where A549 lung epithelial cells exposed *in vitro* to rhinoviral infection resulted in corticosteroid insensitivity. The molecular mechanisms responsible were explored and shown to be due to reduced nuclear translocation of the receptor for corticosteroids – the glucocorticoid-receptor (GR) (Papi et al., 2013). Ligand-activated GR translocation is a critical step in corticosteroid function; without this, GRs are unable to interact in a *cis*- or

trans-manner to exert transcriptional control. Notably, upregulation of a critical, corticosteroid-induced, anti-inflammatory protein – mitogen-activated protein kinase phosphatase 1 (MKP-1) - was attenuated by rhinoviral infection and contributed to corticosteroid insensitivity. While the impact of respiratory viruses on corticosteroid insensitivity have begun to be uncovered (reviewed in (Saturni et al., 2015)), the influence of bacterial infection warrants further investigation.

Accordingly, we have established an *in vitro* cellular model of bacterial exacerbation utilizing the synthetic bacterial lipoprotein Pam3CSK4 (Alkhouri et al., 2014; Hirota et al., 2013; Manetsch et al., 2012c). The impact of this TLR2 ligand on corticosteroid insensitivity and the role played by GR translocation and MKP-1 upregulation was previously unknown. We address this herein and show that Pam3CSK4 induces corticosteroid insensitivity in A549 lung epithelial cells. Interestingly, the mechanism is not via retardation of GR translocation into the nucleus, the mechanism responsible for rhinoviral-induced corticosteroid insensitivity (Papi et al., 2013). Instead we reveal that while the total amount of anti-inflammatory MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was unaffected, a proportion of MKP-1 was rendered inactive by oxidation of the catalytic cysteine. MKP-1 is one of the major ways in which corticosteroids achieve anti-inflammatory action (Che et al., 2014; Issa et al., 2007; King et al., 2009; Manetsch et al., 2012a; Quante et al., 2008; Rahman et al., 2014). Given that this anti-inflammatory pathway is less effective under *in vitro* conditions mimicking bacterial exacerbation, we were compelled to uncover alternative, non-steroidal anti-inflammatory strategies. We focused on activators of ubiquitous serine/threonine phosphatase protein phosphatase 2A (PP2A). PP2A dephosphorylates a number of proteins that control inflammatory cell signalling (Junttila et al., 2008; Miskolci et al., 2003; Shanley et al., 2001) and our recent research has revealed that known and novel PP2A activators can also have substantial anti-inflammatory effect in the

context of respiratory inflammation (Patel et al., 2015; Rahman et al., 2016; Rahman et al., 2015). In this study we are the first to examine the impact of PP2A activators in an *in vitro* model of bacterial exacerbation, and notably, we show that PP2A activators can repress inflammation when corticosteroid insensitivity exists.

2. Material and Methods

2.1 Chemicals

Pam3CSK4 was purchased from InVivoGen (San Diego, CA), tumor necrosis factor α (TNFα) was from R&D Systems (Minneapolis, MN) and FTY720 was from Cayman Chemical Company (Ann Arbor, MI). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell culture

The human alveolar epithelial cell line (A549) was cultured in Ham's F-12K (Kaighn's) medium (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and fetal calf serum (10%). All experiments were performed after an overnight serum-starvation period (14-16 h) in Ham's F-12K supplemented with sterile BSA (0.1%), and cells were stimulated with 4 ng/ml TNFα in accordance with previous publications (Cornell et al., 2009; Rahman et al., 2015). A minimum of three experimental replicates performed on separate days were used for each experiment.

2.3 ELISA

IL-6 ELISA was performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

2.4 Western blotting

To examine GR and p65 NF-κB translocation, cytoplasmic and nuclear protein extraction was performed using NE-PER nuclear and cytosolic extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). Western blotting was performed using rabbit polyclonal IgG antibodies raised against GR (E-20: Santa Cruz

Biotechnology, Santa Cruz, CA) or an XP® rabbit monoclonal antibody against p65 NF-κB (D14E12: Cell Signaling Technologies, Danvers, MA). Mouse monoclonal antibodies to α-tubulin (IgG_1, DM1A: Santa Cruz Biotechnology) and rabbit polyclonal antibodies to lamin A/C (Cell Signaling Technology) were used as loading controls for the cytosolic and nuclear fractions, respectively. To measure MKP-1upregulation in whole cell lysates, MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology). IκB-α degradation and heme oxygenase 1 (HO-1) upregulation were also detected using rabbit polyclonal antibodies from Santa Cruz (C19) or Cell Signaling Technologies (P249), respectively. For all experiments in whole cell lysates, α-tubulin was utilized as the loading control. Antibodies to detect phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total ERK, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK were from Cell Signaling Technology. Primary antibodies were detected with goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.5 MKP-1 oxidation

The method used to measure oxidized MKP-1 was an adaptation of the PROP (purification of reversibly oxidized proteins) technique developed by Templeton *et al.* (Templeton et al., 2010; Victor et al., 2012). A549 cells were subjected to oxidative stress conditions (see experimental text) before commencing the PROP procedure. Briefly, the non-oxidized thiols were blocked using N-ethylmaleimide, then the oxidized thiols were reduced with dithiothrietol and eluted with thiol affinity chromatography (thiopropyl sepharose 6B: GE Healthcare, Little Chlafont, UK). Oxidized MKP-1 was then measured using Western blot

and the degree of oxidation compared to native MKP-1 measured in whole cell lysates run in parallel.

2.6 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way ANOVA with Fisher's post-hoc multiple comparison test or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean+SEM of $n \geq 3$ independent replicates.

3. Results

3.1 TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells

Herein we adapt a clinically-relevant model of corticosteroid insensitivity (Papi et al., 2013) to mimic bacterial exacerbation *in vitro* and demonstrate that exposure to a synthetic bacterial lipoprotein (TLR2/TLR1 ligand; Pam3CSK4) induces relative corticosteroid insensitivity. We chose to use 1 μg/ml Pam3CSK4 based on our earlier studies with this bacterial mimic (Hirota et al., 2013; Manetsch et al., 2012c). When A549 cells were pretreated with Pam3CSK4, the dose-dependent repression of TNFα-induced IL-6 secretion achieved with dexamethasone was significantly altered (Figure 1: $P<0.05$). This is particularly notable at 0.01-1 μM dexamethasone, where the inhibitory impact of dexamethasone on cytokine secretion from A549 cells is significantly attenuated by Pam3CSK4 (Figure 1: $P<0.05$).

3.2 Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus

We then wished to explore the molecular mechanism responsible for corticosteroid insensitivity exerted by the TLR2 ligand. Corticosteroids mediate their actions via interaction with the cytosolic GR that then undergoes translocation into the nucleus. As this is an essential step necessary for corticosteroid action, we examined whether the molecular basis of corticosteroid insensitivity established by Pam3CSK4 is due to a retardation of GR translocation. We address this in Figure 2, where the effect of Pam3CSK4 pretreatment on GR translocation induced by dexamethasone (0.0001-1 μM) was assessed under two experimental conditions: i.e. in the absence (Figure 2A) or presence of stimulation with TNFα (Figure 2B). Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR determined by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. As shown in Figure 2A, GR

resides in the cytosol and is undetectable in the nucleus under unstimulated conditions. After treatment with dexamethasone however, GR undergoes translocation in a concentration-dependent manner. Notably, dexamethasone-induced GR translocation is unaffected by Pam3CSK4 (Figure 2A). These results were independent of stimulation with TNFα; as confirmatory experiments (performed in Figure 2B) show a similar pattern of GR translocation in cells that have been treated with TNFα. Taken together these experiments demonstrate that corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus.

3.3 Corticosteroids have no effect on TNFα-induced IκB-α degradation and p65 NF-κB nuclear entry

Corticosteroids also have the potential to form inhibitory interactions with the pro-inflammatory DNA-binding transcription factors, such as NF-κB (reviewed in (Ammit, 2013)). To exclude the possible inhibition of NF-κB activity as a mechanism for corticosteroid insensitivity we took two experimental approaches. Firstly, we showed that corticosteroids had no effect on TNFα-induced IκB-α degradation (Figure 3). IκB-α is a constitutively expressed protein that forms an inhibitory complex with NF-κB. TNFα can induce the degradation of IκB-α, freeing NF-κB from its inhibitory control and allowing it to exert transcriptional impact. This is shown in Figure 3, where the basal level of inhibitory IκB-α is significantly degraded by TNFα. Notably, corticosteroids do not affect TNFα-induced IκB-α degradation in A549 cells. We examined this after pretreating cells with a range of dexamethasone concentrations (0.0001-1 μM) and, as shown in Figure 3, there was no significant effect on the extent of IκB-α degradation achieved with TNFα in the presence of dexamethasone ($P<0.05$). Secondly, we examined whether dexamethasone repressed the translocation of the p65 NF-κB subunit into the nucleus. Cells were pretreated with a range of

dexamethasone concentrations (0.0001-1 μM) and the effect on TNFα-induced nuclear translocation assessed. As shown in Figure 4, corticosteroids have no effect on the nuclear entry of p65 NF-κB induced by TNFα. Taken together, the results presented in Figures 3 and 4 confirm that corticosteroids do not act to repress the TNFα-induced NF-κB pathway.

3.4 Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1

Thus far we have shown that Pam3CSK4 reduces the anti-inflammatory efficacy of corticosteroids on TNFα-induced cytokine production without affecting GR nor p65 NF-κB translocation. We (Che et al., 2014; Manetsch et al., 2012b; Quante et al., 2008; Rahman et al., 2014) and others (Issa et al., 2007; King et al., 2009) have shown that a major mechanism responsible for the anti-inflammatory action of corticosteroids is via the upregulation of MAPK-deactivating phosphatase – MKP-1; thus, we investigated whether impaired MKP-1 upregulation was responsible for corticosteroid insensitivity induced by bacterial ligand Pam3CSK4. This is explored in Figure 5, where MKP-1 upregulation in whole cell lysates of A549 cells was compared by Western blotting (Figure 5A) and analyzed by densitometry (Figure 5B). We demonstrate that TNFα induces significant upregulation of MKP-1 protein (3.2±0.5-fold compared to vehicle-treated cells (designated as 1) (P<0.05)); in alignment with previous studies in A549 cells (King et al., 2009). MKP-1 is corticosteroid-inducible (King et al., 2009) and accordingly, TNFα-induced MKP-1 in A549 cells pretreated with dexamethasone is significantly enhanced at 0.01-1 μM (Figure 5B: P<0.05). However, when we examine the influence of Pam3CSK4 pretreatment on MKP-1 upregulation there was no significant effect; as demonstrated by Western blotting (Figure 5A) and confirmed by densitometric analysis (Figure 5B). Thus, our hypothesis that the bacterial ligand Pam3CSK4 attenuated upregulation of MKP-1 protein was not supported.

3.5 Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1

However, MKP-1 protein is subject to redox regulation (Hou et al., 2008; Kamata et al., 2005) as the catalytic cysteine responsible for phosphatase action can be oxidized and thus reduce anti-inflammatory function (Bonham and Vacratsis, 2009). Notably, Pam3CSK4 has been shown to increase oxidative stress in a TLR2-mediated manner (Singh et al., 2015). To test this in A549 cells we measured the expression of heme oxygenase 1 as a surrogate measure of oxidative stress. HO-1 is an inducible enzyme that can be detected by Western blotting and a recognised marked of oxidative stress (Kamalvand et al., 2003). We examined the effect of Pam3CSK4, alone and in combination with TNF, on HO-1 protein upregulation, and as shown in Figure 6, Pam3CSK4 significantly increases HO-1 protein upregulation as detected by Western blotting (Figure 6A) and assessed densitometrically (Figure 6B)($P<0.05$). The increases in HO-1 upregulation were similar to those achieved with 100 µM H_2O_2 (Figure 6), a positive control for oxidative stress

We were now intrigued to examine whether MKP-1 expressed under these conditions may be oxidized. To do this, we utilized a technique referred to as PROP (purification of reversibly oxidized proteins) (Templeton et al., 2010; Victor et al., 2012). A549 cells were treated under conditions established above to induce corticosteroid insensitivity (dexamethasone (at 10 nM) + TNFα without/with Pam3CSK4) in order to ask the question - is the MKP-1 produced in cells that have been pretreated with Pam3CSK4 oxidized? In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1. As shown in Figures 7A and 7B, and in confirmation with our earlier data (Figure 5), Pam3CSK4 had no significant effect on the dexamethasone + TNFα-induced

upregulation of MKP-1 detected in whole cell lysates (see native MKP-1 (WCL)). In contrast, there was a significant (P<0.05) increase in oxidized MKP-1 detected by the PROP technique (Figure 7B). To examine whether this oxidized MKP-1 resulted in altered MKP-1 activity and an impact on downstream effectors, we examined the phosphorylation of MAPK superfamily members (p38 MAPK, ERK and JNK) under identical conditions. These data are shown by Western blot in Figure 7C and densitometrically in Figure 7D, where we see a small but significant increase in p38 MAPK phosphorylation in the presence of Pam3CSK4 (P<0.05). ERK phosphorylation was unaffected, while there was some variability in the JNK response. In cells pretreated with the widely-used positive control for oxidative stress – H_2O_2 (Kamata et al., 2005; Templeton et al., 2010; Victor et al., 2012) before TNFα, we can confirm that the PROP technique detects increases in oxidized MKP-1 (Figures 7E and 7F). Taken together these results suggest that MKP-1 oxidation induced by Pam3CSK4 may be a contributing factor in corticosteroid insensitivity.

3.6 Alternative anti-inflammatory approaches to overcome corticosteroid insensitivity: known and novel PP2A activators

By modeling bacterial exacerbation *in vitro* we have demonstrated that exposure to a bacterial ligand induces corticosteroid insensitivity. We revealed, in part, a contributing molecular mechanism (oxidation of MKP-1) and show that translocation of GR and p65 NF-κB is unaffected. We now turn to exploring alternative anti-inflammatory approaches and hypothesize that PP2A activators, being non-steroidal, may prove to inhibit cytokine production when corticosteroid insensitivity exists. To address this we examine the impact of two compounds: FTY720 and theophylline. FTY720 is a well-established activator of PP2A (Perrotti and Neviani, 2013; Rahman et al., 2015); while theophylline has only been recently reported by us (Patel et al., 2015) to function as a novel activator of PP2A. The

concentrations of FTY720 and theophylline utilized in this study were in accordance with previous publications (Patel et al., 2015; Rahman et al., 2015). We show that FTY720 (2.5 μM) in combination with dexamethasone (0.001-1 μM) achieves significant repression of IL-6 with lower concentrations of corticosteroids (Figure 8A), and inhibits IL-6 secretion in cells where corticosteroid insensitivity has been established by exposure to the bacterial ligand Pam3CSK4 (Alkhouri et al., 2014; Manetsch et al., 2012c)(Figure 8B) (P<0.05). At a concentration of 10 μM, theophylline is anti-inflammatory and can repress TNFα-induced IL-6 secretion (Figure 9A); in confirmation of our earlier publication (Patel et al., 2015). It also appeared to have an additive repressive effect together with corticosteroids, although this difference was not significant. However, theophylline can still significantly repress IL-6 secretion even when corticosteroid insensitivity exists (Figure 9B: P<0.05). Thus, PP2A activators are a non-steroidal anti-inflammatory alternative and/or corticosteroid-sparing approach in respiratory inflammation where corticosteroid insensitivity exists.

4. Discussion

Corticosteroid insensitivity is a major factor limiting treatment of chronic respiratory disease today. The relative responsiveness to corticosteroids can be influenced by a range of endogenous and exogenous factors, including respiratory infections. In this study we modeled bacterial infection *in vitro* and showed for the first time that ligation of the TLR2 receptor with a synthetic bacterial lipoprotein induces corticosteroid insensitivity. We then explored the cellular mechanisms responsible and demonstrate that, unlike rhinoviral-induced corticosteroid insensitivity, Pam3CSK4 did not perturb GR nuclear translocation. Notably, while the overall amount of native MKP-1 upregulation protein produced under these conditions was unaffected by Pam3CSK4 pretreatment, a significant proportion of MKP-1 was oxidized and consequently was rendered inactive. Finally, we explored alternative modalities to repress cytokine production and demonstrate the utility of PP2A activators as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

When a person with chronic lung disease (such as asthma and COPD) experiences an infection their lung function deteriorates and they require greater amounts of their anti-inflammatory medicines to treat their respiratory symptoms. This is because viral or bacterial infection changes the molecular pathways by which respiratory medicines act; making them less sensitive. These changes are due to interaction of viral and bacterial products with pattern recognition receptors, particularly TLRs expressed on airway cells (reviewed in (Saturni et al., 2015)). We have focused on mimicking bacterial infection *in vitro* with Pam3CSK4 and have previously shown that TLR2 ligation robustly increased TNFα-induced secretion of cytokines implicated as playing a role in exacerbation (Manetsch et al., 2012c). Moreover, TLR2 activation causes tachyphylaxis to bronchodilatory β_2-agonists *in vitro* and *ex vivo* (Alkhouri et al., 2014). In the current study, we reveal an additional detrimental

consequence of bacterial infection beyond amplification of inflammation and β_2-adrenergic desensitization; namely, we show that Pam3CSK4 induces corticosteroid insensitivity. This *in vitro* evidence is in support of a recent study examining clinical samples from people with COPD that linked increased TLR2 with this corticosteroid insensitive condition (Simpson et al., 2013).

However, the molecular mechanisms responsible differ from those reported for corticosteroid insensitivity induced in A549 cells by rhinoviral infection (Papi et al., 2013). Corticosteroids initiate their effects on gene expression by interacting with cytoplasmic GRs. Translocation of GR into the nucleus is a requisite step to allow GR to act as a ligand-dependent transcription factor. Once in the nucleus, ligand-activated GRs then repress inflammation by: (i) activation of glucocorticoid response elements and upregulation of anti-inflammatory genes, including MKP-1 and glucocorticoid-inducible leucine zipper 1; (ii) inhibitory interactions with proinflammatory DNA-binding transcription factors, such as NF-κB; and (iii) effects on recruitment of transcriptional co-activators, co-repressors and the chromatin machinery (reviewed in (Ammit, 2013)). Papi *et al.* (Papi et al., 2013) showed that rhinoviral infection reduced GR nuclear translocation, thus levels of MKP-1 upregulation was lower. Additionally, they demonstrated induction of MAPK signaling pathways (JNK in particular) and NF-κB activation. In contrast, we show that GR translocation was unaffected by Pam3CSK4 and that the upregulation of MKP-1 in whole cell lysates was unchanged. We also confirm that corticosteroids have no effect on TNFα-induced IκB-α degradation and p65 NF-κB nuclear entry, excluding the possibility that corticosteroid insensitivity is due to inhibitory interactions with the pro-inflammatory DNA-binding transcription factors, particularly NF-κB.

However, just examining the overall protein levels of MKP-1 may not reveal the entire story. It is increasingly recognized that the MAPK-deactivating ability of dual-specificity

phosphatases (with MKP-1, aka DUSP1, being a founding member of this family) can be regulated post-translationally by oxidation (Bonham and Vacratsis, 2009). The catalytic cysteine of MKP-1 can be modified by oxidation, thereby reducing its function as phosphatase. Redox regulation of MKP-1 has been demonstrated *in vitro* (Hou et al., 2008; Kamata et al., 2005) and highlighted as a potential mechanism responsible for corticosteroid insensitivity in our recent *in vivo* study exposing MKP-1 wild-type and knock-out mice to ozone (Pinart et al., 2014). Notably, Pam3CSK4 can increase oxidative stress in a TLR2-mediated manner (Singh et al., 2015) and herein we show that Pam3CSK4 increases production of a surrogate marker for oxidative stress, HO-1. Therefore we utilized the PROP technique to show that a proportion of MKP-1 expressed under these conditions is oxidized. This results in altered MKP-1 activity with impact on downstream effectors; notably p38MAPK phosphorylation is increased under these conditions. Taken together, these results suggest that Pam3CSK4-mediated oxidation of MKP-1 may contribute to corticosteroid insensitivity.

Finally, we explored alternative means of repressing cytokines when corticosteroid insensitivity exists. We focused on small molecules that are known and novel activators of the ubiquitous serine-threonine phosphatase – PP2A. PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling, including MAPKs and NF-κB (Junttila et al., 2008; Miskolci et al., 2003; Rahman et al., 2015; Shanley et al., 2001). A number of small molecules can activate PP2A (Perrotti and Neviani, 2013). The best known of these is FTY720, and we recently showed that it activates TNFα-induced PP2A enzymatic activity and represses cytokine production in A549 cells (Rahman et al., 2015). In the current study we examine its efficacy when used as an adjunct therapy with corticosteroids *in vitro* and show that FTY720 can act additively in a corticosteroid-sparing manner. Most importantly in the context of the bacterial exacerbation, FTY720 can significantly repress cytokine

production when corticosteroid insensitivity exists. Lastly, we explored the potential effects of theophylline. Theophylline has beneficial effects in asthma and COPD and acts via a number of different molecular pathways (reviewed in (Barnes, 2013)). We recently reported a new anti-inflammatory function for theophylline; it increases TNFα-induced PP2A enzymatic activity in A549 cells, as well as primary cultures of airway smooth muscle (Patel et al., 2015). Thus, we examined its impact on corticosteroid insensitivity and although the impact was not as great as FTY720, theophylline was able overcome corticosteroid insensitivity. It is important to note however, that we believe that these repressive effects on cytokine function exerted by FTY720 and theophylline are anti-inflammatory effects acting via alternative, PP2A-dependent pathways, rather than restoration of corticosteroid sensitivity, although further investigation is warranted.

Collectively, our study shows that, like rhinoviral infection modeled *in vitro*, bacterial ligands exert corticosteroid insensitivity. These models allow us to explore the molecular mechanisms responsible and highlight potential therapeutic strategies to combat inflammation in infectious exacerbation. We reveal that oxidation of anti-inflammatory MKP-1, rather than retardation of nuclear translation, occurs via TLR2-mediated pathways. Finally, we suggest that activating PP2A may prove to be efficacious, non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in the context of bacterial exacerbation.

Acknowledgements

This research was supported by National Health and Medical Research Council of Australia (APP1025637) and philanthropic funding from Mr Maurice Renshaw. Md. Mostafizur Rahman was supported by an Endeavour Postgraduate Award; Pavan Prabhala received scholarship support from Mr Renshaw; Nowshin Nowaz Rumzhum received an International Postgraduate Research Scholarship; Brijeshkumar S. Patel was supported by an Australian Postgraduate Award.

References

Alkhouri, H., Rumzhum, N.N., Rahman, M.M., FitzPatrick, M., de Pedro, M., Oliver, B.G., Bourke, J.E., Ammit, A.J., 2014. TLR2 activation causes tachyphylaxis to beta2 -agonists in vitro and ex vivo: modelling bacterial exacerbation. Allergy 69(9), 1215-1222.

Ammit, A.J., 2013. Glucocorticoid insensitivity as a source of drug targets for respiratory disease. Curr Opin Pharmacol 13(3), 370-376.

Barnes, P.J., 2013. Theophylline. Am. J. Respir. Crit. Care Med. 188(8), 901-906.

Bonham, C.A., Vacratsis, P.O., 2009. Redox Regulation of the Human Dual Specificity Phosphatase YVH1 through Disulfide Bond Formation. The Journal of Biological Chemistry 284(34), 22853-22864.

Che, W., Parmentier, J., Seidel, P., Manetsch, M., Ramsay, E.E., Alkhouri, H., Ge, Q., Armour, C.L., Ammit, A.J., 2014. Corticosteroids inhibit sphingosine 1-phosphate-induced interleukin-6 secretion from human airway smooth muscle via mitogen-activated protein kinase phosphatase 1-mediated repression of mitogen and stress-activated protein kinase 1. Am. J. Respir. Cell Mol. Biol. 50(2), 358-368.

Chung, K.F., 2013. New treatments for severe treatment-resistant asthma: targeting the right patient. The Lancet Respiratory Medicine 1(8), 639-652.

Cornell, T.T., Hinkovska-Galcheva, V., Sun, L., Cai, Q., Hershenson, M.B., Vanway, S., Shanley, T.P., 2009. Ceramide-dependent PP2A regulation of TNFalpha-induced IL-8 production in respiratory epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 296(5), L849-856.

Hirota, J.A., Im, H., Rahman, M.M., Rumzhum, N.N., Manetsch, M., Pascoe, C., Bunge, K., Alkhouri, H., Oliver, B.G., Ammit, A.J., 2013. The NLRP3 Inflammasome is not

Activated in Airway Smooth Muscle Upon TLR2 Ligation. Am. J. Respir. Cell Mol. Biol. 49(4), 517-524.

Hou, N., Torii, S., Saito, N., Hosaka, M., Takeuchi, T., 2008. Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. Endocrinology 149(4), 1654-1665.

Issa, R., Xie, S.P., Khorasani, N., Sukkar, M., Adcock, I.M., Lee, K.Y., Chung, K.F., 2007. Corticosteroid inhibition of growth-related oncogene protein-alpha via mitogen-activated kinase phosphatase-1 in airway smooth muscle cells. J. Immunol. 178(11), 7366-7375.

Junttila, M.R., Li, S.-P., Westermarck, J., 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 22(4), 954-965.

Kamalvand, G., Pinard, G., Ali-Khan, Z., 2003. Heme-oxygenase-1 response, a marker of oxidative stress, in a mouse model of AA amyloidosis. Amyloid 10(3), 151-159.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., Karin, M., 2005. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120(5), 649-661.

King, E.M., Holden, N.S., Gong, W., Rider, C.F., Newton, R., 2009. Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. J. Biol. Chem. 284(39), 26803-26815.

Manetsch, M., Che, W., Seidel, P., Chen, Y., Ammit, A.J., 2012a. MKP-1: a negative feedback effector that represses MAPK-mediated pro-inflammatory signaling pathways and cytokine secretion in human airway smooth muscle cells. Cell. Signal. 24(4), 907-913.

Manetsch, M., Ramsay, E., King, E., Seidel, P., Che, W., Ge, Q., Hibbs, D., Newton, R., Ammit, A., 2012b. Corticosteroids and beta(2) -agonists upregulate mitogen-activated protein kinase phosphatase 1: in vitro mechanisms. Br. J. Pharmacol. 166(7), 2049-2059.

Manetsch, M., Seidel, P., Heintz, U., Che, W., Hughes, J.M., Ge, Q., Sukkar, M.B., Ammit, A.J., 2012c. TLR2 ligand engagement upregulates airway smooth muscle TNFalpha-induced cytokine production. Am J Physiol Lung Cell Mol Physiol 302(9), L838-845.

Marwick, J.A., Chung, K.F., 2010. Glucocorticoid insensitivity as a future target of therapy for chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 5, 297-309.

Miskolci, V., Castro-Alcaraz, S., Nguyen, P., Vancura, A., Davidson, D., Vancurova, I., 2003. Okadaic acid induces sustained activation of NFκB and degradation of the nuclear IκBα in human neutrophils. Arch. Biochem. Biophys. 417(1), 44-52.

Papi, A., Contoli, M., Adcock, I.M., Bellettato, C., Padovani, A., Casolari, P., Stanciu, L.A., Barnes, P.J., Johnston, S.L., Ito, K., Caramori, G., 2013. Rhinovirus infection causes steroid resistance in airway epithelium through nuclear factor κB and c-Jun N-terminal kinase activation. J. Allergy Clin. Immunol. 132(5), 1075-1085.e1076.

Patel, B.S., Rahman, M.M., Rumzhum, N.N., Oliver, B.G., Verrills, N.M., Ammit, A.J., 2015. Theophylline Represses IL-8 Secretion From ASM Cells Independently of PDE Inhibition: Novel Role as a PP2A Activator. Am. J. Respir. Cell Mol. Biol.

Perrotti, D., Neviani, P., 2013. Protein phosphatase 2A: a target for anticancer therapy. Lancet Oncol 14(6), e229-e238.

Pinart, M., Hussain, F., Shirali, S., Li, F., Zhu, J., Clark, A.R., Ammit, A.J., Chung, K.F., 2014. Role of mitogen-activated protein kinase phosphatase-1 in corticosteroid insensitivity of chronic oxidant lung injury. Eur. J. Pharmacol. 744, 108-114.

Quante, T., Ng, Y.C., Ramsay, E.E., Henness, S., Allen, J.C., Parmentier, J., Ge, Q., Ammit, A.J., 2008. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. Am. J. Respir. Cell Mol. Biol. 39(2), 208-217.

Rahman, M.M., Alkhouri, H., Tang, F., Che, W., Ge, Q., Ammit, A.J., 2014. Sphingosine 1-phosphate induces neutrophil chemoattractant IL-8: repression by steroids. PLoS One 9(3), e92466.

Rahman, M.M., Rumzhum, N.N., Hansbro, P.M., Morris, J.C., Clark, A.R., Verrills, N.M., Ammit, A.J., 2016. Activating protein phosphatase 2A (PP2A) enhances tristetraprolin (TTP) anti-inflammatory function in A549 lung epithelial cells. Cell. Signal. 28(4), 325-334.

Rahman, M.M., Rumzhum, N.N., Morris, J.C., Clark, A.R., Verrills, N.M., Ammit, A.J., 2015. Basal protein phosphatase 2A activity restrains cytokine expression: role for MAPKs and tristetraprolin. Scientific reports 5, 10063.

Saturni, S., Contoli, M., Spanevello, A., Papi, A., 2015. Models of Respiratory Infections: Virus-Induced Asthma Exacerbations and Beyond. Allergy Asthma Immunol Res 7(6), 525-533.

Shanley, T.P., Vasi, N., Denenberg, A., Wong, H.R., 2001. The Serine/Threonine Phosphatase, PP2A: Endogenous Regulator of Inflammatory Cell Signaling. J. Immunol. 166(2), 966-972.

Simpson, J.L., McDonald, V.M., Baines, K.J., Oreo, K.M., Wang, F., Hansbro, P.M., Gibson, P.G., 2013. Influence of age, past smoking, and disease severity on TLR2, neutrophilic inflammation, and MMP-9 levels in COPD. Mediators Inflamm. 2013, 462934.

Singh, A., Singh, V., Tiwari, R.L., Chandra, T., Kumar, A., Dikshit, M., Barthwal, M.K., 2015. The IRAK-ERK-p67phox-Nox-2 axis mediates TLR4, 2-induced ROS

production for IL-1beta transcription and processing in monocytes. Cellular & molecular immunology.

Templeton, D.J., Aye, M.S., Rady, J., Xu, F., Cross, J.V., 2010. Purification of reversibly oxidized proteins (PROP) reveals a redox switch controlling p38 MAP kinase activity. PLoS One 5(11), e15012.

Victor, K.G., Rady, J.M., Cross, J.V., Templeton, D.J., 2012. Proteomic profile of reversible protein oxidation using PROP, purification of reversibly oxidized proteins. PLoS One 7(2), e32527.

Figure Legends

Figure 1. TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 µg/ml), followed by vehicle or dexamethasone (0.0001-1 µM) for 30 min. After stimulation with TNFα (4 ng/ml) for 24 h, IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant reduction of corticosteroid-mediated repression by Pam3CSK4 (P<0.05)). Data are mean+SEM values from n=9 independent experiments.

Figure 2. Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 µg/ml), followed by vehicle or dexamethasone (0.0001-1 µM) for 30 min. GR translocation was examined in the (A) absence or (B) or presence of stimulation with TNFα (4 ng/ml) for 1 h. Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR determined by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. Data are representative blots from n=3 independent experiments.

Figure 3. Corticosteroids have no effect on TNFα-induced IκB-α degradation. A549 cells were pretreated with vehicle or dexamethasone (0.0001-1 µM) for 30 min. Cells were then stimulated without and with TNFα (4 ng/ml) for 1 h and the impact on IκB-α degradation examined by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of IκB-α degradation (expressed as a percentage of IκB-α measured in vehicle-treated cells, normalized to α-tubulin). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple

comparison test (where * denotes significant degradation of IκB-α induced by TNFα (P<0.05); there was no significant effect of dexamethasone). Data are mean+SEM values from n=3 independent experiments.

Figure 4. Corticosteroids have no effect on TNFα-induced p65 NF-κB nuclear translocation. A549 cells were pretreated with vehicle or dexamethasone (0.0001-1 μM) for 30 min. Cells were then stimulated without and with TNFα (4 ng/ml) for 1 h, nuclear protein extracts prepared and entry of p65 NF-κB into the nucleus determined by Western blotting, compared with lamin A/C as a loading control. (A) Results are representative Western blots and (B) densitometric analysis of p65 NF-κB nuclear translocation (expressed as fold increase in p65 NF-κB in the nucleus of vehicle-treated cells, normalized to lamin A/C). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant p65 NF-κB nuclear translocation induced by TNFα (P<0.05); there was no significant effect of dexamethasone). Data are mean+SEM values from n=3 independent experiments.

Figure 5. Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1. A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle or dexamethasone (0.0001-1 μM) for 30 min. Cells were treated without and with TNFα (4 ng/ml) for 1 h and the impact on MKP-1 upregulation compared by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to vehicle-treated cells). Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant

increase in MKP-1 protein upregulation induced by TNFα, and § denotes significant potentiation of TNFα-induced MKP-1 by dexamethasone (P<0.05); there was no effect of Pam3CSK4). Data are mean+SEM values from n=3 independent experiments.

Figure 6. Pam3CSK4 increases heme oxygenase 1 (HO-1) protein upregulation: a marker of oxidative stress. A549 cells were treated for 2 h with either vehicle, Pam3CSK4 (1 µg/ml) or H_2O_2 (100 µM) as a positive control for oxidative stress. HO-1 upregulation was examined by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometric analysis of HO-1 protein upregulation (expressed as a fold increase compared to vehicle-treated cells, normalized to α-tubulin). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant upregulation of HO-1 induced by both Pam3CSK4 and H_2O_2 (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 7. Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1. (A-D) A549 cells were pretreated for 30 min with either vehicle or Pam3CSK4 (1 µg/ml), followed by dexamethasone (0.01 µM) for 30 min then TNFα (4 ng/ml) for 1 h. (A, B) In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates (WCL) to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1 and Western blotting performed. (C, D) To demonstrate the impact of oxidized MKP-1 on downstream effectors, the phosphorylation of MAPK family members (p38 MAPK, ERK and JNK) in WCL were measured by Western blotting (normalized to total p38 MAPK, ERK and JNK, respectively). (E, F) As a positive control for oxidative stress, A549 cells were pretreated for 30 min

without and with 100 μM H_2O_2 before stimulation with TNFα (4 ng/ml) for 1 h and processed as above (A, B). (A, C, E) Results are representative Western blots and (B, D, F) densitometric analysis (results expressed as fold increase compared to cells treated without (A-D) Pam3CSK4 or (E-F) H_2O_2). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test or Student's unpaired *t* test (where * denotes significant increase (P<0.05). Data are mean+SEM values from n=3-4 independent experiments.

Figure 8. FTY720 is corticosteroid-sparing and overcomes corticosteroid insensitivity. A549 were pretreated for 6 h with vehicle or FTY720 (2.5 μM), before 30 min with (A) vehicle or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min. After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of FTY720 (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 9. Theophylline is anti-inflammatory and can overcome corticosteroid insensitivity. A549 were pretreated for 30 min with (A) vehicle or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min, without or with theophylline (10 μM). After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of theophylline (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

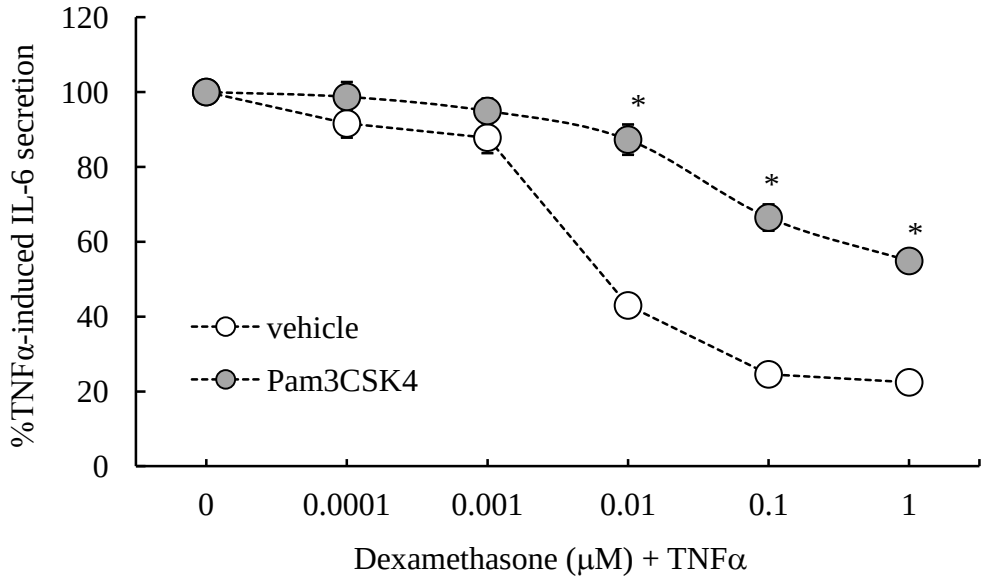

120
100
80
60
40
20
0
%TNFα-induced IL-6 secretion
vehicle
Pam3CSK4
*
*
*
0
0.0001
0.001
0.01
0.1
1
Dexamethasone (μM) + TNFα

Figure 1

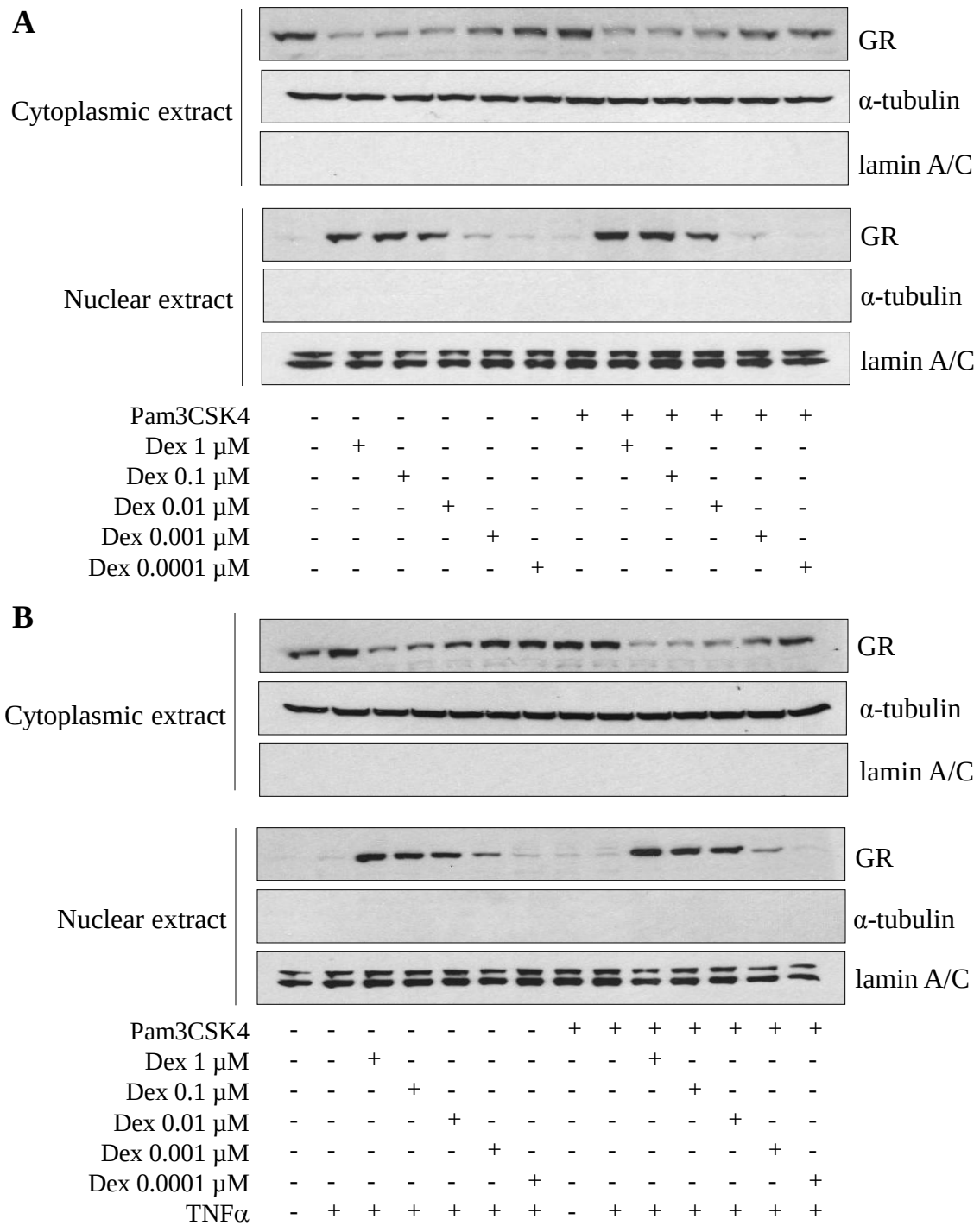

A
Cytoplasmic extract
GR
α-tubulin
lamin A/C
Nuclear extract
GR
α-tubulin
lamin A/C
Pam3CSK4 - - - - - - + + + + + +
Dex 1 μM - + - - - - - + - - - -
Dex 0.1 μM - - + - - - - - + - - -
Dex 0.01 μM - - - + - - - - - + - -
Dex 0.001 μM - - - - + - - - - - + -
Dex 0.0001 μM - - - - - + - - - - - +
B
Cytoplasmic extract
GR
α-tubulin
lamin A/C
Nuclear extract
GR
α-tubulin
lamin A/C
Pam3CSK4 - - - - - - - + + + + + + +
Dex 1 μM - - + - - - - - - + - - - -
Dex 0.1 μM - - - + - - - - - - + - - -
Dex 0.01 μM - - - - + - - - - - - + - -
Dex 0.001 μM - - - - - + - - - - - - + -
Dex 0.0001 μM - - - - - - + - - - - - - +
TNFα - + + + + + + - + + + + + +

Figure 2

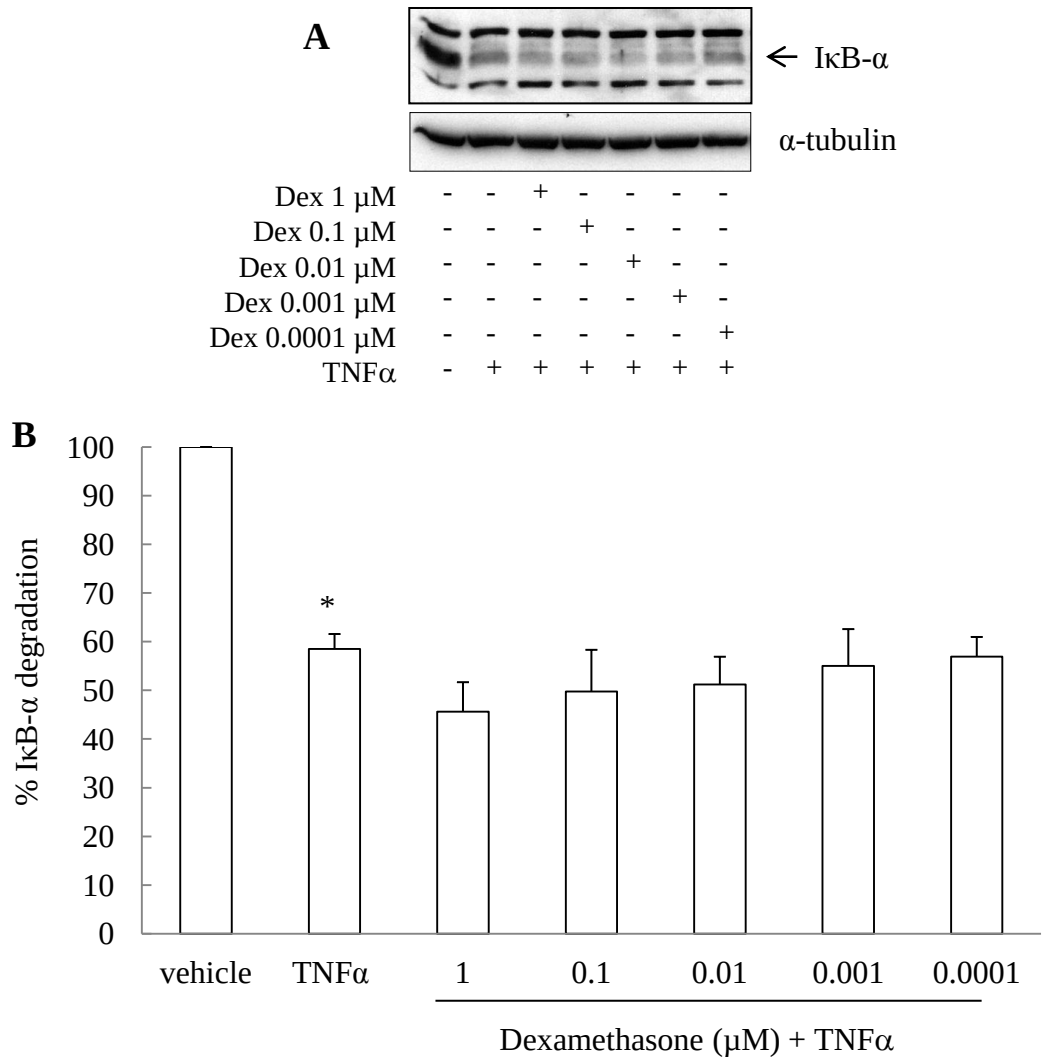

A
← IκB-α
α-tubulin
Dex 1 µM - - + - - - -
Dex 0.1 µM - - - + - - -
Dex 0.01 µM - - - - + - -
Dex 0.001 µM - - - - - + -
Dex 0.0001 µM - - - - - - +
TNFα - + + + + + +
B
% IκB-α degradation
100
90
80
70
60
50
40
30
20
10
0
*
vehicle
TNFα
1
0.1
0.01
0.001
0.0001
Dexamethasone (µM) + TNFα

Figure 3

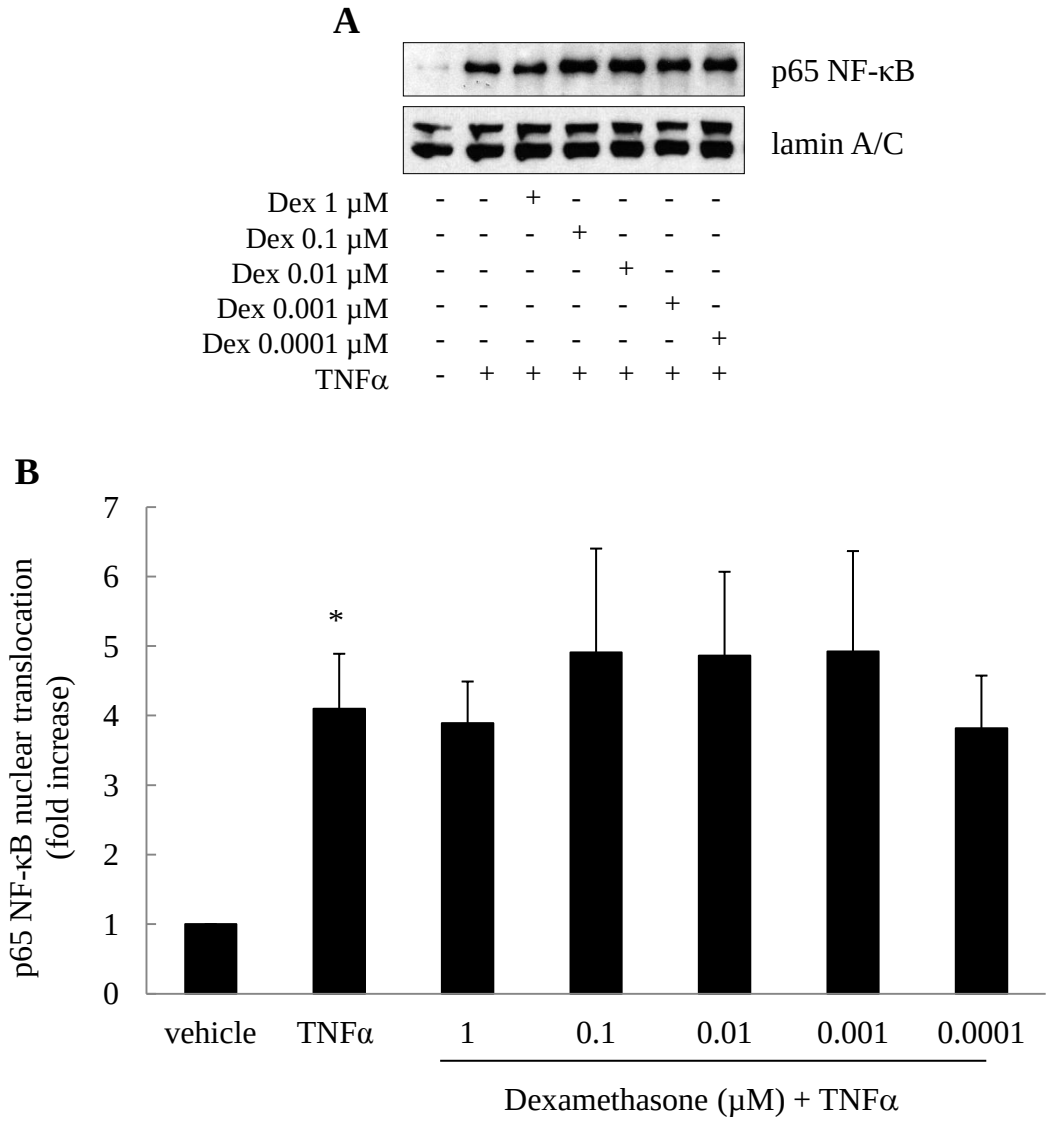

A
p65 NF-κB
lamin A/C
Dex 1 μM - - + - - - -
Dex 0.1 μM - - - + - - -
Dex 0.01 μM - - - - + - -
Dex 0.001 μM - - - - - + -
Dex 0.0001 μM - - - - - - +
TNFα - + + + + + +
B
p65 NF-κB nuclear translocation (fold increase)
0
1
2
3
4
5
6
7
*
vehicle
TNFα
1
0.1
0.01
0.001
0.0001
Dexamethasone (μM) + TNFα

Figure 4

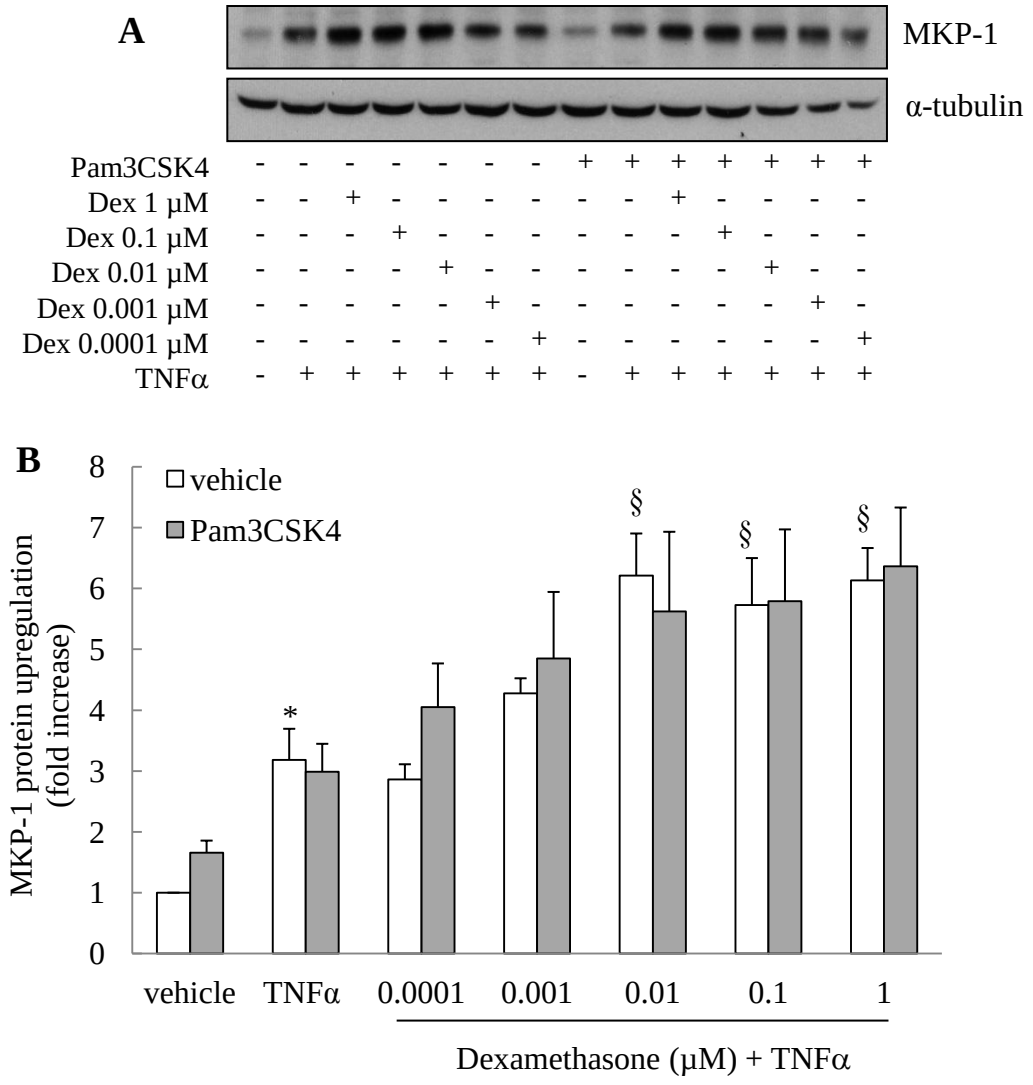

A
MKP-1
α-tubulin
Pam3CSK4 - - - - - - - + + + + + + +
Dex 1 µM - - + - - - - - - + - - - -
Dex 0.1 µM - - - + - - - - - - + - - -
Dex 0.01 µM - - - - + - - - - - - + - -
Dex 0.001 µM - - - - - + - - - - - - + -
Dex 0.0001 µM - - - - - - + - - - - - - +
TNFα - + + + + + + - + + + + + +
B
vehicle
Pam3CSK4
MKP-1 protein upregulation (fold increase)
0 1 2 3 4 5 6 7 8
*
§
§
§
vehicle TNFα 0.0001 0.001 0.01 0.1 1
Dexamethasone (µM) + TNFα

Figure 5

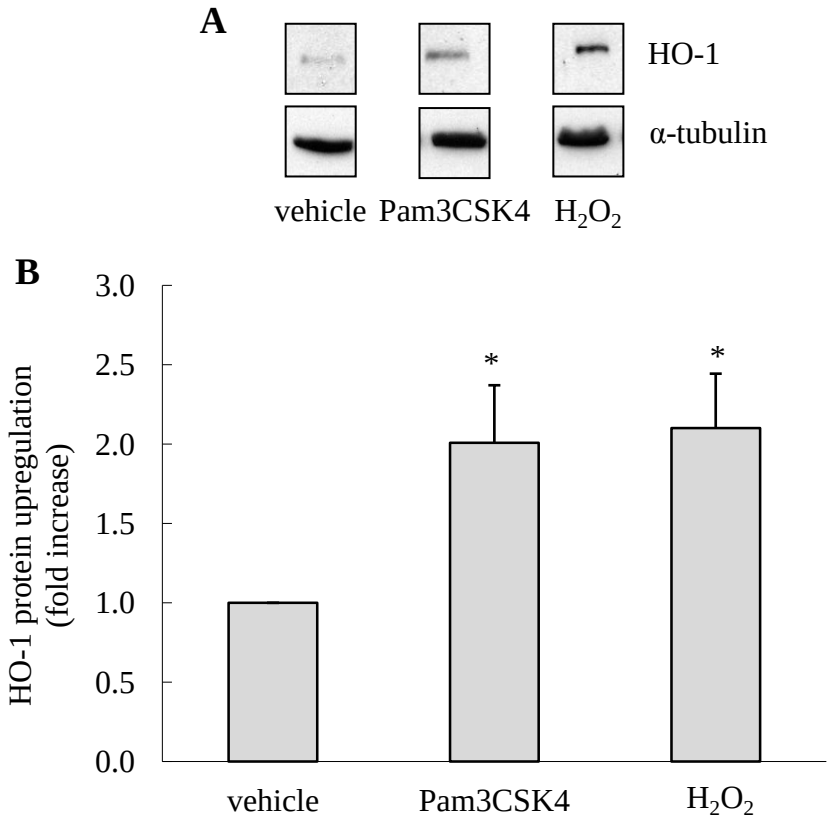

A
HO-1
α-tubulin
vehicle
Pam3CSK4
H2O2
B
HO-1 protein upregulation
(fold increase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
vehicle
Pam3CSK4
H2O2

Figure 6

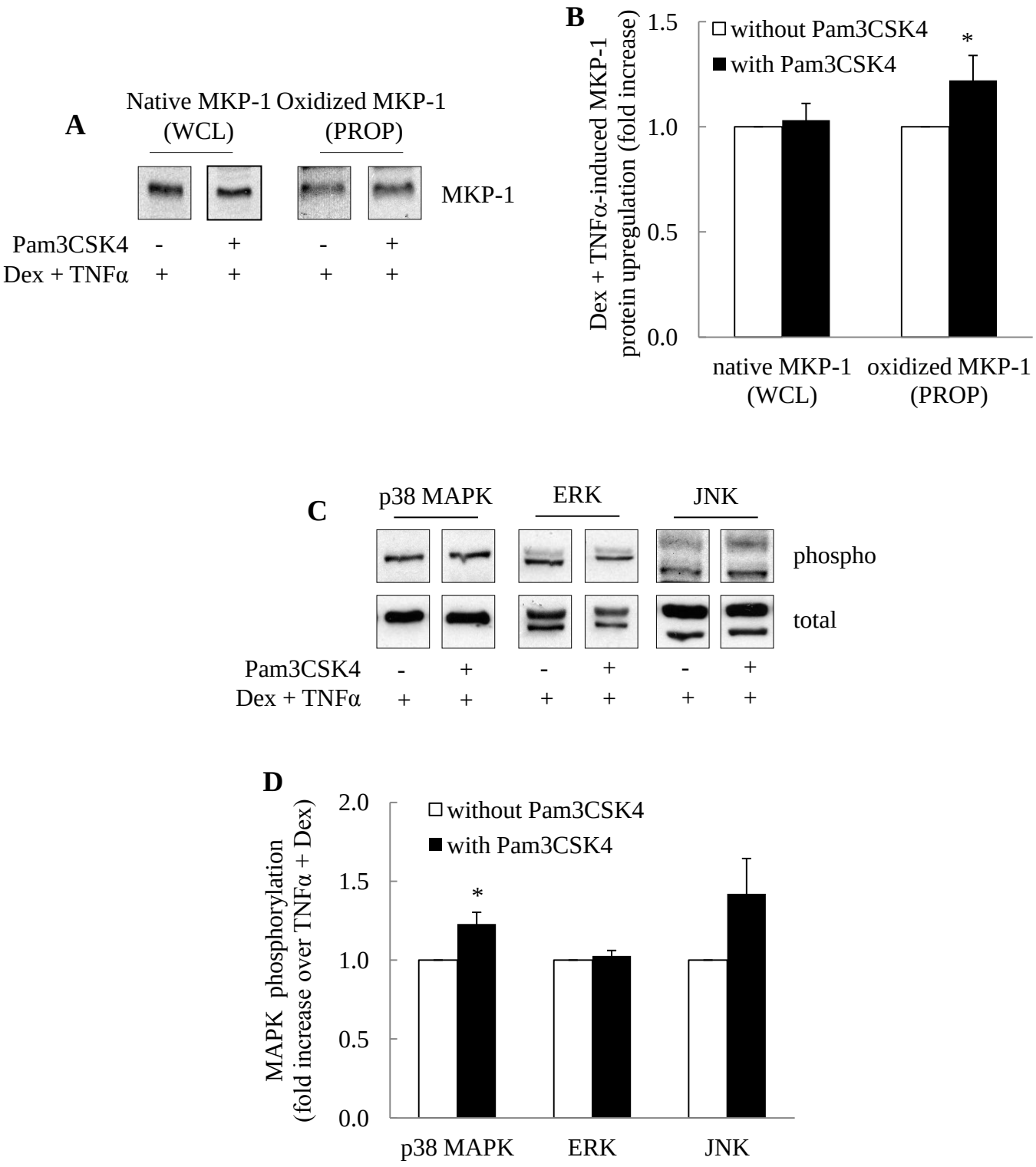

A
Native MKP-1 (WCL)
Oxidized MKP-1 (PROP)
MKP-1
Pam3CSK4 - + - +
Dex + TNFα + + + +
B
Dex + TNFα-induced MKP-1 protein upregulation (fold increase)
without Pam3CSK4
with Pam3CSK4
1.5
1.0
0.5
0.0
*
native MKP-1 (WCL)
oxidized MKP-1 (PROP)
C
p38 MAPK
ERK
JNK
phospho
total
Pam3CSK4 - + - + - +
Dex + TNFα + + + + + +
D
MAPK phosphorylation (fold increase over TNFα + Dex)
without Pam3CSK4
with Pam3CSK4
2.0
1.5
1.0
0.5
0.0
*
p38 MAPK
ERK
JNK

Figure 7

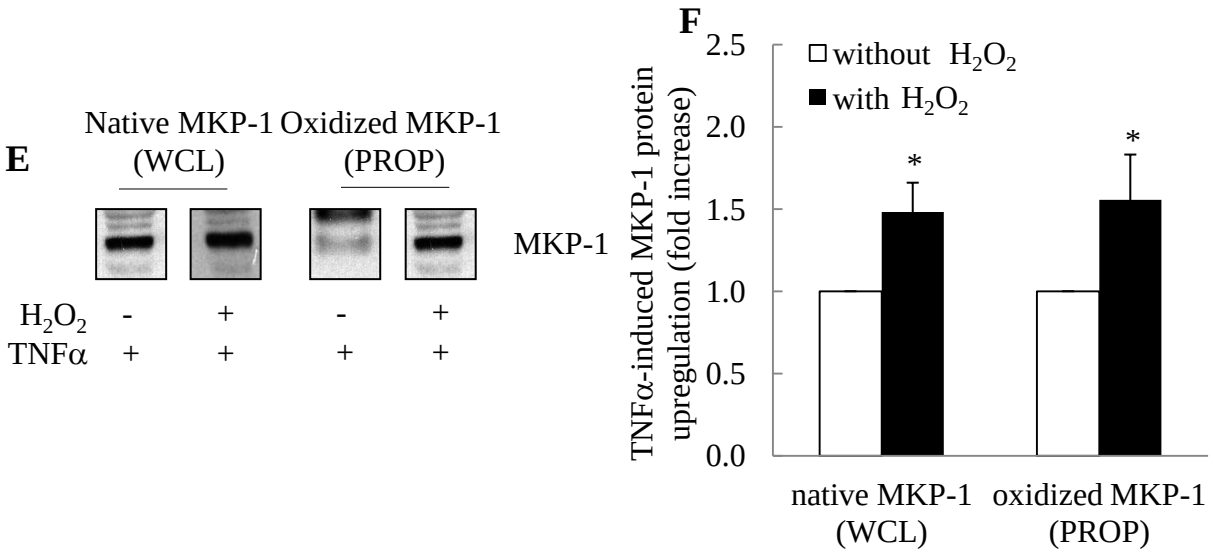


Figure 7 (continued)

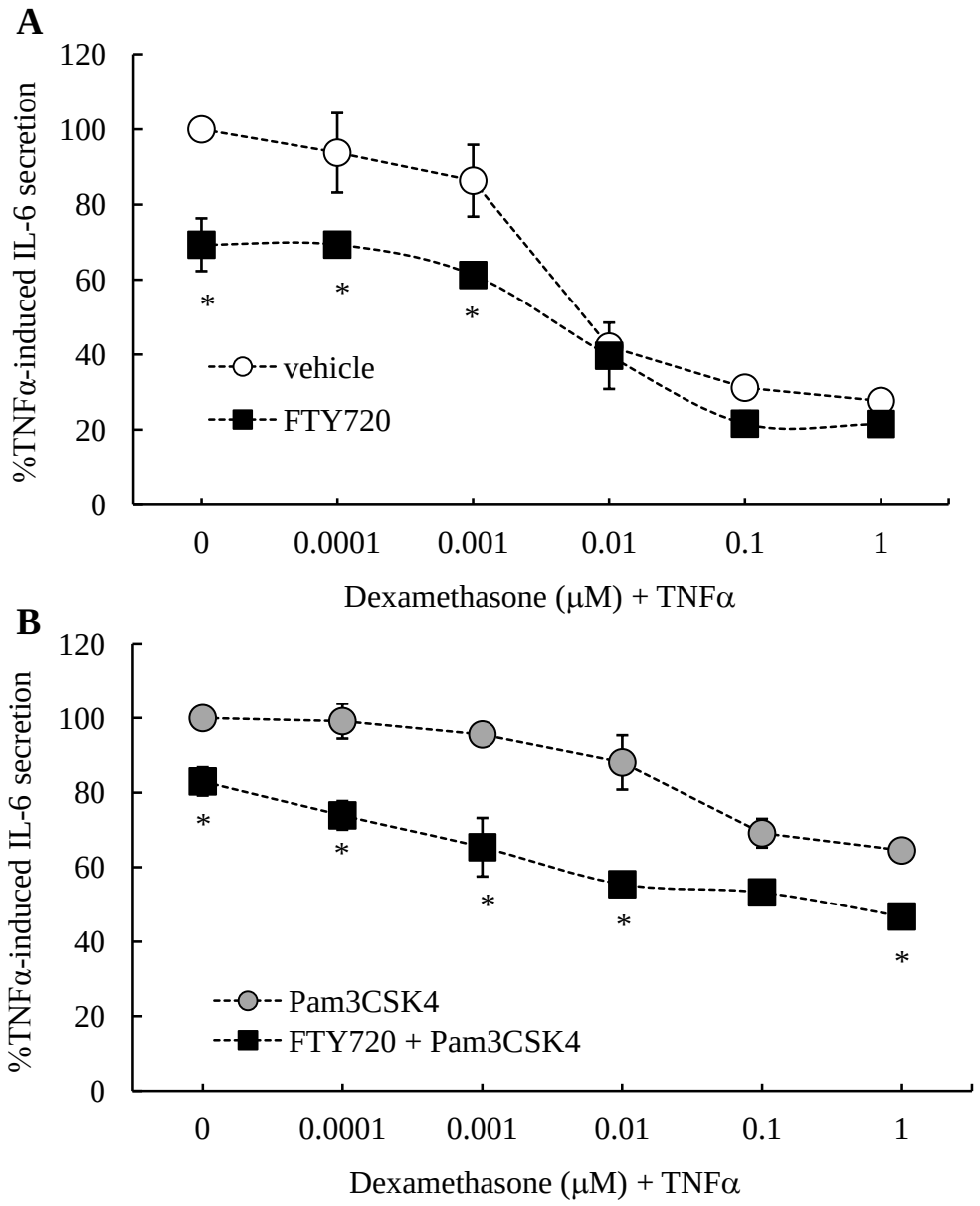

A
%TNFα-induced IL-6 secretion
120
100
80
60
40
20
0
vehicle
FTY720
*
*
*
0
0.0001
0.001
0.01
0.1
1
Dexamethasone (μM) + TNFα
B
%TNFα-induced IL-6 secretion
120
100
80
60
40
20
0
Pam3CSK4
FTY720 + Pam3CSK4
*
*
*
*
*
0
0.0001
0.001
0.01
0.1
1
Dexamethasone (μM) + TNFα

Figure 8

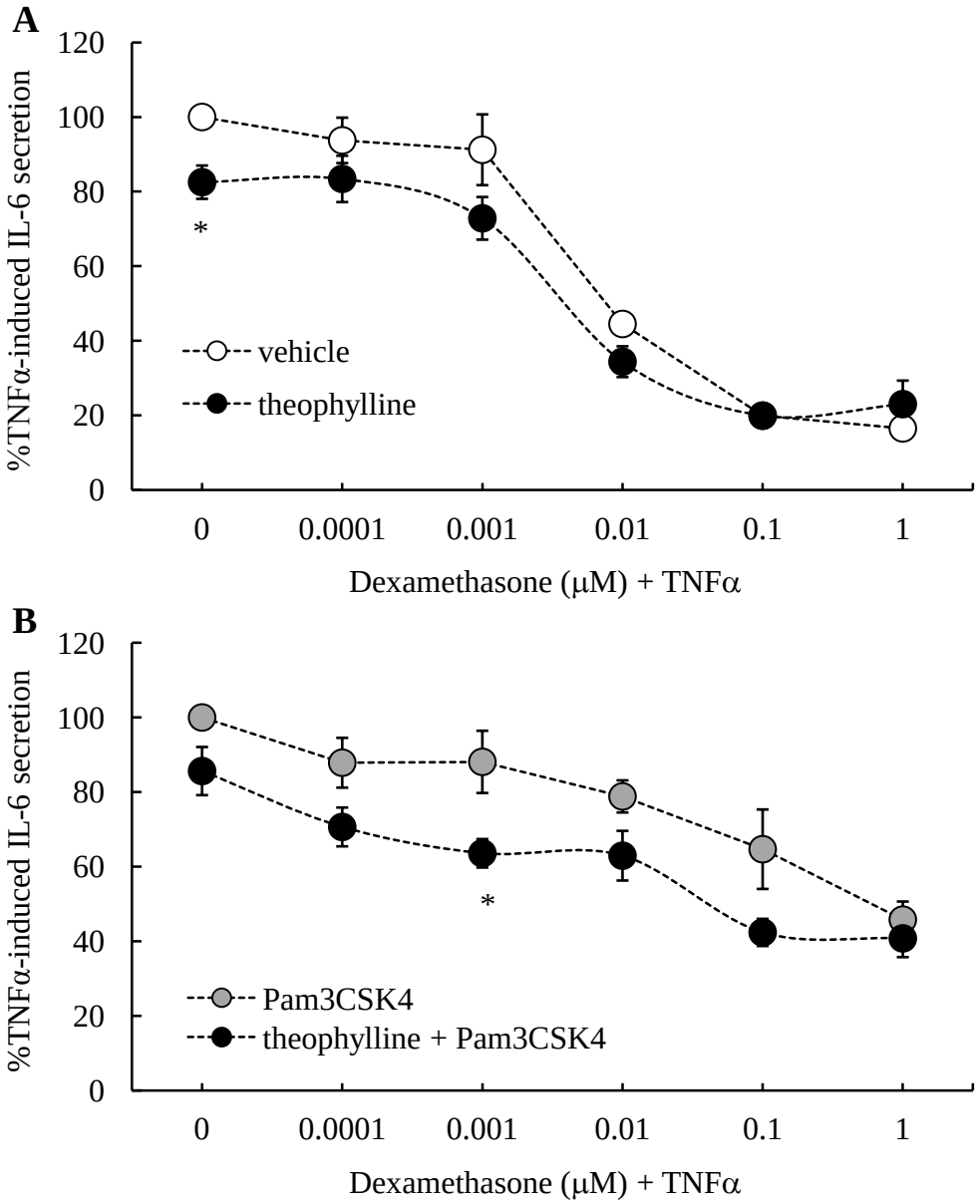

A
%TNFα-induced IL-6 secretion
120
100
80
60
40
20
0
*
vehicle
theophylline
0
0.0001
0.001
0.01
0.1
1
Dexamethasone (μM) + TNFα
B
%TNFα-induced IL-6 secretion
120
100
80
60
40
20
0
*
Pam3CSK4
theophylline + Pam3CSK4
0
0.0001
0.001
0.01
0.1
1
Dexamethasone (μM) + TNFα

Figure 9