Research article

Glucocorticoids synergize with IL-1β to induce TLR2 expression via MAP Kinase Phosphatase-1-dependent dual Inhibition of MAPK JNK and p38 in epithelial cells

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Abstract

Background: Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, their role in enhancing host immune and defense response against invading bacteria is poorly understood. Toll-like receptor 2 (TLR2) has recently gained importance as one of the major host defense receptors. The increased expression of TLR2 in response to bacteria-induced cytokines has been thought to be crucial for the accelerated immune response and resensitization of epithelial cells to invading pathogens.

Results: We show that IL-1β, a key proinflammatory cytokine, greatly up-regulates TLR2 expression in human epithelial cells via a positive IKKβ-IκBα-dependent NF-κB pathway and negative MEKK1-MKK4/7-JNK1/2 and MKK3/6-p38 α/β pathways. Glucocorticoids synergistically enhance IL-1β-induced TLR2 expression via specific up-regulation of the MAP kinase phosphatase-1 that, in turn, leads to dephosphorylation and inactivation of both MAPK JNK and p38, the negative regulators for TLR2 induction.

Conclusion: These results indicate that glucocorticoids not only suppress immune and inflammatory response, but also enhance the expression of the host defense receptor, TLR2. Thus, our studies may bring new insights into the novel role of glucocorticoids in orchestrating and optimizing host immune and defense responses during bacterial infections and enhance our understanding of the signaling mechanisms underlying the glucocorticoid-mediated attenuation of MAPK.

Background

Epithelial cells are often situated at host-environment boundaries, and thus act as the first line of defense against bacterial pathogens [1]. The epithelial cells are not merely a passive barrier but can detect foreign pathogens and generate a range of mediators that play important roles in the

activation of innate and adaptive immunity. For effective host defense, the epithelial cells recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) play a critical role in early innate immunity to invading microorganisms by sensing PAMPs [2]. Stimulation of TLRs by PAMPs initiates a signaling cascade that induces the production and secretion of proinflammatory cytokines [3]. Moreover, stimulation of TLRs also induces the production of effector cytokines that leads to activation of adaptive immunity.

Mammalian TLRs were originally found as homologues of the *Drosophila* Toll [4]. TLRs are type I transmembrane receptors, that possess extracellular leucine-rich repeats domains flanked by cytoplasmic domains [4,5]. Although at least 10 TLRs have been identified, only two TLRs, TLR2 and TLR4, have been well-studied. While TLR4 is mainly involved in Gram-negative bacteria lipopolysaccharide (LPS) signaling, TLR2 can respond to a variety of Gram-positive bacterial products, including peptidoglycan, lipoprotein, lipoteichoic acid and lipoarabinomannan. In addition to Gram-positive bacterial PAMPs, TLR2 also recognizes factors released by Gram-negative bacteria including nontypeable *Haemophilus influenzae* (NTHi) [6,7] and coccobacilli, *Neisseria meningitidis* [8] as well as the *Mycoplasma* lipopeptides [9,10]. The importance of TLR2 in host defense was further demonstrated by studies of knockout mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive *Staphylococcus aureus* [11]. Thus, it is clear that TLR2 plays a crucial role in host defense against a variety of microbial pathogens.

In contrast to its relatively high level of expression in lymphoid tissues, TLR2 is expressed at low levels in epithelial cells. A key issue that has thus been raised is whether the low amount of TLR2 expressed in epithelial cells is sufficient for mediating the host defense response against invading microbial pathogens. Our recent studies revealed that TLR2, although expressed at very low level in unstimulated human epithelial cells, is greatly up-regulated by NTHi via a positive IKKβ-IκBα-dependent NF-κB pathway and a negative MKK3/6-p38α/β pathway [12]. Moreover, glucocorticoids synergistically enhance NTHi-induced expression of TLR2 via a negative cross-talk with p38 MAP kinase pathway, supporting the notion that glucocorticoids plays an important role in orchestrating and optimizing immune functions, including host defense, during bacterial infections [13,14]. However, still unclear is whether up-regulation of TLR2 expression in epithelial cells can also be generalized to other key mediators such as proinflammatory cytokines, e.g. interleukin 1-β (IL-1β)

and if so, whether the cytokine-mediated up-regulation of TLR2 can also be further enhanced by glucocorticoids.

IL-1β, a proinflammatory cytokine, is produced by various cell types including epithelial cells and can be strongly induced during bacterial infections [15]. It has been recognized as one of the key mediators of the host response to microbial invasion, inflammation, immunological reactions and tissue injury [16]. Although it has been shown to induce the expression of a variety of non-structural, function-associated genes that are expressed during inflammation, particularly other cytokines, whether IL-1β also regulates the expression of host defense receptor TLR2 in human epithelial cells is still unknown. In addition, it is still unclear whether the IL-1β-mediated TLR2 expression can also be enhanced by glucocorticoids.

Here we show that IL-1β up-regulates TLR2 via a positive IKKβ-IκBα-dependent NF-κB signaling pathway and negative MKK3/6-p38α/β and MEKK1-MKK4/7-JNK1/2 pathways in human epithelial cells. Surprisingly, glucocorticoids synergistically enhance the IL-1β-induced TLR2 expression via a MKP-1-dependent dual inhibition of MAPK p38 and JNK signaling pathways. These studies may provide novel insights into the role of IL-1β and glucocorticoids in orchestrating and optimizing host immune and defense responses and lead to new therapeutic strategies for modulating innate immune and inflammatory responses for bacterial infections.

Results
IL-1β selectively up-regulates TLR2, but not TLR4, in human epithelial cells
We first examined whether IL-1β up-regulates TLR2 expression in human epithelial cells. Human epithelial HeLa cells were first treated with IL-1β and the relative quantity of TLR2 mRNA was then measured by performing real-time quantitative PCR analysis. As shown in Figure 1a and 1b, IL-1β greatly up-regulated the TLR2 mRNA, but not TLR4 mRNA, in HeLa cells in a time- and dose-dependent manner. Similar results were also observed in primary human bronchial epithelial (NHBE) cells (Figure 1c). To further determine whether up-regulation of TLR2 mRNA is accompanied by elevated TLR2 protein, Western blot analysis was performed using antibodies against human TLR2. As shown in Figure 1d, up-regulation of TLR2 was also observed at protein level. These data demonstrate that TLR2, but not TLR4, is greatly up-regulated by IL-1β in human epithelial cells.

Activation of NF-κB via IKKβ-IκBα signaling pathway is required for IL-1β-induced TLR2 up-regulation
We next sought to determine which intracellular signaling pathways are involved in IL-1β-induced TLR2 up-regulation. Based on the essential involvement of NF-κB in
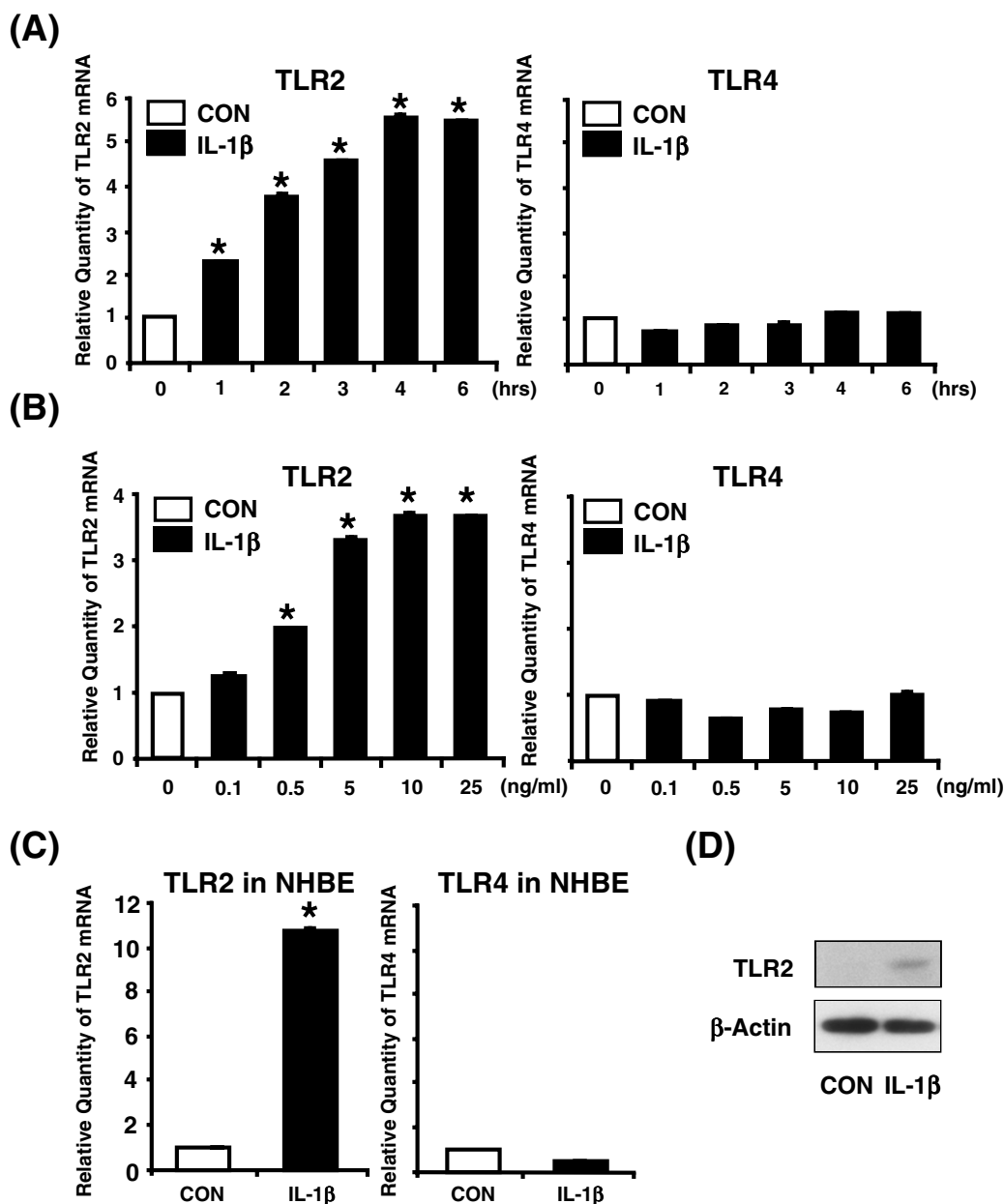
Figure 1
IL-1β up-regulates TLR2, but not TLR4, in human epithelial cells including primary bronchial epithelial cells. *A*, IL-1β up-regulates TLR2 mRNA, but not TLR4 mRNA, in HeLa cells in a time-dependent manner. Cells were treated with IL-1β (10 ng/ml) for the indicated time periods. The expression of TLR2 and TLR4 at mRNA level was then measured by real-time quantitative PCR in IL-1β-treated and -untreated human cervix epithelial HeLa cells. TLR2 and TLR4 mRNA levels were normalized to the level of cyclophilin that served as an internal control for the amount of RNA used in each reaction. Values are the mean ± SD; *n* = 3. The symbols (*) indicate results significantly different (p < 0.01) from unstimulated condition. *B*, IL-1β up-regulates TLR2 mRNA, but not TLR4 mRNA, in HeLa cells in a dose-dependent manner. HeLa cells were treated with the indicated concentration of IL-1β for 3 h. Values are the mean ± SD; *n* = 3. The symbols (*) indicate results significantly different (p < 0.01) from unstimulated condition. *C*, IL-1β up-regulates TLR2 mRNA, but not TLR4 mRNA, in primary human bronchial epithelial NHBE cells. NHBE cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols (*) indicate results significantly different (p < 0.01) from unstimulated condition. *D*, IL-1β also induces expression of TLR2 at protein level in HeLa cells. HeLa cells were treated for 5 h with IL-1β (10 ng/ml). Protein level of TLR2 was then assessed by Western blot analysis using an anti-hTLR2 antibody. β-Actin was used as a protein loading control.
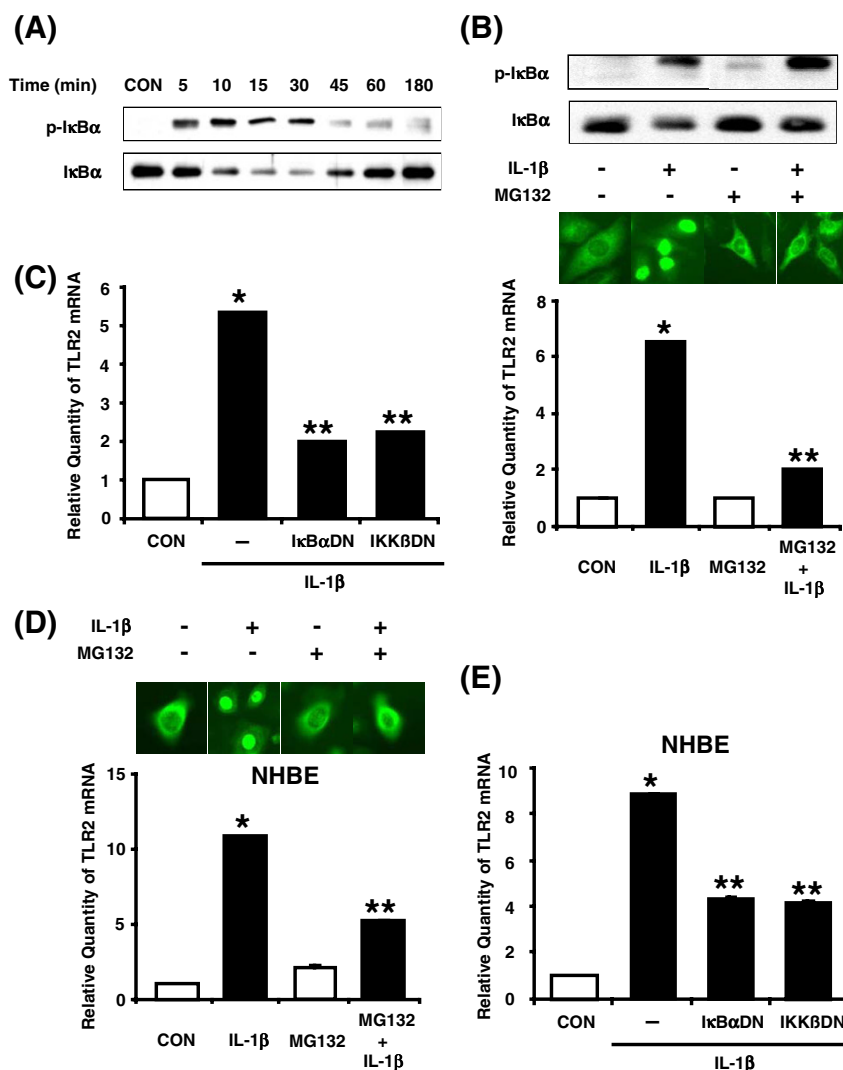
Figure 2
IKKβ-IκBα-dependent activation of NF-κB is required for IL-1β-induced TLR2 up-regulation. *A*, IL-1β induces IκBα phosphorylation and degradation in HeLa cells as assessed by Western blot analysis. Cells were treated with IL-1β (10 ng/ml) for the indicated time periods. *B*, MG 132 inhibits IL-1β-induced NF-κB nuclear translocation, IκBα degradation and TLR2 up-regulation in HeLa cells. For Western blotting and immunofluorescent staining, cells were pretreated with MG132 (1 μM) for 2 h prior to treatment with IL-1β (10 ng/ml) for 20 min. For the measurement of TLR2 mRNA, cells were treated with IL-1β (10 ng/ml) for 3 h after MG132 pretreatment. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without MG132)(**). *C*, Overexpression of a transdominant mutant of IκBα [IκBα [(S32A, S36A)] and a dominant-negative mutant of IKKβ [IKKβ (K44A)] inhibit IL-1β-induced TLR2 up-regulation in HeLa cells. HeLa cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**). *D*, MG 132 also inhibits IL-1β-induced NF-κB nuclear translocation and TLR2 up-regulation in NHBE cells. For immunofluorescent staining, cells were pretreated with MG132 (1 μM) for 2 h prior to treatment with IL-1β (10 ng/ml) for 20 min. For the measurement of TLR2 mRNA, cells were treated with IL-1β (10 ng/ml) for 3 h after MG132 pretreatment. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without MG132)(**). *E*, Overexpression of a transdominant mutant of IκBα (S32A, S36A) and a dominant-negative mutant of IKKβ (K44A) reduce IL-1β-induced TLR2 up-regulation in NHBE cells. Cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**).

regulating the expression of large numbers of genes involved in immunity and inflammation and the fact that NF-κB can be activated by IL-1β, we investigated the involvement of NF-κB signaling in TLR2 induction. We first investigated whether IL-1β induces phosphorylation and degradation of IκBα by performing Western blot analysis using antibodies against phosphorylated and total IκBα, respectively. IκBα phosphorylation became evident at 5 min, peaked at 10 min, and declined thereafter (Figure 2a). Consistent with this result, the IL-1β-induced degradation of IκBα was observed at 5 min and returned to baseline levels by 3 h (Figure 2a). We then determined the requirement of IκBα degradation in IL-β-induced TLR2 expression. As shown in Figure 2b, MG132, a proteasome inhibitor that prevents the degradation of IκBα, completely blocked the NF-κB nuclear translocation induced by IL-1β (*middle panel*). As expected, the IL-1β-induced IκBα degradation was also blocked by MG132 (Figure 2b, *upper panel*). Accordingly, phosphorylated IκBα was no longer degraded in the presence of MG-132, thus persisting in the cytoplasm. Concomitantly, IL-1β-induced TLR2 up-regulation was also inhibited by MG132 (Figure 2b, *lower panel*). To further confirm the involvement of IκBα degradation in IL-1β-induced TLR2 expression, we next transfected HeLa cells with a transdominant mutant of IκBα (S32A, S36A). As shown in Figure 2c, overexpression of the IκBα transdominant mutant inhibited IL-1β-induced TLR2 up-regulation. On the basis of a recent report that IκB kinase β (IKKβ) acts as an immediate kinase of IκBα, we investigated the role of IKKβ in IL-1β-induced TLR2 induction. Overexpression of a dominant-negative mutant form of IKKβ inhibited IL-1β-induced TLR2 up-regulation (Figure 2c). Likewise, the involvement of IKKβ-IκBα in IL-1β-induced TLR2 expression was also confirmed in human primary bronchial epithelial NHBE cells (Figure 2d and 2e). Taken together, these data demonstrate that IKKβ-IκBα-dependent activation of NF-κB is required for IL-1β-induced TLR2 up-regulation in human epithelial cells.

Activation of MKK3/6–p38 α/β MAPK pathway is negatively involved in IL-1β-induced TLR2 expression

Many cellular stress stimuli can simultaneously activate both NF-κB and p38 MAPK modules. Because of this overlap as well as the evidence for the activation of p38 by IL-1β [17], we explored the possible involvement of p38 MAPK pathway in IL-1β-induced TLR2 up-regulation. We first confirmed whether IL-1β induces phosphorylation of p38 MAPK in HeLa cells. As shown in Figure 3a, p38 MAPK is strongly phosphorylated by IL-1β in a time-dependent manner. To determine whether the activation of p38 MAPK is involved in IL-1β-induced TLR2 up-regulation, we assessed the effect of the pyridinyl imidazole, SB203580, a highly specific inhibitor for p38 MAPK, on TLR2 induction. Interestingly, SB203580 greatly

enhanced IL-1β-induced TLR2 up-regulation, suggesting that activation of p38 MAPK is negatively involved in IL-1β-induced TLR2 expression (Figure 3b). Moreover, overexpression of a dominant-negative mutant form of either p38α [fp38α(AF)] or p38β [fp38β(AF)] also enhanced the IL-1β-induced TLR2 expression, whereas overexpression of a wild-type p38α or p38β reduced it, thereby confirming the negative involvement of p38 MAPK in HeLa cells (Figure 3c). Similarly, SB203580 and overexpression of a dominant-negative mutant form of either p38α[fp38α(AF)] or p38β [fp38β(AF)] also enhanced the IL-1b-induced TLR2 expression in primary bronchial epithelial NHBE cells (Figure 3d and 3e). As immediate upstream kinases of p38 α and β, two MAPK kinases (MKK3 and MKK6) have been identified [18-20]. To further investigate whether activation of MKK3/6 is also negatively involved in IL-1β-induced TLR2 expression, we first sought to determine whether IL-1β induces activation of MKK3/6. As shown in Figure 3f, IL-1β strongly induced phosphorylation of MKK3/6 in HeLa cells. We next investigated the effect of overexpressing a dominant-negative mutant form of either MKK3 [MKK3b(A)] or MKK6 [MKK6b(A)] on TLR2 induction in HeLa cells. The IL-1β-induced TLR2 up-regulation was enhanced by blocking MKK3/6 signaling (Figure 3g). Similar results were also obtained in primary NHBE cells (Figure 3h). Collectively, our data suggest that activation of MKK3/6-p38 α/β MAPK signaling pathway is negatively involved in IL-1β-induced TLR2 up-regulation in human epithelial cells.

Activation of MEKK1-MKK4/7-JNK1/2 pathway is also negatively involved in IL-1β-induced TLR2 expression

Besides the p38 MAPK pathway, the c-Jun N-terminal kinase (JNK) pathway represents another key MAPK pathway involved in the regulation of immune response genes. JNK is activated by a variety of environmental stresses including pro-inflammatory cytokine IL-1β [18]. Therefore, we next sought to investigate the involvement of JNK pathway in IL-1β-induced TLR2 expression. We first determined whether IL-1β induces phosphorylation of JNK in HeLa cells. As shown in Figure 4a, JNK is strongly phosphorylated by IL-1β in a time-dependent manner. To determine the involvement of JNK pathway in TLR2 up-regulation, a dominant-negative mutant form of either JNK1 [JNK1(AF)] or JNK2 [JNK2(AF)] was transfected into HeLa cells. Overexpression of a dominant-negative mutant of either JNK1 or JNK2 enhanced the IL-1β-induced TLR2 expression (Figure 4b). Similar result was also observed in primary NHBE cells (Figure 4c). These results suggest that JNK1/2 pathway is also negatively involved in IL-1β-induced TLR2 expression in human epithelial cells.

On the basis that JNK activation is mediated by two upstream MAPK kinases (MKKs), MKK4 and MKK7, and
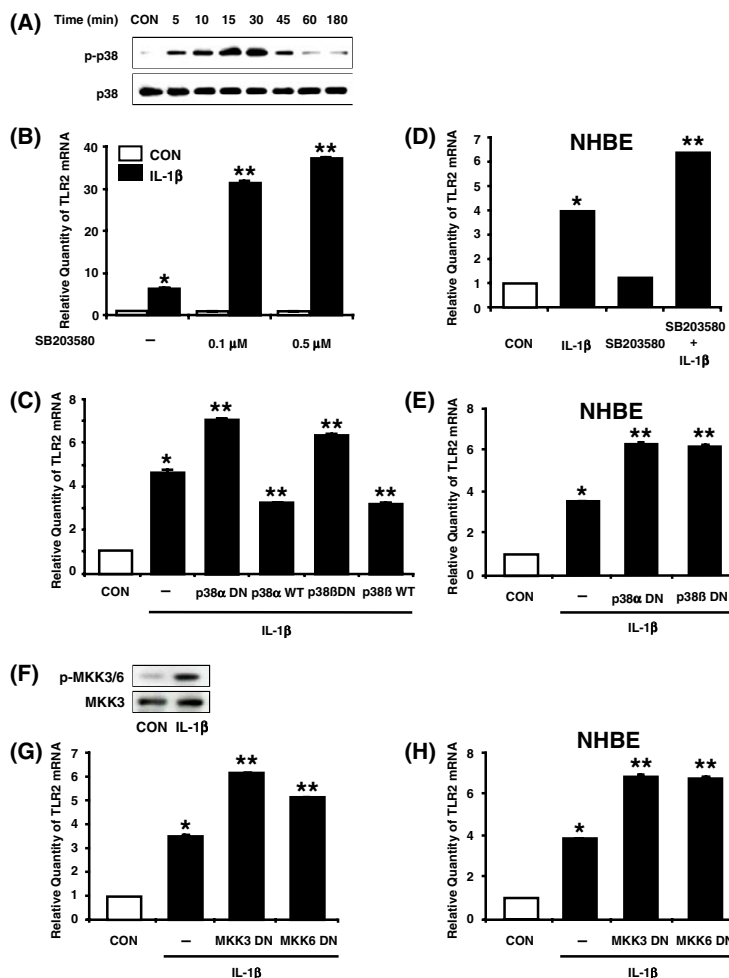
Figure 3
Activation of MKK3/6-p38 α/β MAPK pathway is negatively involved in IL-1β-induced TLR2 expression. *A*, IL-1β induces p38 phosphorylation in HeLa cells in a time-dependent manner as assessed by Western blot analysis. Cells were treated with IL-1β (10 ng/ml) for the indicated time periods. *B*, SB203580 greatly enhances IL-1β-induced TLR2 up-regulation in HeLa cells. Cells were pretreated for 2 h with the indicated concentration of SB203580 prior to treatment with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without SB203580)(**). *C*, Overexpression of a dominant-negative mutants of either p38α [fp38α(AF)] or p38β [fp38β (AF)] enhances, whereas overexpression of a wild-type p38α or p38β reduces, the IL-1β-induced TLR2 expression in HeLa cells. Cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without wild-type or dominant-negative construct)(**). *D*, SB203580 greatly enhances IL-1β-induced TLR2 up-regulation in NHBE cells. Cells were pretreated for 2 h with SB203580 (20 μM) prior to treatment with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without SB203580)(**). *E*, Overexpression of a dominant-negative mutant of p38α or p38β enhances the IL-1β-induced TLR2 expression in NHBE cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant negative construct)(**). *F*, IL-1β induces MKK3/6 phosphorylation in HeLa cells. Cells were treated with IL-1β (10 ng/ml) for 20 min. *G*, Overexpression of a dominant-negative mutant form of either MKK3 [MKK3b (A)] or MKK6 [MKK6b (A)] also enhances IL-1β-induced TLR2 up-regulation in HeLa cells. Cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**). *H*, Overexpression of a dominant-negative mutant form of either MKK3 or MKK6 also enhances IL-1β-induced TLR2 up-regulation in NHBE cells. Cells were treated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**).
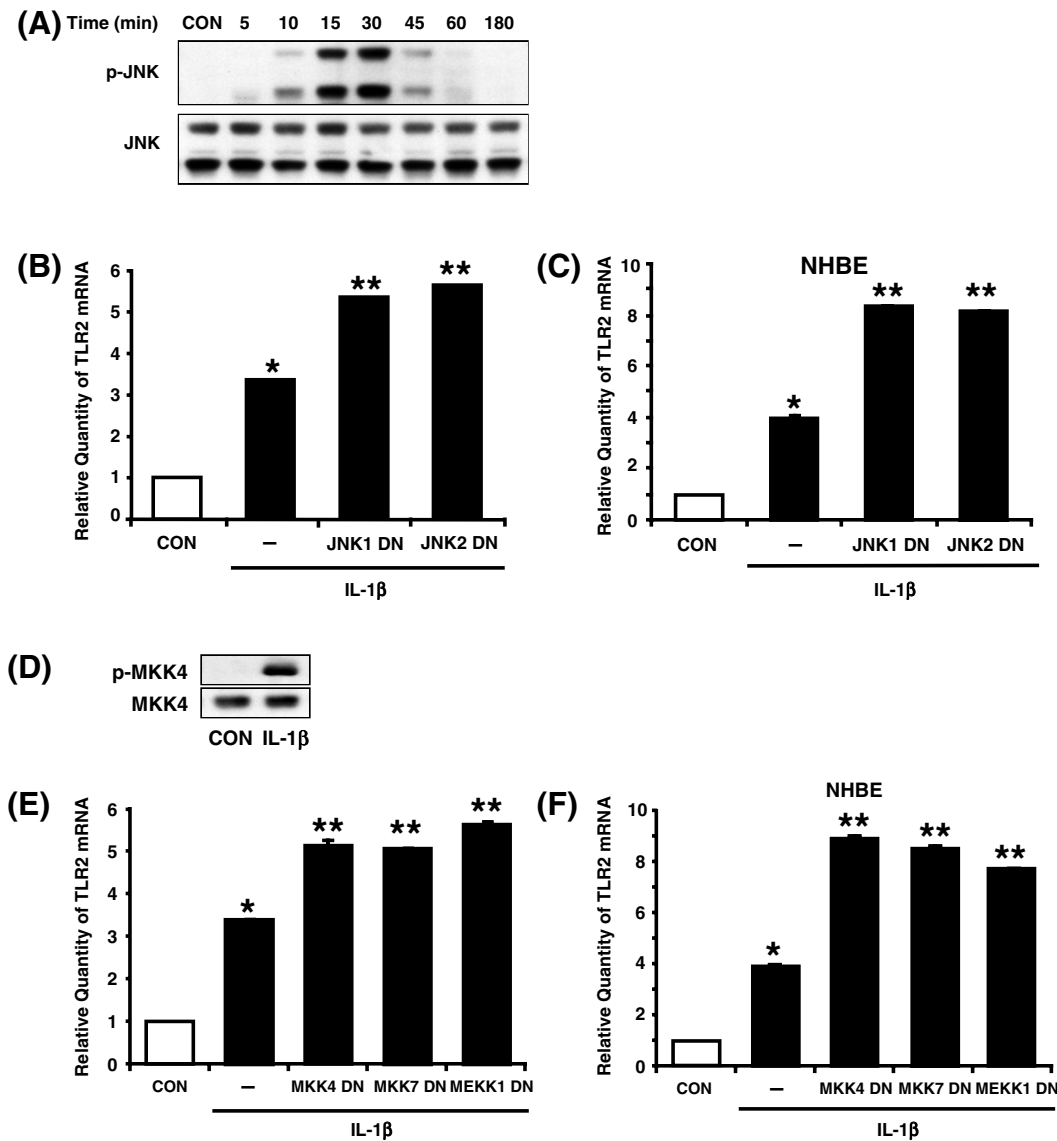
Figure 4
Activation of MEKK1-MKK4/7-JNK1/2 pathway is also negatively involved in IL-1β-induced TLR2 expression. *A*, IL-1β induces JNK phosphorylation in HeLa cells in a time-dependent manner as assessed by Western blot analysis. Cells were treated with IL-1β (10 ng/ml) for the indicated time periods. *B*, Overexpression of a dominant-negative mutants of either JNK1 [JNK1 (AF)] or JNK2 [JNK2 (AF)] enhances the IL-1β-induced TLR2 expressionin HeLa cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**). *C*, Overexpression of a dominant-negative mutant of either JNK1 or JNK2 enhances the IL-1β-induced TLR2 in NHBE cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**). *D*, IL-1β induces MKK4 phosphorylation in HeLa cells. Cells were treated with IL-1β (10 ng/ml) for 20 min. *E*, Overexpression of a dominant-negative mutant form of MKK4 or MKK7 or MEKK1 enhances IL-1β-induced TLR2 up-regulation in HeLa cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant negative construct)(**). *F*, Overexpression of a dominant-negative mutant of MKK4 or MKK7 or MEKK1 also enhances IL-1β-induced TLR2 up-regulation in NHBE cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without dominant-negative construct)(**). For all the TLR2 mRNA measurement, cells were treated with IL-1β (10 ng/ml) for 3 h.

that both MKKs are activated by the further upstream MAPK/Erk kinase kinase 1 (MEKK1), we next sought to investigate whether the MEKK1-MKK4/7 signaling pathway is also negatively involved in TLR2 induction. We first confirmed whether MKK4 is activated by IL-1β in HeLa cells. Figure 4d showed a strong phosphorylation of MKK4 induced by IL-1β. To further determine the involvement of MEKK1-MKK4/7 pathway, HeLa cells were transfected with a dominant-negative mutant of either MKK4 or MKK7 or MEKK1. As shown in Figure 4e, overexpression of these dominant-negative mutants enhanced IL-1β-induced TLR2 expression (Figure 4e). Similar results were also observed in primary human bronchial epithelial NHBE cells (Figure 4f). Together, these results indicate that activation of MEKK1-MKK4/7-JNK1/2 signaling pathway is also negatively involved in IL-1β-induced TLR2 expression in human epithelial cells.

Glucocorticoids synergistically enhance IL-1β-induced TLR2 expression likely via negative cross-talk with MAPK p38 and JNK pathways

Glucocorticoids are well known for their anti-inflammatory, immune suppressive and anti-allergic activities [21]. They exert their anti-inflammatory effects by inhibiting the activity of transcription factors such as AP-1 and NF-κB that are involved in regulation of a variety of genes involved in the inflammatory responses [22,23]. Because we have shown that activation of NF-κB is required for IL-1β-induced TLR2 expression, it is logical that glucocorticoids should suppress the IL-1β-induced TLR2 up-regulation. Surprisingly, dexamethasone (DEX), a glucocorticoid analog, synergistically enhanced IL-1β-induced TLR2 expression at the mRNA level in both HeLa cells and primary human bronchial epithelial NHBE cells (Figure 5a and 5b). The enhancing effect of DEX on TLR2 up-regulation was counteracted by RU486, a synthetic antiglucocorticoid that acts as a competitor against binding to the glucocorticoid receptor (GR), suggesting the enhancing effect of DEX is mediated by the GR. Concomitantly, Western blot analysis showed that IL-1β also synergistically enhanced TLR2 expression at protein level, thereby confirming that up-regulation of TLR2 mRNA is indeed accompanied by elevated TLR2 protein (Figure 5c). These results indicate that DEX synergistically enhances IL-1β-induced TLR2 up-regulation at both mRNA and protein levels in human epithelial cells.

Because glucocorticoids have been shown to inhibit a number of MAPK family members, we next sought to determine whether DEX inhibits the IL-1β-induced activation of p38 or JNK by performing Western blot analysis. As shown in Figure 5d and 5e, DEX inhibited IL-1β-induced phosphorylation of both p38 and JNK, but not MKK3/6 or MKK4 and the inhibitory effects of DEX were blocked by GR antagonist RU486. Therefore, it is likely

that DEX may synergistically enhance IL-1β-induced TLR2 up-regulation via negative cross-talk with p38 and JNK, but not their upstream kinases MKK3/6 or MKK4.

Glucocorticoids may enhance IL-1β-induced TLR2 up-regulation via up-regulation of MAP kinase phosphatase 1 (MKP-1)

Based on recent reports that glucocorticoids inhibit the activation of p38 and JNK by up-regulating the expression of MKP-1 [24-26], we investigated whether MKP-1 is involved in the enhancing effect of DEX on IL-1β-induced TLR2 expression. We first confirmed that MKP-1 protein expression is indeed induced by DEX, and the DEX-induced MKP-1 expression was counteracted by the GR antagonist RU486 in HeLa cells (Figure 6a). We next investigated whether overexpression of MKP-1 mimics DEX. As expected, overexpression of wild-type MKP-1 inhibited IL-1β-induced p38 and JNK phosphorylation, whereas mutated MKP-1 had almost no effect on their phosphorylation (Figure 6b). Moreover, overexpression of wild-type MKP-1 enhanced IL-1β-induced TLR2 expression, whereas mutated MKP-1 slightly inhibited the TLR2 induction in both HeLa cells (Figure 6c) and primary NHBE cells (Figure 6d). Thus, it is clear that overexpression of wild-type MKP-1 mimics the enhancing effect of DEX on IL-1β-induced TLR2 up-regulation, supporting the hypothesis that glucocorticoids may enhance the IL-1β-induced TLR2 expression via a MKP-1-dependent inhibition of p38 and JNK.

Inhibition of MKP-1 expression attenuates glucocorticoids-mediated inhibition of IL-1β-induced p38 and JNK phosphorylation and enhancement of TLR2 expression

Having demonstrated that overexpression of MKP-1 inhibits IL-1β-induced p38 and JNK phosphorylation and enhances TLR2 induction, we next sought to determine whether inhibition of MKP-1 expression blocks the enhancing effect of DEX on TLR2 induction via negative cross-talks with p38 and JNK. We first confirmed that Ro-31-8220, an inhibitor for MKP-1 expression, inhibited DEX-mediated MKP-1 up-regulation at protein level (Figure 7a). We then determined whether Ro-31-8220 blocks the DEX-mediated inhibition of IL-1β-induced p38 or JNK phosphorylation. As shown in Figure 7b, the inhibitory effect of DEX on IL-1β-induced phosphorylation of p38 or JNK was blocked by Ro-31-8220. Moreover, DEX-mediated synergistic enhancement of IL-1β-induced TLR2 expression was greatly inhibited by Ro-31-8220 in both HeLa cells (Figure 7c) and primary NHBE cells (Figure 7d). These data suggest that DEX-mediated synergistic enhancement of TLR2 induction is mediated by up-regulation of MKP-1.
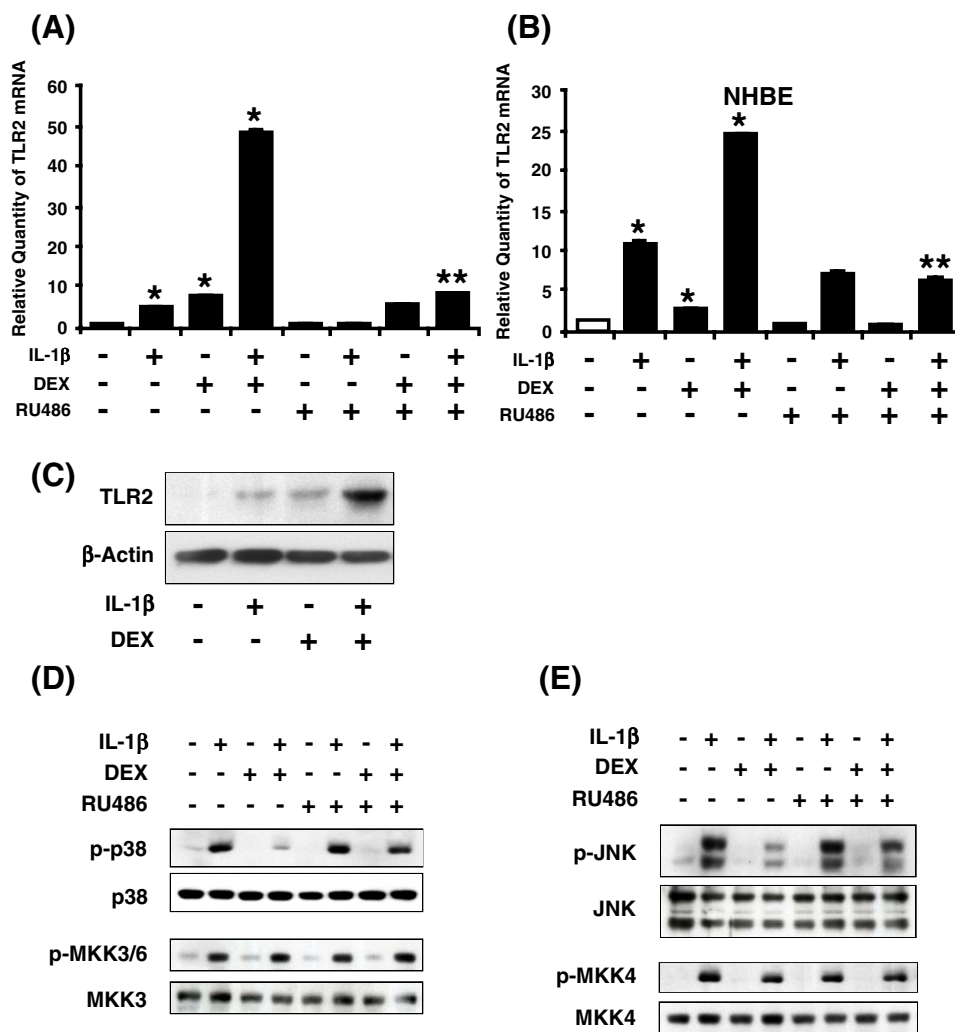
Figure 5

Glucocorticoids synergistically enhance IL-1β-induced TLR2 up-regulation via negative cross-talks with MAPK p38 and JNK1/2 pathways. *A*, Dexamethasone (DEX) synergistically enhances IL-1β-induced TLR2 up-regulation and RU486 counteracts the enhancing effect of DEX on IL-1β-induced TLR2 up-regulation at mRNA level in HeLa cells. HeLa cells were first pretreated with RU486 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 3 h, and the TLR2 mRNA levels were then assessed by real-time quantitative PCR. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without RU486)(**). *B*, DEX (10^{-6} M) synergistically enhances IL-1β-induced TLR2 up-regulation and RU486 (10^{-6} M) counteracts the enhancing effect of DEX on IL-1β-induced TLR2 up-regulation at mRNA level in NHBE cells. Cells were first pretreated with RU486 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 3 h, and the TLR2 mRNA levels were then assessed. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without RU486)(**). *C*, Glucocorticoids also synergistically enhance the induction of TLR2 at protein level in HeLa cells. HeLa cells were first treated for 2 h with DEX (10^{-6} M). The cells were then stimulated with IL-1β (10 ng/ml) for 5 h. Protein level of TLR2 was assessed by Western blot analysis using an anti-hTLR2 antibody. β-Actin was used as a protein loading control. *D*, DEX inhibits IL-1β-induced phosphorylation of p38, but not MKK3/6, and the inhibitory effect of DEX is blocked by RU486. HeLa cells were first treated with RU486 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 20 min. IL-1β-induced phosphorylation of p38 MAPK and MKK3/6 was then assessed by Western blot analysis. *E*, DEX inhibits IL-1β-induced phosphorylation of JNK, but not MKK4, and the inhibitory effect of DEX is blocked by RU486. HeLa cells were first treated with RU486 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 20 min.
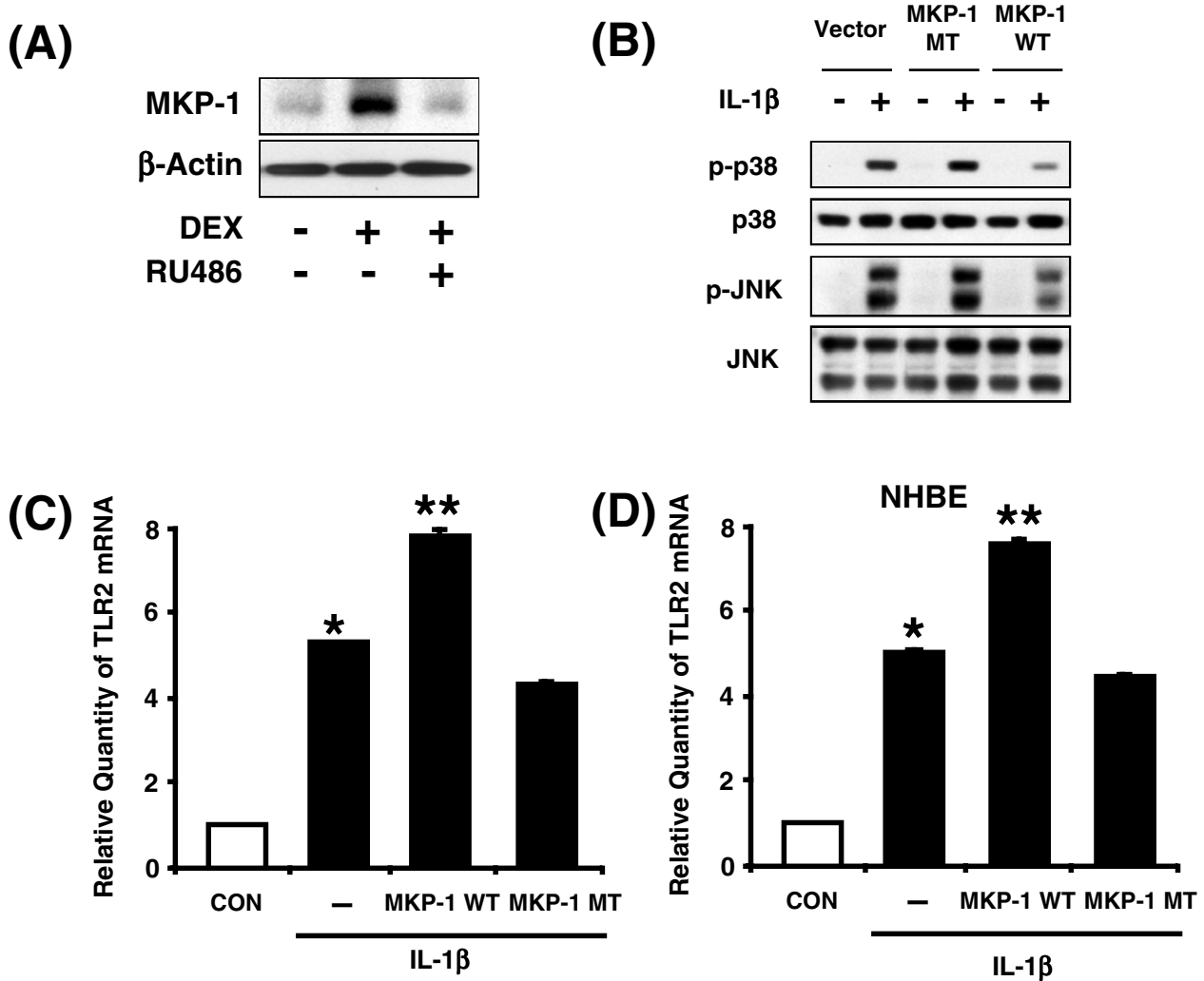
Figure 6
Glucocorticoids enhance IL-1β-induced TLR2 up-regulation likely via upregulation of MKP-1. *A*, MKP-1 protein expression is induced by DEX and the MKP-1 induction is counteracted by RU486 in HeLa cells. HeLa cells were first treated with RU486 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. Protein level of MKP-1 was assessed by Western blot analysis. β-Actin was used as a protein loading control. *B*, Overexpression of wild-type MKP-1 inhibits IL-1β-induced p38 and JNK phosphorylation, whereas overexpression of MKP-1 mutant has almost no effect on IL-1β-induced p38 and JNK phosphorylation. Transfected HeLa cells were stimulated with IL-1β (10 ng/ml) for 20 min. IL-1β-induced phosphorylation of p38 MAPK and JNK was then assessed by Western blot analysis. *C*, Overexpression of wild-type MKP-1 enhances the IL-1β-induced TLR2 expression, whereas overexpression of MKP-1 mutant has almost no effect on the IL-1β-induced TLR2 expression in HeLa cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without MKP-1 construct)(**). *D*, Overexpression of wild-type MKP-1 enhances the IL-1β-induced TLR2 expression, whereas overexpression of MKP-1 mutant has almost no effect on the IL-1β-induced TLR2 expression in NHBE cells. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-stimulated condition (without MKP-1 construct)(**). For all of the TLR2 mRNA measurement, cells were treated with IL-1β (10 ng/ml) for 3 h.
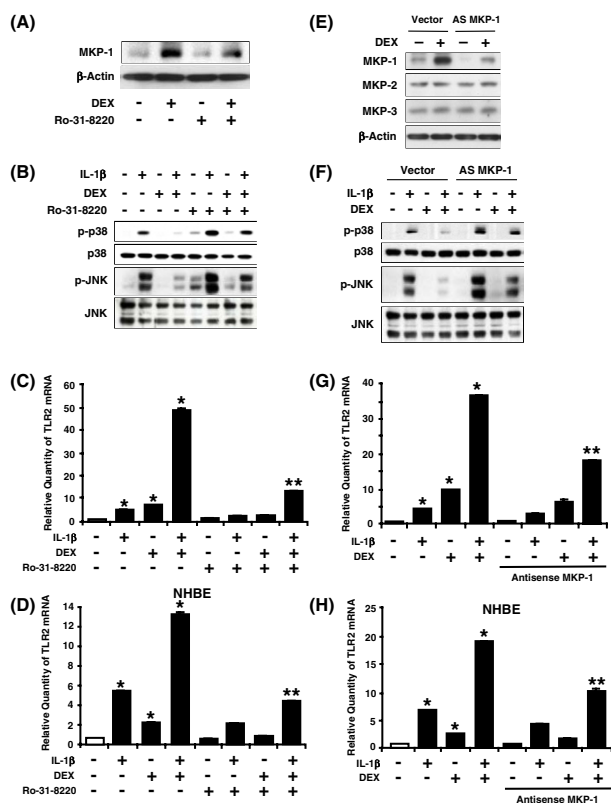
Figure 7

Inhibition of MKP-1 attenuates dexamethasone-mediated inhibition of IL-1β-induced p38 and JNK phosphorylation and enhancement of TLR2 expression. *A*, MKP-1 protein expression is induced by DEX and the MKP-1 induction is inhibited by Ro-31-8220, an inhibitor for MKP-1 expression, in HeLa cells. HeLa cells were first treated with Ro-31-8220 (10^{-6} M) for 2 h, and were then treated with DEX (10^{-6} M) for 2 h. Protein level of MKP-1 was assessed by Western blot analysis. β-Actin was used as a protein loading control. *B*, The inhibitory effect of DEX on IL-1β-induced p38 or JNK phosphorylation is antagonized by Ro-31-8220. HeLa cells were first treated with Ro-31-8220 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 20 min. IL-1β-induced phosphorylation of p38 or JNK was then assessed by Western blot analysis. *C*, DEX-mediated enhancement of IL-1β-induced TLR2 expression is greatly inhibited by Ro-31-8220 in HeLa cells. HeLa cells were first treated with Ro-31-8220 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 3 h, and the TLR2 mRNA levels were then assessed by real-time quantitative PCR. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without Ro-31-8220)(**). *D*, DEX-mediated enhancement of IL-1β-induced TLR2 expression is greatly inhibited by Ro-31-8220 in NHBE cells. NHBE cells were first treated with Ro-31-8220 (10^{-6} M) for 2 h and were then treated with DEX (10^{-6} M) for 2 h. The cells were stimulated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without Ro-31-8220)(**). *E*, Overexpression of the antisense MKP-1 inhibited MKP-1 protein expression, but not MKP-2 or MKP-3 expression. Transfected HeLa cells were treated with DEX (10^{-6} M) for 2 h. Protein level of MKP-1, -2 and -3 were assessed by Western blot analysis. β-Actin was used as a protein loading control. *F*, Overexpression of antisense MKP-1 blocks the inhibitory effect of DEX on IL-1β-induced p38 or JNK phosphorylation in HeLa cells. Transfected HeLa cells were first pretreated with DEX (10^{-6} M) for 2 h. The cells were then stimulated with IL-1β (10 ng/ml) for 20 min. IL-1β-induced phosphorylation of p38 or JNK was next assessed by Western blot analysis. *G*, DEX-mediated enhancement of IL-1β-induced TLR2 expression is attenuated by overexpression of antisense MKP-1 in HeLa cells. Transfected HeLa cells were pretreated with DEX (10^{-6} M) for 2 h, and then stimulated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without antisense MKP-1 construct)(**). *H*, DEX-mediated enhancement of IL-1β-induced TLR2 expression is also attenuated by overexpression of antisense MKP-1 in NHBE cells. Transfected cells were pretreated with DEX (10^{-6} M) for 2 h, and then stimulated with IL-1β (10 ng/ml) for 3 h. Values are the mean ± SD; *n* = 3. The symbols indicate results significantly different (p < 0.01) from unstimulated condition (*) and IL-1β-DEX-stimulated condition (without antisense MKP-1 construct)(**).

To further confirm whether the up-regulation of MKP-1 by glucocorticoids is indeed required for the enhancement of IL-1β-induced TLR2 expression, we investigated the effect of overexpressing an antisense full-length MKP-1 construct on the glucocorticoid-mediated enhancement of TLR2 induction [27]. As shown in Figure 7e, we first confirmed that overexpression of the antisense MKP-1 construct specifically inhibited MKP-1 protein expression, but not MKP-2 or MKP-3 expression. In addition, overexpression of the antisense MKP-1 construct antagonized the inhibitory effect of DEX on IL-1β-induced phosphorylation of p38 or JNK (Figure 7f). Moreover, overexpression of the same antisense MKP-1 also inhibited the DEX-mediated enhancement of IL-1β-induced TLR2 expression in both HeLa cells and primary human bronchial epithelial NHBE cells (Figure 7g and 7h). Taken together, our data demonstrate that glucocorticoids synergistically enhance IL-1β-induced TLR2 expression via up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 and JNK, the negative regulators for TLR2 expression in human epithelial cells.

Discussion

Despite the importance of glucocorticoids in suppressing immune and inflammatory responses, their role in enhancing host immune and defense response against invading bacterial pathogens is poorly understood [13,14,28,29]. Although there are reports that glucocorticoids may have a beneficial role in certain bacterial infections, the molecular basis for their beneficial effects still remains unclear. In the present study, we provided evidence that glucocorticoids synergistically enhance the TLR2 expression, a key receptor that has been shown to play a crucial role in host defense response, as evidenced by the studies of TLR2 knockout mice showing decreased survival after infection with Gram-positive *S. aureus* [11]. Thus, it appears that glucocorticoids may not only suppress but also likely enhance host immune and defense response. In addition, our study may provide the molecular basis for the explanation of the beneficial role of glucocorticoids in certain bacterial infections. Given the fact that TLR2 expression is low in unstimulated epithelial cells, the synergistic enhancement of IL-1β-induced TLR2 expression by glucocorticoids will probably contribute to the accelerated immune response by epithelial cells as well as resensitization of epithelial cells to invading pathogens. It should be noted that glucocorticoids enhance the TLR2 expression induced not only by IL-1β, but also by a variety of other pathogenic inducers including the Gram-negative bacterium nontypeable *Haemophilus influenzae*, the Gram-positive bacterium *Streptococcus pneumoniae*, and the proinflammatory cytokines TNF-α and IL-1α (data not shown). Therefore, up-regulation of the host defense receptor, TLR2, may be one of the important positive immune-regulatory mechanisms widely involved in the glucocorticoid-mediated host defense against many microbial pathogens. Our studies may bring new insights into the novel role of glucocorticoids in orchestrating and optimizing immune functions, including host defense, during bacterial infections.

Another interesting finding in this study is that glucocorticoids synergistically enhance the IL-1β-induced TLR2 expression via a MKP-1-mediated inactivation of both MAPK p38 and JNK, the negative regulators for TLR2 induction. Although several MAPKs have been identified as potential targets for negative regulation by glucocorticoids [25,30-36], the signaling mechanisms underlying the inhibition of MAPKs by glucocorticoids still remain largely unknown. Our finding that glucocorticoids inhibit the IL-1β-induced phosphorylation and activation of MAPKs p38 and JNK via up-regulation of MKP-1 may enhance our understanding of the signaling mechanisms underlying the glucocorticoid-mediated attenuation of MAPKs. In agreement with recent reports that glucocorticoids inhibit p38 via up-regulation of MKP-1 [24,26], our data showed that glucocorticoids enhanced IL-1β-induced TLR2 expression via up-regulating MKP-1 expression, that, in turn, leads to the dephosphorylation and inactivation of p38 in HeLa cells and primary NHBE cells. However, our finding that glucocorticoids inhibit the IL-1β-induced phosphorylation of JNK via an up-regulation of MKP-1 is rather unexpected, because it is in sharp contrast with the previous report that glucocorticoid-mediated inhibition of JNK does not require any *de novo* protein synthesis and independent of the transactivation function of GR [31]. Similar to the glucocorticoid-mediated inactivation of p38, glucocorticoids require ongoing MKP-1 synthesis to inhibit phosphorylation of ERK1/2 [25,37]. Hence, multiple signaling mechanisms are likely to underlie the regulation of MAPKs by glucocorticoids.

The molecular mechanisms underlying p38 and JNK-mediated inhibition of TLR2 induction by IL-1β still remain unclear. The human TLR2 promoter contains multiple transcription factor binding elements including Sp1 and Ets-1 [38]. Sp1 and Ets-1 have been shown to be regulated by p38 signaling pathway [39,40]. Activation of Sp1 and Ets family through JNK has been also reported [41,42]. Thus, Sp1 and Ets-1 could be involved in p38 and JNK-mediated inhibition of TLR2 expression. TLR2 promoter region also contains some uncharacterized regulatory elements [38]. These elements may also play a role in transcriptional inhibition of TLR2 mediated by p38 and JNK signaling. In addition to these transcription factors, the possible involvement of the regulation of mRNA stability by p38 and JNK can not be ruled out because both have been shown to be involved in regulating the mRNA stability of certain genes [43-46].

Although we have shown the involvement of multiple signaling pathways including the positive IKKβ-IκBα-dependent NF-κB pathway and the negative MKK3/6-p38 α/β and MEKK1-MKK4/7-JNK1/2 pathways, our data do not preclude the involvement of other signaling mechanisms such a direct transcriptional activation of TLR2 by glucocorticoids, or direct cross-talk between GR and other transcription factors that can be activated by the above signaling pathways. Future studies will focus on elucidating the signaling mechanisms underlying the glucocorticoid-mediated synergistic enhancement of IL-1β-induced TLR2 at the transcriptional level by taking advantage of the recent success in cloning the regulatory region of human TLR2. In addition, the role of MKP-1 in mediating the enhancement of TLR2 induction by glucocorticoids will also be confirmed *in vivo* by using an MKP-1 knockout mouse strain. Moreover, it is also interesting to determine whether glucocorticoids also enhance antibacterial defense responses.

Conclusion
We provided direct evidence that glucocorticoids synergistically enhance the IL-1β-induced TLR2 expression, a key host defense receptor, via specific up-regulation of MKP-1 expression, which, in turn, leads to the inactivation of both p38 and JNK signaling pathways, the negative regulators for TLR2 induction (Figure 8). These studies may bring new insights into the role of glucocorticoids in enhancing host defense response against bacterial invasion and lead to novel therapeutic intervention for modulating host defense and immune responses in bacterial infections.

Methods
Reagents
Human IL-1β was purchased from PIERCE ENDOGEN (Woburn, MA). MG-132, SB203580 and Ro-31-8220 were purchased from Calbiochem (La Jolla, CA). Dexamethasone and RU486 were purchased from Sigma (St. Louis, MO).

Cell culture
The human cervix epithelial cell line, HeLa, was maintained as described [6]. Primary human bronchial epithelial cells (NHBE) (Clonetics, Walkersville, MD) were maintained in Bronchial Epithelial Basal Medium (BEBM) (Clonetics) according to the manufacturer's instructions.

Real-time quantitative PCR analysis of TLR2 and TLR4
Total RNA was isolated from the cells using a TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) following manufacturer's instruction. For the RT reaction, TaqMan Reverse Transcription Regents (Applied Biosystems, Foster City, CA) were used. The real-time quantitative PCR was performed using an ABI 7700 sequence detector (Applied Biosystems). The primers and probes used for the PCR amplification of TLR2 were; primers (5'-GGCCAG-CAAATTACCTGTGTG-3' and 5'-AGGCGGACATCCT-GAACCT-3') and a probe (5'-TCCATCCCATGTGCGTGGCC-3'). The primers and probes used for the PCR amplification of TLR4 were; primers (5'-CCAGTGAGGATGATGCCAGGAT-3' and 5'-GCCATGGCTGGGATCAGAGT-3') and a probe (5'-TGTCTGCCTCGCGCCTGGC-3'). All primers and probes were synthesized by Applied Biosystems Custom Oligo Synthesis Service. Relative quantity of TLR2 and TLR4 mRNA were obtained using Comparative CT Method, and was normalized using Pre-Developed TaqMan Assay Reagent Human Cyclophilin (Applied Biosystems) as an endogenous control as previously described [24].

Plasmids and transfections
The expression plasmids IκBα(S32/36A), IKKβ (K49A), fp38α (AF) and wild-type (WT), fp38β2 (AF) and WT, MKK3b (A), MKK6b (A), JNK1 (AF), JNK2 (AF), MKK4 DN, MKK7 DN and MEKK1 DN were previously described [6,47]. The expression plasmid of the wild-type and mutant MKP-1 were kindly provided by Dr. N. Tonks (Cold Spring Harbor Laboratory, NY) [48]. The antisense full-length MKP-1 construct was kindly provided by Dr. Desbois-Mouthon (INSERM U-402, Faculté de Médecine Saint-Antoine, 75571 Paris, France) and was described previously [27]. All transient transfections were carried out in duplicate for RT-PCR analysis using TransIT-LT1 reagent (Mirus, Madison, WI) following manufacturer's instruction, unless otherwise indicated. In all transfections with expression plasmids of signaling molecules, an empty vector was used as a control.

Western Blot analysis
Phosphorylation of IκBα, JNK, p38, MKK4 and MKK3/6 was assessed using antibodies against phospho-IκBα (Ser32), IκBα, phospho-JNK (Thr183/Tyr185), JNK, phospho-p38 (Thr180/182), p38, phospho-MKK4 (Thr261), phospho-MKK3/MKK6 (Ser189/207) and MKK3 (New England Biolabs, Beverly, MA). Anti-MKK4 (K-18) was purchased from Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Phosphorylation of of IκBα, JNK, p38, MKK4 and MKK3/6 was detected as described [49]. Anti-MKP-1 (V-15), anti-MKP-2 (H-67) and anti-MKP-3 (C-20) antibodies were purchased from Santa Cruz, and used to assess protein expression. TLR2 expression was assessed with an anti-TLR2 antibody (Imgenex Corporation, San Diego, CA). Monoclonal anti-β-Actin antibody (Sigma) was used as a protein loading control for Western blot analyses of MKP-1, MKP-2, MKP-3 and TLR2.
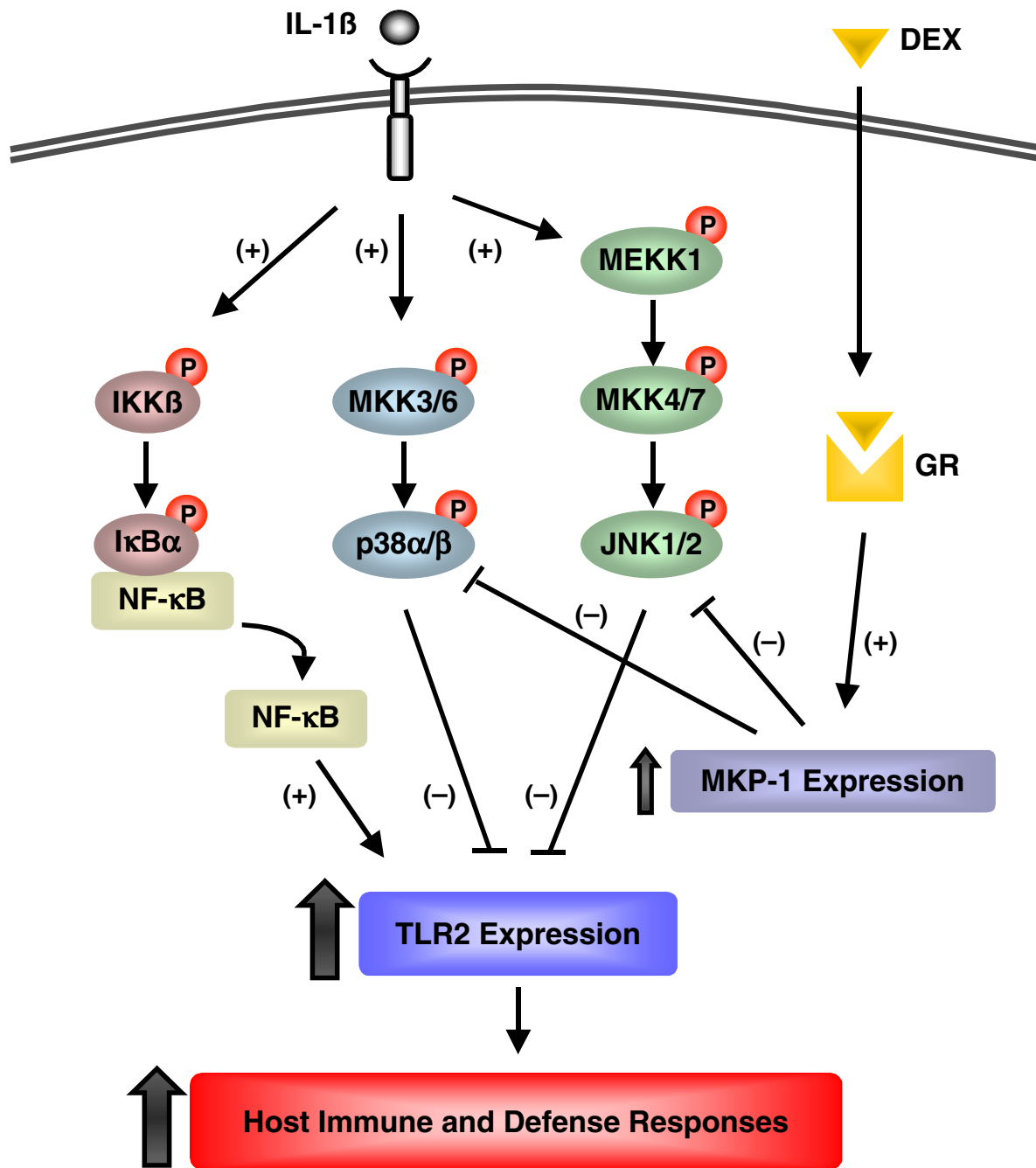
Figure 8
Schematic representation of the signaling pathways involved in glucocorticoid-mediated enhancement of IL-1β-induced TLR2 expression via MKP-1-dependent inhibition of p38 and JNK in human epithelial cells. IL-1β up-regulates TLR2 expression via a positive IKKβ-IκBα-dependent NF-κB pathway and negative MKK3/6-p38α/β and MEKK1-MKK4/7-JNK1/2 signaling pathways. Glucocorticoids synergistically enhance IL-1β-induced TLR2 expression via up-regulation of MKP-1 that, in turn, leads to dephosphorylation and inactivation of p38 and JNK pathways, the negative regulators for TLR2 expression. Abbreviations: DEX, dexamethasone; GR, glucocorticoids receptor; IκBα, Inhibitor of NF-κBα ; IKKβ, IκB kinase β ; IL-1β, interleukin 1 β ; JNK, c-Jun N-terminal kinase;MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEKK1, MAPK/Erk kinase kinase 1; MKP-1, MAP kinase phosphatase-1; NF-κB, nuclear factor κB; TLR2, Toll-like receptor 2.

Immunofluorescent staining

Cells were cultured on 4-chamber microscopeslides. After IL-1β treatment, the cells were fixed in paraformaldehyde solution (4%), incubated with mouse anti-p65 NF-κB monoclonal antibodies for 1 h (Santa Cruz Biotechnology, Inc.). Primary antibody was detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Samples were viewed and photographed using a Zeiss Axiophot microscope.

Statistical analysis

Data were analyzed using Student's *t* test or ANOVA and Tukey's post hoc test. A value of $p < 0.05$ was considered significant.

Authors' contributions

AS carried all the experiments described. JH was involved in advising and designing the experiments to investigate the role of p38 MAPK and JNK in TLR2 induction; AC was involved in advising the experiments to explore the role of MKP-1 in mediating GCs-mediated enhancement of TLR2; SA was involved in advising the experiments to explore the molecular details of GCs-mediated TLR2 induction. JDL was involved in hypothesizing and designing the experiments to test that IL-1 induces TLR2 via a complex signaling network involving both NF-κB and MPAK pathways. All authors read and approved the final manuscript.

Acknowledgements

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