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The Irreversible Inhibition of the MAPK^{p38} Pathway Downregulates LPS-augmented Release of Interleukin-Related Inflammatory Cytokines (IL-1β, IL-6): Immune Surveillance Unraveling IκB-α/NF-κB Phosphorylation State-independent Mechanism *in vitro*

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Abstract **Background:** The participation of signaling pathways involving the mitogen-activated protein kinases (MAPKs) in regulating the inflammatory response characterized by the release of cytokines is not well established in the alveolar epithelium. We have previously examined the effect of MAPK^{p38} blockade on the *in vitro* release of TNF-α, indicating the likely involvement of other pro-inflammatory cytokines. **Methods:** This study investigated the selective inhibition of MAPK^{p38} in modulating the release of interleukin-related inflammatory cytokines, including IL-1β and IL-6. LPS-mediated release of cytokines is closely associated with the blockade of MAPK by the compound SB203580, an irreversible and selective inhibitor of MAPK^{p38}, independent of MAPK$^{ERK\ (p42/p44)}$ and MAPK^{JNK}. **Results:** Pre-treatment with ascending concentrations of SB203580 (0.1 – 100 μM) prior to LPS administration downregulated/attenuated the release of IL-1β, IL-6 and TNF-α in a dose-dependent and dose-independent manners. Furthermore, unraveling the immune molecular pathways likely involved with MAPK^{p38}-mediated secretion of cytokines revealed that SB203580 increased IκB-α phosphorylation, where IκB-α is considered the main cytosolic inhibitor of the transcription factor involved with regulating the processes of cellular inflammation, NF-κB. This upregulation of the phosphorylation status of IκB-α was accompanied by downregulating the cytosolic accumulation of the non-phosphorylated form of IκB-α, indicating normal nuclear translocation of the associated transcription factor. **Conclusions:** These results show that MAPK^{p38} is required, at least in part, for the release of inflammatory cytokines induced by LPS, a mechanism that is independent of the phosphorylation of IκB-α. In addition, the MAPK^{p38}-dependent release of cytokines seemingly does not require the activation of the NF-κB pathway.

Keywords: *cytokines, electrophoresis, ELISA, IκB-α, IL-1β, IL-6, inflammation, MAPKs, NF-κB*

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1. Introduction

The mitogen-activated protein kinases (MAPKs) are regulatory enzymes that belong to a family of immune regulators that modulates myriad cellular functions in physiological and pathophysiological conditions [1]. Of those cellular activities governed by MAPKs, perhaps the regulation of inflammation and ensuing inflammatory conditions is the most prominent [2].

Furthermore, it is well established that the cellular inflammatory pathways are strictly regulated by transcription factors, specifically nuclear factor-κB (NF-κB). In the inactive state, NF-κB is held in the cytosol by inhibitory molecules, mainly IκB, the phosphorylation of which liberates the transcription factor ability to undergo nuclear translocation, DNA binding and activation, following which the inflammatory mechanism ensues [3]. The ensuing milieu of inflammatory markers include the secretion of pro-inflammatory cytokines, such as interleukins (ILs) and tumor necrosis factor (TNF).

Another regulatory mechanism involved with inflammation that is intertwined with the NF-κB pathway is MAPK^{p38}. This mechanism is perhaps a major regulatory lynchpin that ostensibly connects MAPK signaling with NF-κB nuclear translocation and activation [4]. Interestingly, blockade of the MAPK signaling pathway attenuated, but did not abrogate, the inflammatory reaction *in vitro* and *in vivo*, indicating that there is, at least in part,

a crosstalk between the two major pathways governing the process of inflammation and its ramifications [5].

The inflammatory signaling pathway as regulated by the NF-κB/MAPK nexus is essentially not well characterized in alveolar epithelial cells [5,6,7,8]. Moreover, the role of pro-inflammatory cytokines in mediating the inflammatory signal controlled by NF-κB/MAPK is not clear either [6]. This study is designed to unravel the likely involvement of the NF-κB/MAPK pathway in regulating the release of inflammatory cytokines augmented by lipopolysaccharide (LPS; endotoxin).

Importantly, selective inhibition of $MAPK^{p38}$ attenuated the release of IL-1β and IL-6, in a manner similar to reported observations with TNF-α [2], and that this downregulation is seemingly independent of the phosphorylation/activation states of IκB-α/NF-κB. These observations unprecedentedly separate the NF-κB/MAPK pathways in mediating LPS-induced production of inflammatory cytokines *in vitro*.

2. Materials and Methods

2.1. Chemicals and Reagents

In all experiments carried out, and unless otherwise specifically stated, chemicals, including $MAPK^{p38}$ inhibitor, of standardized analytical grade were obtained from Sigma-Aldrich. Technical procedures using live animals (*Sprague-Dawley* rats) were strictly followed and upheld in all experimental setups under the Animals (Scientific Procedures) Act, 1986 (UK).

2.2. Primary Cultures of Alveolar Epithelial Cells

For the preparation of cell cultures, fetal alveolar type II epithelial cells (FATEII) were isolated from lungs obtained from the fetuses of pregnant rats at day 19 of gestation, essentially as described elsewhere [2,6]. Epithelial cells were harvested and then grown (5×10^6) at ≈ 21% O_2/5% CO_2 atmosphere in pre-equilibrated serum-free PC-1 media for 24h at 37°C. The approximate adenylate energy charge (AEC), considered a reliable index of cell viability and competence, was determined at ≥ 0.7 and transepithelial monolayer resistance was subsequently monitored constant at ≥ 250-300 Ωcm^2 [2,6].

2.3. Lipopolysaccharide (LPS)-mediated Activation of Cytosolic IκB-α Phosphorylation and Selective Inhibition of $MAPK^{p38}$ by SB203580

The effect of LPS/SB203580 treatments on threonine/tyrosine phosphorylation of IκB-α was determined by pre-incubating cells for 1h with physiologic concentrations of SB203580 (0, 0.1, 1, 10, 100 μM) (Calbiochem, UK), a selective, irreversible inhibitor of $MAPK^{p38}$. Following pre-treatment with SB203580, cells were exposed to LPS (1 μg/ml) for 24h. Cell culture filters were washed and proteins (≈ 20 μg) were loaded per lanes and separated by SDS-PAGE (15%), then transferred by electrophoresis to nitrocellulose membranes, which were blotted with a specific antibody to *p*IκB-α. Blots were subsequently stripped/reprobed with phosphorylation-state independent IκB-α to determine the baseline levels [2]. The constitutive expression of β-actin as evidence of semi-quantitative loading per lane has also been determined. Cellular viability has been confirmed [6] and toxicity due to pre-treatments and/or treatments has been minimal and relatively insignificant [2].

2.4. Selective Inhibition of $MAPK^{p38}$ and LPS-induced Release of pro-inflammatory Cytokines Quantified by Sandwich ELISA

Cells were pre-incubated for 1h with SB203580 (0, 0.1, 1, 10, 100 μM), as indicated above. Following change and removal of cell culture medium, cells were exposed to freshly prepared LPS (1 μg/ml) for 24h and cell-free supernatants were collected for pro-inflammatory cytokine release analysis for IL-1β and IL-6 by highly specific and non-cross-reactive sandwich, solid-phase, enzyme-linked immunosorbent assay (ELISA; R&D Systems), as per manufacturer's instructions and as described in detail elsewhere [2,6].

In brevity, the following experimental setup was undertaken: The bioactivity of extracellular cytokines was measured by a two-site, solid-phase, developed sandwich ELISA. Immunoaffinity purified polyclonal rabbit anti-rat IL-1β and IL-6 primary antibodies were utilized to coat microtiter plates with high-binding characteristics (MaxiSorp, Nunc). Moreover, recombinant rat and biotinylated immunoaffinity purified sheep anti-rat cytokine polyclonal antibodies (R&D Systems) were, respectively, used as standard and recognition antibodies. The color was then developed by using streptavidin-poly-horseradish peroxidase (HRP) coupled reaction with the chromogen 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (TMB), and the optical density (O.D.) was measured at 450 nm. The inter-assay and intra-assay coefficients of variations (CVs) were determined at < 10%; moreover, the minimum sensitivity detected for cytokines (IL-1β, IL-6, and TNF-α) was ≤ 2 pg/ml. The interpolated results obtained from the linear regression of the standard curve were expressed as pg/ml, as indicated above [6].

2.5. Statistical Analysis and Data Presentation

The obtained data are the means and the error bars the SEM. In addition, statistical evaluation of the difference in mean separation was performed by the established one-way analysis of variance (ANOVA), which was followed by the *post hoc* Tukey's test. The *a priori* level of significance at 95% confidence level was considered at $P < 0.05$.

3. Results

3.1. Selective Inhibition of $MAPK^{p38}$ and the Release of Pro-inflammatory Cytokines *in vitro*

To unravel the connection between regulatory pathways involving $MAPK^{p38}$ and the *in vitro* secretion of pro-inflammatory cytokines, cell cultures were pretreated with SB203580, a selective inhibitor of $MAPK^{p38}$, followed by a determined period for LPS treatment. The supernatant

cell culture levels of IL-1β, quantified by ELISA, are shown in Figure 1A. LPS upregulated the secretion of IL-1β almost more than 15 folds, as compared with control baseline levels. Furthermore, pre-treatment with SB203580 reduced, in a dose-dependent manner, the LPS-mediated release of IL-1β, almost by 5 – 12 folds (Figure 1A).

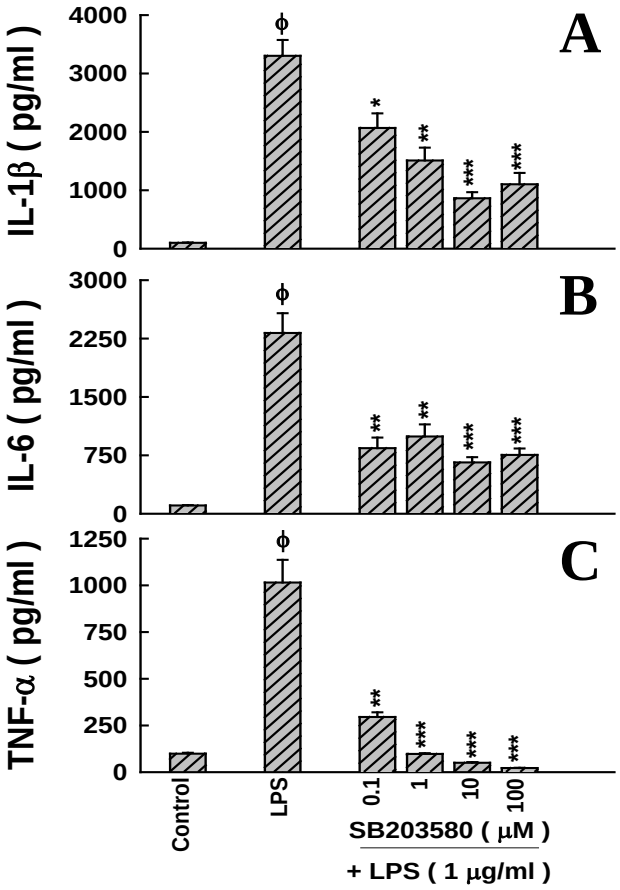

Figure 1. Histogram analysis of the cell culture supernatant levels of pro-inflammatory cytokines with selective MAPK^{p38} inhibition. **(A)** The levels of IL-1β in the presence or absence of LPS/SB203580. **(B)** The levels of IL-6 in the presence or absence of LPS/SB203580. **(C)** The levels of TNF-α in the presence or absence of LPS/SB203580, shown here for comparison purposes with interleukins, mainly IL-1β and IL-6 [2]. The number of experimental observations is n = 3 – 5, for separate and independently prepared cell cultures of alveolar epithelial cells with or without pre-treatments with LPS/SB203580. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with LPS. ϕ $P < 0.001$ (LPS), as compared with control baseline

In understanding the role of IL-6, the supernatant cell culture levels of this cytokine are shown in Figure 1B. As noted with IL-1β (Figure 1A), LPS upregulated the secretion of IL-6 almost more than 20 folds, as compared with control baseline levels. Furthermore, pre-treatment with SB203580 reduced, in a dose-independent manner, the LPS-induced release of IL-6, almost by 8 folds (Figure 1B).

The levels of TNF-α are shown here for comparison purposes. Similarly, the supernatant cell culture levels of TNF-α are shown in Figure 1C. As noted with IL-1β (Figure 1A) and IL-6 (Figure 1B), LPS upregulated the secretion of TNF-α almost 10 folds, as compared with control baseline levels. Pre-treatment with SB203580 significantly reduced, in a dose-independent manner, the LPS-induced release of TNF-α, almost by 4 folds (Figure 1C).

3.2. Selective Inhibition of MAPK^{p38} and the Cytosolic Phosphorylation of IκB-α *in vitro*

Whether the IκB-α/NF-κB pathway is likely involved with MAPK^{p38}-dependent secretion of inflammatory cytokines is not well established in alveolar epithelial cells. This assumption is because the transcription factor NF-κB is ostensibly a major player in regulating the inflammatory process, *in vitro* and *in vivo* [5]. In brevity, the nuclear translocation and activation of NF-κB is strictly dependent on the cytosolic phosphorylation of IκB-α, the major inhibitor of this transcription factor. Therefore, the degree of IκB-α phosphorylation correlates with that of NF-κB activation.

The levels of IκB-α phosphorylation *in vitro* with or without pretreatment with SB203580 in cell cultures infused with LPS are collectively shown in Figure 2A. As noted, the phosphorylated level of IκB-α increased, in a dose-dependent manner, and the cytosolic accumulation of the non-phosphorylated form of IκB-α decreased in parallel. This indicates that the inhibition of NF-κB imposed by IκB-α is no longer in effect, thereby allowing its nuclear translocation. To ensure semi-quantitative loading per gel lane, the relative constitutive expression of β-actin (20 μg) has been used as previously examined, and essentially as reported elsewhere [2,7]. The histogram analysis of the relative levels of phosphorylation of IκB-α in the presence or absence of SB203580 is shown in Figure 2B.

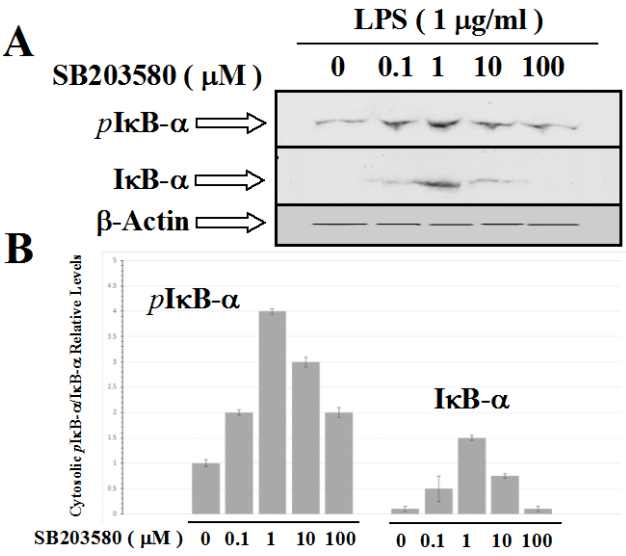

Figure 2. Electrophoretic typical gel analysis for the effect of LPS/SB203580 on IκB-α cytosolic phosphorylation. **(A)** Cell cultures were pretreated with ascending concentrations of SB203580 for 1h, followed by incubation with LPS for 24h. The phosphorylated (*p*IκB-α) and non-phosphorylated forms of IκB-α were subsequently determined. Semi-quantitative loading per lane is verified by the consistent expression of the constitutive form of β-actin [2,7] **(B)** Histogram analysis of the relative levels of *p*IκB-α and IκB-α with selective MAPK^{p38} inhibition. The number of experimental observations is n = 3 – 5, for separate and independently prepared cell cultures of alveolar epithelial cells with or without pre-treatments with LPS/SB203580

Hypothetical putative pathways depicting the intertwined signaling crosstalk between the MAPK^{p38} and NF-κB pathways are shown in Figure 3. The involvement of upstream and downstream kinases in regulating either pathway is also shown. In addition, the selective inhibition

of the MAPK^{p38} pathway attenuated the release of inflammatory cytokines, however, it contributes to phosphorylation of IκB-α and subsequent NF-κB activation. These pathways governing the release of inflammatory cytokines revolve around the activation by incoming signals of membrane-bound receptors and cytosolic cofactors. In brevity, LPS/endotoxin ostensibly activates downstream MEKK/NIK, the primary regulatory kinases in MAPK and NF-κB activation, respectively. This bifurcation leads to two separate pathways: One involving MAPKs and downstream cofactors, and the other involves IκB-α phosphorylation and NF-κB activation. Either pathways can lead to increase in DNA-binding activity and subsequent release of inflammatory cytokines. The selective inhibition of MAPK^{p38} by SB203580 separated the abovementioned pathways, independently of IκB-α phosphorylation, indicating MAPK-dependent regulation of cytokine release (Figure 3).

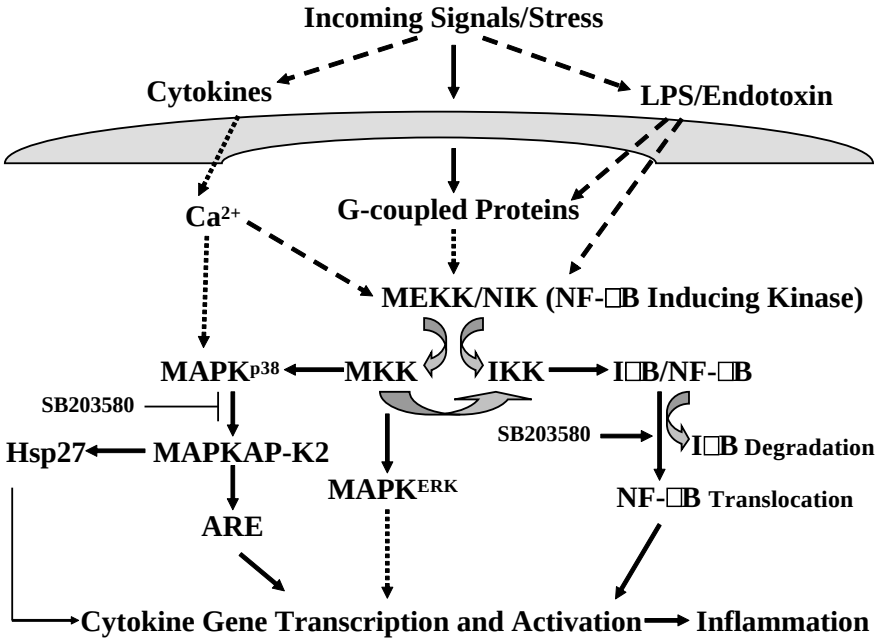

Figure 3. Hypothetical putative pathways depicting the intertwined signaling crosstalk between the MAPK^{p38} and NF-κB pathways. The involvement of upstream and downstream kinases in regulating either pathway is also shown. In addition, the selective inhibition of the MAPK^{p38} pathway attenuated the release of pro-inflammatory cytokines, however, it contributes to phosphorylation of IκB-α and subsequent NF-κB activation (see Results section for further details)

4. Discussion

Immunologically a typical orchestrated event, the cellular inflammatory process is a complicated network of events that ostensibly involve a plethora of peptides, cofactors and transcription factors [3]. Among those biological response modifiers that are key players during the onset of inflammation are the released cytokines, which regulate various mechanisms of injury and tissue remodeling [8]. Although the inflammatory so-called 'soup' is not strictly mediated by just pro-inflammatory cytokines, the involvement of interleukins in the process is unparalleled in that they regulate a large magnitude of cellular events that are integral components of the inflammatory process [4,9].

On the molecular mechanisms implicated in inflammation, furthermore, mainly two separate but interrelated pathways transcriptionally control the events that mark the inflammatory process: i) NF-κB signaling and its related inhibitory molecules, such as IκB, and ii) MAPK signaling which is essentially regulated by similar but different phosphorylation mechanisms (refer to Figure 3) [10]. The link that holds the two pathways of NF-κB/MAPK together via an active crosstalk is not well identified in the alveolar epithelium *in vitro* and *in vivo*. The observations therein reported point in the direction of independency in controlling the release of interleukin-related inflammatory cytokines, but partial involvement of either pathway cannot be ruled out from this report alone. Further studies are, therefore, warranted to decipher the underlying mechanisms involved.

Inhibition of the MAPK signaling pathway using selective and specific regulators is a key control to understanding the MAPK dependency in mediating pro-inflammatory cytokines release [8,11]. The choice of using the specific inhibitor SB203580 is based on the assumption that this regulatory molecule at low, yet effective, concentrations is strictly specific to MAPK$^{p38\alpha}$ and MAPK$^{p38\beta}$, without the eligibility of affecting other MAPKs, under the same physiologic conditions, including MAPK^{ERK} and MAPK^{JNK} [2]. Moreover, this inhibitor blocks the downstream signaling pathway involving MAPKs and heat shock protein (HSP)-27, which is transcriptionally active with the regulatory mechanisms involving MAPKs [2]. Therefore, the specificity of this molecular cannot be undermined. According to Sigma-Aldrich website, "*SB203580 is a compound that belongs to the pyridinyl imidazole family, known to suppress the activation of MAPKAP kinase-2 and inhibit the phosphorylation of HSP-27 in response to cytokines (IL-1), cellular stresses and bacterial endotoxin in vivo. This compound is well known not to inhibit MAPK^{JNK} or MAPK^{p42} and therefore, is useful for studying the physiological roles and targets of MAPK^{p38} and MAPKAP kinase-2. It has also been shown to induce the activation*

of the serine/threonine kinase Raf-1 and has been reported to inhibit cytokine production." In corroboration with the aforementioned, we have previously reported an effective attenuating mechanism in SB203580-dependent downregulation of the phosphorylation of HSP-27, a key element in the $MAPK^{p38}$ pathway [2,9,11].

Interestingly, using cellular non-toxic concentrations of SB203580 (0.1 – 100 μM) pre-treatments, prior to LPS administration effectively attenuated the *in vitro* secretion of IL-1β and IL-6, both of which are key players in the inflammatory process, jibing with other investigations [12,13]. Of note, however, the attenuation of IL-1β and IL-6 shown in this study is not as prominent and inhibitory when compared with that of TNF-α, as previously indicated [2]. However well known the early involvement of TNF-α in the inflammatory process prior to the release of other cytokines [8,14], including IL-1β and IL-6, the results undoubtedly mark a clear and definitive involvement of late onset released cytokines, when compared with early onset released cytokines such as TNF-α. In brevity, the cytokine milieu whether involving early or late phases of inflammation is unprecedentedly controlled, at least in part, by upstream kinases such as $MAPK^{p38}$ [15].

The probable crosstalk between the $MAPK^{p38}$ pathway and the upstream signaling mechanism controlling the regulation of NF-κB is not well understood [16]. The choice of using SB203580, the selective inhibitor of $MAPK^{p38}$, in understanding the role of IκB-α, the major NF-κB cytosolic inhibitor, has certainly shed light on deciphering the code of the NF-κB/MAPK crosstalk. To that end, it is only reasonable to suggest that unraveling the degree of cytosolic phosphorylation of IκB-α can help understand the involvement of NF-κB in this bidirectional crosstalk. Moreover, it is expected that under controlled, physiologic or supraphysiologic conditions of inflammation, the inhibition of one pathway ($MAPK^{p38}$) would lead to either inhibition or at least attenuation of the other pathway (NF-κB) [5]. However, the results therein reported do not jibe well with this theory. On the contrary, selective inhibition of $MAPK^{p38}$ induced the phosphorylation of IκB-α, thereby allowing NF-κB nuclear translocation and activation [5,7]. Although the DNA-binding activity of NF-κB following the phosphorylation of IκB-α has not been detected in this study, previous work has shown that that is the case in the alveolar epithelium [2]. This conundrum is certainly shedding light on the fact that although cellular signaling pathways involved with inflammation are intertwined, the $MAPK^{p38}$ pathway is overshadowing that of NF-κB in regulating the release of inflammatory cytokines [17].

In brevity, this study has shown for the first time and beyond any shred of doubt that the supposedly ironclad crosstalk resembling two intertwined regulatory pathways (NF-κB/MAPK) can be separated when it comes to understanding the inflammatory process *in vitro*, at least within the perimeter of the experimental setup used in this work. The importance of this work is prominent at the following strata of identification: i) Selective inhibition of $MAPK^{p38}$ attenuated, but did not abrogate, the LPS-induced release of pro-inflammatory cytokines (IL-1β and IL-6), as opposed to almost total inhibition of TNF-α; ii) The selective inhibition of $MAPK^{p38}$ does not preclude any potential involvement of other MAPKs, including $MAPK^{JNK}$ and $MAPK^{ERK}$; iii) Selective inhibition of $MAPK^{p38}$ upregulated IκB-α phosphorylation, thereby allowing the nuclear translocation and activation of NF-κB; and iv) The release of pro-inflammatory cytokines in the alveolar epithelium, and at least within this experimental setup, is partially involving $MAPK^{p38}$ and seemingly independent of NF-κB.

Declaration of Competing Interests

The author declares that there are no competing interests associated with the bearings of this research work.

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Author's Contributions

This work in its entirety is the conception and undertaking of Dr. John J. Haddad, with laboratory technical assistance from the aforementioned laboratory managers. Solely the author wrote the manuscript and all illustrations were professionally created using Microsoft Office 2013.

Author's Fields of Interest

Dr. John J. Haddad is a research scientist and investigator in immunology and molecular biology, specialized in inflammation and the governing signaling pathways in physiology and pathophysiology.

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