

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

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Abstract

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that plays an important proinflammatory role in asthmatic airways. Corticosteroids are first-line antiinflammatories in asthma; however, their repressive effects on S1P-induced cytokine secretion have not been investigated. To address this, our *in vitro* study reveals the molecular mechanisms by which corticosteroids inhibit S1P-induced IL-6 expression in the pivotal immunomodulatory cell type, airway smooth muscle (ASM). We first uncover the cellular signaling pathways responsible: S1P activates a cyclic adenosine monophosphate/cAMP response-element-binding protein (CREB)/CRE-dependent pathway to induce IL-6 transcription, concomitant with stimulation of the mitogen-activated protein kinase (MAPK) superfamily and downstream mitogen and stress-activated protein kinase 1 (MSK1) and histone H3 phosphorylation. In this way, S1P stimulates parallel signaling pathways to induce IL-6 secretion via CRE-driven transcription of the IL-6 gene promoter in a relaxed chromatin environment achieved through histone H3 phosphorylation. Second, we investigated how corticosteroids mediate their repressive effects. The corticosteroid dexamethasone inhibits S1P-induced IL-6 protein secretion and mRNA expression, but CREB/CRE transrepression, inhibition of IL-6 mRNA stability, or subcellular relocation of MSK1 were not responsible for the repressive effects of dexamethasone. Rather, we show that

dexamethasone rapidly induces up-regulation of the MAPK deactivator MAPK phosphatase 1 (MKP-1) and that MKP-1 blocks the MAPK-driven activation of MSK1 and phosphorylation of histone H3. This was confirmed by treatment with triptolide, an inhibitor of MKP-1 up-regulation, where repressive effects of corticosteroids were reversed. Our study reveals the molecular mechanism underlying the antiinflammatory capacity of corticosteroids to repress proinflammatory functions induced by the potent bioactive sphingolipid S1P in the lung.

Keywords: lipid mediators; mitogen-activated protein kinases; DUSP; asthma; sphingosine 1-phosphate

Clinical Relevance

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that plays an important proinflammatory role in asthmatic airways. Corticosteroids are first-line antiinflammatories in asthma; however, their repressive effects on S1P-induced cytokine secretion have not been investigated to date. To address this, our *in vitro* study reveals the molecular mechanisms by which corticosteroids inhibit S1P-induced IL-6 expression in the pivotal immunomodulatory cell type airway smooth muscle.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that is elevated in the airways of patients with asthma (1).

Recent evidence in mouse models of asthma have demonstrated that S1P plays a crucial role in the development of airway

inflammation (2) and hyperresponsiveness (3); thus, S1P-mediated pathways have emerged as legitimate targets for drug

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discovery to combat asthma. The regulation of the S1P-induced proasthmatic phenotype by the first-line antiinflammatory medications (i.e., corticosteroids), has not been explored. Thus, an understanding of the molecular mechanisms that underlie S1P-induced proinflammatory effects and the regulation of these functions by antiasthma drugs is important.

In 2001 we demonstrated that S1P modulates myriad airway smooth muscle (ASM) functions that promote inflammation and remodeling in asthma (1). S1P increased the cytokine IL-6 in a concentration-dependent manner via activation of S1P receptors linked to adenylate cyclase. However, the mechanisms of IL-6 transcriptional up-regulation are unknown, and we need to demonstrate the antiinflammatory capacity of the corticosteroids against this potent bioactive sphingolipid in the lung. Furthermore, understanding the molecular mechanisms by which corticosteroids perform their antiinflammatory actions will yield crucial information for further refinement of corticosteroid regimens in pharmacotherapy.

Our previous work (1) demonstrated that S1P-induced secretion of IL-6 occurred via cyclic adenosine monophosphate (cAMP) accumulation. We have also recently shown that S1P activates members of the mitogen-activated protein kinase (MAPK) (p38 MAPK, p42/p44 MAPK [ERK] and c-Jun N-terminal kinase [JNK]) superfamily in a cell-type specific manner (4) and that S1P-induced IL-6 secretion is ERK- and p38 MAPK-dependent (4). Herein we extend our earlier studies to uncover the parallel cellular signaling pathways stimulated by S1P. We show that (1) a cAMP/cAMP response-element-binding protein (CREB)/CRE-dependent pathway is responsible for S1P-induced IL-6 transcription (i.e., S1P rapidly and robustly leads to phosphorylation of CREB at Ser¹³³ and the CRE binding site of the IL-6 promoter, but not AP-1, C/EBP, or NF- κ B, is essential for S1P-induced IL-6 transcription) and (2) S1P activates the MAPK superfamily in a temporally specific manner. ERK and p38 phosphorylation then contributes to activation of the downstream mitogen and stress-activated protein kinase 1 (MSK1) (via phosphorylation at Thr⁵⁸¹) to induce phosphorylation of histone H3 at Ser¹⁰. It is

this latter pathway that is targeted for repression by corticosteroids. We show that MAPK-driven phosphorylation of histone H3 is repressed by corticosteroid-mediated up-regulation of the endogenous MAPK deactivator mitogen-activated protein kinase phosphatase 1 (MKP-1).

Materials and Methods

ASM Cell Culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or from lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified, and cultured as previously described (5). A minimum of three different ASM primary cell lines were used for each experiment.

Chemicals

S1P (Biomol) was purchased through Enzo Life Sciences (Exeter, UK). Unless otherwise specified, all chemicals were from Sigma-Aldrich (St. Louis, MO).

ELISA

Cell supernatants were collected and stored at -20°C for later analysis by ELISA. IL-6 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

Transfection

ASM cells were transfected with the parental IL-6 promoter construct, pIL-6-luc 651, a pGL3 luciferase reporter vector (Promega, Madison, WI) containing a 651-bp fragment of the human IL-6 gene promoter (kindly provided by Oliver Eickelberg, Ludwig-Maximilians-Universität and Helmholtz Zentrum München, Germany) [6], with permission from Shigeru Katamine, Nagasaki University, Nagasaki, Japan [7]); a series of constructs in which the consensus binding sites for AP-1, C/EBP- β , and NF- κ B had been inactivated by site-directed mutagenesis (pIL-6-luc 651 Δ AAP-1, pIL-6-luc 651 Δ C/EBP- β , or pIL-6-luc 651 Δ NF- κ B, respectively) (6); or a 5'-deletion construct (pIL-6-luc158) in which the AP-1 and the CRE binding sites had been deleted (8). Transient transfection of ASM cells was performed using Lipofectamine 2000 (Invitrogen Corp.,

Carlsbad, CA) as previously described (9). Cells were harvested, and luciferase and β -galactosidase activities were assessed according to manufacturer's instructions (Promega). Data are presented as normalized luciferase activity relative to vehicle-treated cells (expressed as fold difference).

Details about real-time RT-PCR, mRNA stability, and Western blotting are provided in the online supplement.

Small Interfering RNA Knock Down

ASM cells were transiently transfected using nucleofection with 1 μg MSK1-specific SMART pool small interfering RNA (siRNA), consisting of a pool of four individual siRNA from Dharmacon (Lafayette, CO) or a scrambled control, using the methods established in our previous publication (10). Briefly, ASM cells were transfected with the Nucleofector (Amaxa, Koln, Germany) using the basic kit for primary smooth muscle cells with the manufacturer's optimized protocol of P-024. ASM cells were plated for 16 hours after transfection before being growth-arrested for a further 24 hours. Cells were then treated in accord with the experimental protocol before supernatants were removed for IL-6 protein measurement by ELISA, and lysates were used for MSK1 or MKP-1 Western blotting.

Translocation

Cytoplasmic and nuclear protein extraction was performed using a NE-PER nuclear and cytosolic extraction kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. MSK1 was quantified by Western blotting and compared with α -tubulin and lamin A/C (rabbit polyclonal IgG antibodies; Cell Signaling Technology, Danvers, MA) as loading controls for the cytosolic and nuclear fractions, respectively.

Statistical Analysis

Statistical analysis was performed using Student's unpaired *t* test, one-way ANOVA, Fisher's *post hoc* multiple comparison test or two-way ANOVA, then Bonferroni's post-test. *P* values less than 0.05 were sufficient to reject the null hypothesis for all analyses.

Results

S1P Induces IL-6 Secretion from ASM Cells via Transcriptional Regulation of IL-6 Gene Expression

To confirm our earlier report of proinflammatory effects of S1P on ASM synthetic function (1), growth-arrested ASM cells were treated with vehicle or S1P (1 μ M) for 24 hours, and secreted IL-6 was measured by ELISA. S1P induces a significant increase in IL-6 secretion ($P < 0.05$) (Figure 1A). This increase in IL-6 secretion from ASM cells in response to S1P occurred via transcriptional regulation (Figure 1B); S1P (1 μ M) induced a 2.9 ± 0.4 -fold increase in IL-6 promoter activity in ASM cells transiently transfected with pIL-6-luc 651 ($P < 0.05$), a pGL3 luciferase reporter vector containing a 651-bp fragment of the human IL-6 gene promoter.

Dexamethasone Inhibits S1P-Induced IL-6 Protein Secretion and mRNA Expression at the Transcriptional, but Not Posttranscriptional, Level of Gene Expression

We examined the inhibitory efficacy of the corticosteroid dexamethasone on S1P-induced IL-6 protein secretion and mRNA expression. Growth-arrested ASM cells were pretreated with vehicle or with increasing concentrations of dexamethasone (0.0001–1 μ M) and stimulated for 24 hours with S1P (1 μ M), and IL-6 protein secretion was measured by ELISA. Dexamethasone inhibited S1P-induced IL-6 protein secretion in a concentration-related manner ($P < 0.05$) (Figure 2A). S1P induced a significant 12.3 ± 2.0 -fold increase in IL-6 mRNA expression ($P < 0.05$) (Figure 2B). Moreover, S1P-induced IL-6 mRNA expression was significantly inhibited by

100 nM dexamethasone ($P < 0.05$) (Figure 2B), where dexamethasone pretreatment reduced S1P-induced IL-6 mRNA expression 1.9 ± 0.3 -fold.

After demonstrating that dexamethasone inhibits S1P-induced IL-6 protein secretion and mRNA expression, we examined whether this inhibition occurred at the transcriptional or posttranscriptional level. We transfected ASM cells with the pIL-6-luc 651 promoter and pretreated with vehicle or 100 nM dexamethasone, before stimulation for 24 hours with S1P (Figure 2C). Dexamethasone significantly inhibited S1P-induced IL-6 promoter activity ($P < 0.05$), reducing the luciferase activity to levels equivalent to those observed in vehicle-treated cells. We next examined whether dexamethasone acts at the posttranscriptional level to reduce the stability of S1P-induced IL-6 mRNA transcript. Growth-arrested ASM cells were pretreated for 1 hour with dexamethasone before stimulation with S1P for 9 hours. Transcription was then halted using actinomycin D, and real-time RT-PCR was used to measure IL-6 mRNA degradation over time to determine the kinetics of decay. We found that pretreatment of ASM cells with dexamethasone had no effect on the stability of the S1P-induced IL-6 transcript (Figure 2D). That is, the decay kinetics of the S1P-induced IL-6 transcript induced in the presence (-0.0933 ± 0.0420 ; $t_{1/2}=7.4$ h) or absence (-0.0529 ± 0.0231 ; $t_{1/2}=13.1$ h) of dexamethasone were not significantly different (results expressed as decay constant [k] and half-life [h]; $P > 0.05$).

S1P-Induced IL-6 Transcriptional Regulation Is CRE Dependent, but Dexamethasone Does Not Inhibit S1P-Induced CREB Phosphorylation

We previously demonstrated that S1P-induced secretion of IL-6 is achieved via a S1P receptor-mediated increase in cAMP accumulation (1). We now extend our earlier studies to examine the transcriptional regulation of IL-6 by S1P using a series of luciferase reporter vectors containing full-length, mutated, or truncated constructs of a 651-bp fragment of the human IL-6 gene promoter. ASM cells were transiently transfected with the parental construct pIL-6-luc 651 or with a series of constructs in which the consensus binding sites for AP-1, C/EBP, and NF- κ B have been inactivated by site-

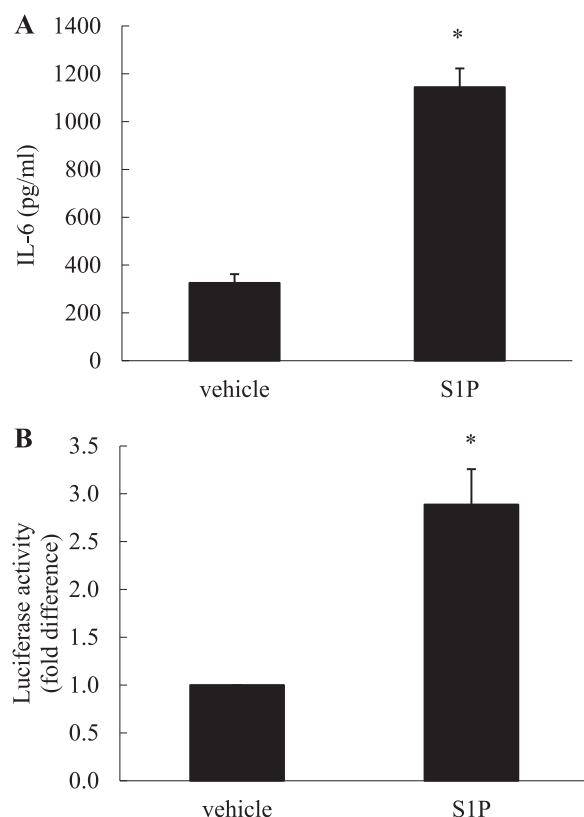


Figure 1. Sphingosine 1-phosphate (S1P) induces IL-6 secretion from airway smooth muscle (ASM) cells via transcriptional regulation of IL-6 gene expression. (A) Growth-arrested ASM cells were treated with vehicle or S1P (1 μ M) for 24 hours. Secreted IL-6 was measured by ELISA. (B) ASM cells were transfected with pIL-6-luc 651, a pGL3 luciferase reporter vector containing a 651-bp fragment of the human IL-6 gene promoter, and then growth-arrested and treated with vehicle or S1P (1 μ M) for 24 hours. Cells were harvested, and luciferase and β -galactosidase activities were assessed. Data represent normalized luciferase activity relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student's unpaired t test. *Significant effect of S1P ($P < 0.05$). Data are mean + SEM values from (A) $n = 12$ and (B) $n = 22$ primary ASM cell lines.

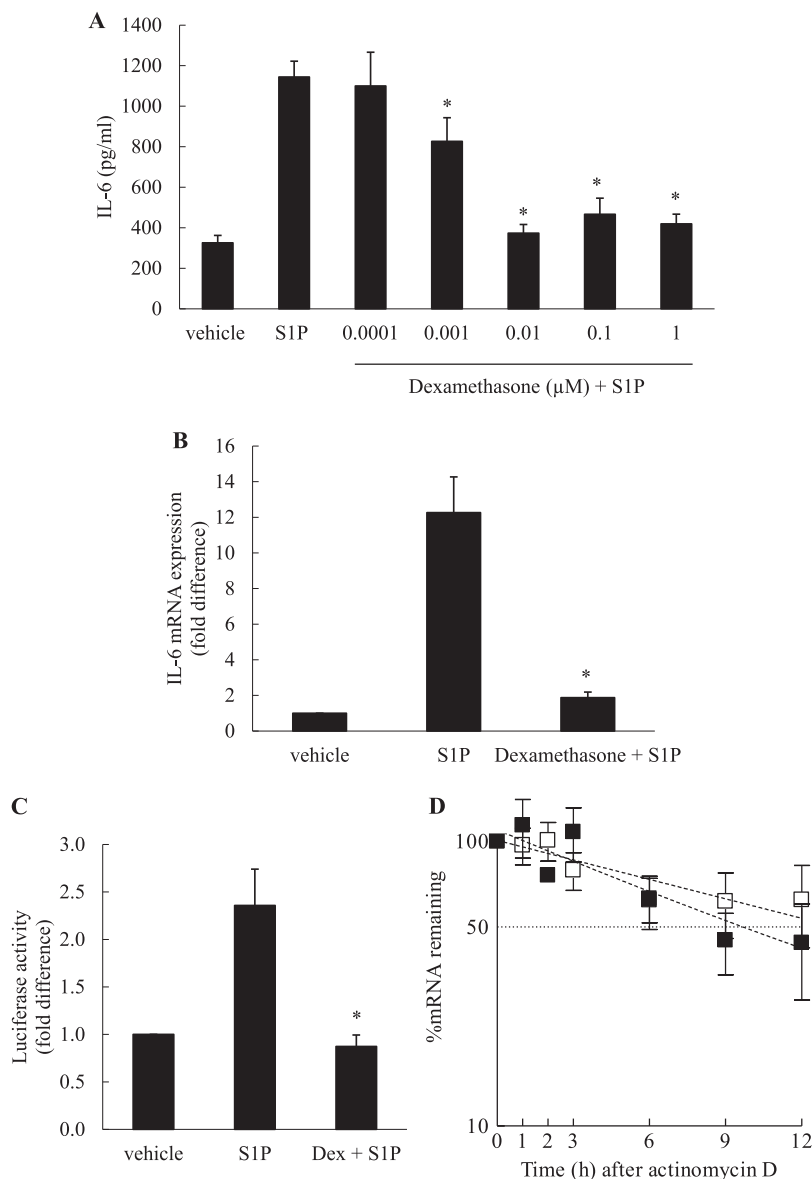


Figure 2. Dexamethasone inhibits S1P-induced IL-6 protein secretion and mRNA expression at the transcriptional, but not posttranscriptional, level of gene expression. (A) Growth-arrested ASM cells were pretreated for 1 hour with vehicle or dexamethasone (0.0001–1 μ M) and then stimulated for 24 hours with S1P (1 μ M). Secreted IL-6 was measured by ELISA. Statistical analysis was performed using one-way ANOVA followed by Fisher's *post hoc* multiple comparison test. *Significant effect of dexamethasone ($P < 0.05$). Data are mean + SEM values from $n = 12$ primary ASM cell lines. (B) Growth-arrested ASM cells pretreated for 1 hour with vehicle or 100 nM dexamethasone were stimulated with S1P (1 μ M) for 9 hours, and IL-6 mRNA expression was quantified by real-time RT-PCR (results expressed as fold difference compared with vehicle-treated cells). (C) ASM cells transfected with pIL-6-luc 651 were pretreated for 1 hour with vehicle or 100 nM dexamethasone (Dex) and then stimulated for 24 hours with S1P (1 μ M). Data represent normalized luciferase activity relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student's unpaired *t* test. *Significant effect of dexamethasone ($P < 0.05$). Data are mean + SEM values from (B) $n = 3$ and (C) $n = 9$ primary ASM cell lines. (D) Growth-arrested ASM cells pretreated for 1 hour with vehicle (open squares) or 100 nM dexamethasone (closed squares) were stimulated with S1P (1 μ M) for 9 hours and then washed and incubated with actinomycin D (5 μ g/ml) to inhibit further transcription. Total RNA was extracted after 0, 1, 2, 3, 6, 9, and 12 h, and IL-6 mRNA expression was quantified. Results are expressed as % mRNA remaining over time (dashed lines represent nonlinear regression of the % mRNA remaining vs. time after actinomycin D) (41). Data are mean \pm SEM values from $n = 3$ primary ASM cell lines.

directed mutagenesis or a 5'-deletion construct (pIL-6-luc158) in which the AP-1 and CRE binding sequences have been deleted. Mutations in the AP-1, C/EBP, or NF- κ B binding sequences had no significant effect on S1P-induced IL-6 promoter activity (Figure 3A). However, in cells transfected with pIL-6-luc158, a 5' truncated version of the parental construct deficient in AP-1 and CRE binding sequences, S1P-induced IL-6 promoter activity was significantly reduced ($P < 0.05$) (Figure 3A). Taken together, these results suggest that binding to CRE, but not to AP-1, NF- κ B, or C/EBP, mediates S1P-induced IL-6 gene expression in ASM via a cAMP-mediated pathway.

Because we have shown that S1P transcriptional regulation occurred via activation of the CRE region of the IL-6 promoter (Figure 3A), we wished to demonstrate S1P-induced phosphorylation of the transcription factor CREB and to examine the possibility that the mechanism of inhibition by dexamethasone was via repression of S1P-induced CREB activation. Growth-arrested ASM cells were pretreated for 1 hour with vehicle or 100 nM dexamethasone and then stimulated with S1P (1 μ M) for up to 1 hour. S1P rapidly phosphorylates CREB (at Ser¹³³) by 5 minutes, and the degree of phosphorylation was sustained for up to 1 hour (Figure 3B). There was no difference in the temporal kinetics of S1P-induced CREB phosphorylation in cells pretreated with dexamethasone compared with those pretreated with vehicle alone.

Taking the results from this section together, these data demonstrate (1) that S1P induces cAMP (1), (2) that S1P rapidly and robustly phosphorylates the transcriptional factor CREB at Ser¹³³, and (3) that the CRE binding site of the IL-6 promoter is essential for S1P-induced IL-6 transcriptional regulation. However, dexamethasone does not inhibit S1P-induced CREB phosphorylation.

S1P-Induced MAPK Phosphorylation Is Inhibited by Dexamethasone-Induced MKP-1

S1P activates members of the MAPK superfamily in a temporally specific manner (4). We now wished to investigate whether the repressive action of corticosteroids on S1P-induced IL-6 occurs in a similar manner to that recently observed for TNF- α -induced IL-6 (11) and IL-1 β -induced

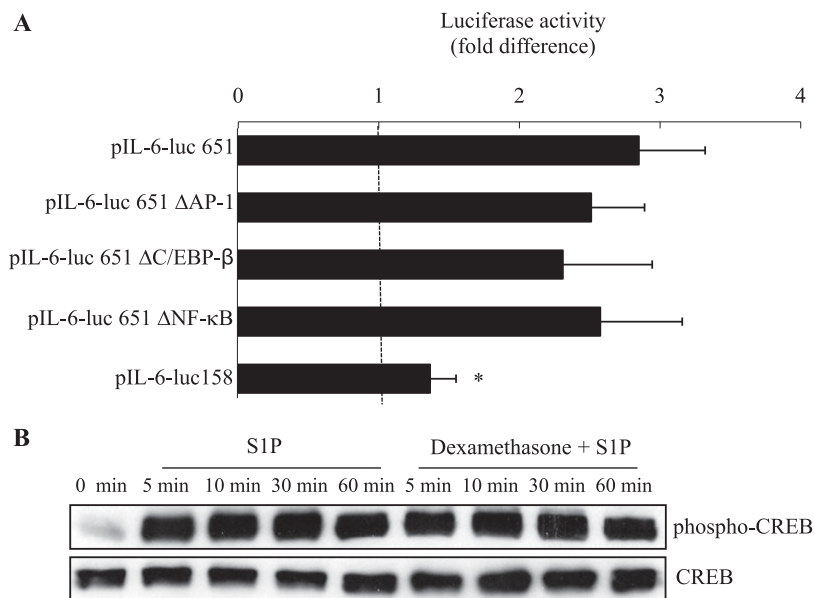


Figure 3. S1P-induced IL-6 transcriptional regulation is CRE dependent, but dexamethasone does not inhibit S1P-induced cyclic adenosine monophosphate response-element-binding protein (CREB) phosphorylation. (A) ASM cells were transfected with the parental construct pIL-6-luc 651, with a series of constructs in which the consensus binding sites for AP-1, C/EBP, and NF-κB had been inactivated by site-directed mutagenesis, or with a 5'-deletion construct (pIL-6-luc158) in which the AP-1 and CREB binding sites had been deleted. Cells were growth arrested and treated with vehicle or S1P (1 μM) for 24 hours. The data represent normalized luciferase activity relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student's unpaired *t* test. *Significant effect of promoter truncation ($P < 0.05$). Data are mean + SEM values from $n = 10$ primary ASM cell lines. (B) Growth-arrested ASM cells pretreated for 1 hour with vehicle or 100 nM dexamethasone were stimulated with S1P (1 μM) for 0, 5, 10, 30, and 60 minutes. Cells were lysed and then analyzed by Western blotting using specific antibodies against phosphorylated (Ser¹³³) and total CREB. Results are representative Western blots (from $n = 6$ primary ASM cell lines).

growth-related oncogene protein α (11) via corticosteroid-induced up-regulation of the endogenous MAPK deactivator, MKP-1. When ASM cells were pretreated for 1 hour with dexamethasone before S1P stimulation, up-regulated MKP-1 was evident at 30 and 60 minutes (Figure 4A). Densitometric analysis demonstrated that dexamethasone significantly enhances S1P-induced MKP-1 protein up-regulation over time ($P < 0.05$) (Figure 4B). The increase in MKP-1 after corticosteroid treatment mirrored the decrease in S1P-induced p38 MAPK phosphorylation at 30 minutes, suggesting that dexamethasone-induced MKP-1 shortens the temporal phosphorylation of MAPKs in response to S1P. This was confirmed by densitometry, where dexamethasone significantly inhibited S1P-induced p38 MAPK (Figure 4C) and ERK (Figure 4D) phosphorylation ($P < 0.05$). Although S1P-induced JNK phosphorylation appears on the Western blot (Figure 4A) to

be inhibited by dexamethasone pretreatment at 30 minutes, this difference was not statistically significant (Figure 4E).

Dexamethasone Inhibits S1P-Induced Activation of MSK1 and Induction of Histone H3 Phosphorylation

Because S1P-induced MAPK phosphorylation was inhibited by dexamethasone (mirrored by up-regulation of MKP-1), we hypothesized that the mechanism responsible for the inhibition of S1P-induced IL-6 by dexamethasone was the MKP-1-mediated repression of the MAPK pathways responsible for cytokine secretion. To address this further, we focused on the p38 MAPK and ERK signaling pathways because these MAPK family members are responsible for S1P-induced IL-6 secretion in ASM cells (4). We examined the activation of two important downstream phosphoproteins, MSK1 and histone H3. MSK1 (mitogen and stress-activated protein kinase) is a key signaling

molecule downstream of p38 MAPK and ERK and is known to induce phosphorylation of histone H3 (at Ser¹⁰) (12). In parallel with dexamethasone, we pretreated ASM cells with inhibitors of p38 MAPK and ERK pathways (using SB203580 and PD98059, respectively) before stimulation with S1P. S1P rapidly and robustly induced phosphorylation of MSK1 at (Thr⁵⁸¹) within 5 minutes, was sustained for 10 to 30 minutes, and subsided to basal levels by 60 minutes (Figures 5A–5C). We noted subsequent phosphorylation of histone H3 (at Ser¹⁰), with a peak generally observed at 30 minutes. Pretreatment of ASM cells with dexamethasone (Figure 5A) repressed S1P-induced MSK1 phosphorylation and histone H3 activation in a manner similar to those in cells pretreated with inhibitors of p38 MAPK (Figure 5B) or ERK (Figure 5C). That is, the temporal kinetics of MSK1 phosphorylation were considerably abridged, and the degree of activation of histone H3 at 30 minutes was substantially repressed. This was confirmed by densitometry (Figures 5D and 5E), where dexamethasone, SB203580, or PD98059 had a significant effect on S1P-induced MSK1 and histone H3 phosphorylation ($P < 0.05$). Phosphorylated histone H3 contributes to a relaxed chromatin environment and is associated with enhanced cytokine expression in ASM cells (13–14). These results demonstrate that the repression of S1P-induced cytokine secretion by dexamethasone may occur via inhibition of MSK1-mediated histone H3 phosphorylation by MKP-1.

S1P Induces IL-6 Secretion via a MSK1-Dependent Pathway, but Inhibition by Dexamethasone Is Not Due to Altered Subcellular Distribution of MSK1

To confirm the important role played by MSK1 signaling in S1P-induced cytokine secretion in ASM cells, we knocked down MSK1 with siRNA and measured the effect on S1P-induced IL-6 secretion. Nucleofection of ASM cells with siRNA against MSK1 substantially reduced MSK1 protein levels in comparison to cells transfected with scrambled control (Figure 6A). We then measured the IL-6 secreted into the cellular supernatants and found that in the absence of MSK1, the amount of IL-6 secreted by ASM cells in

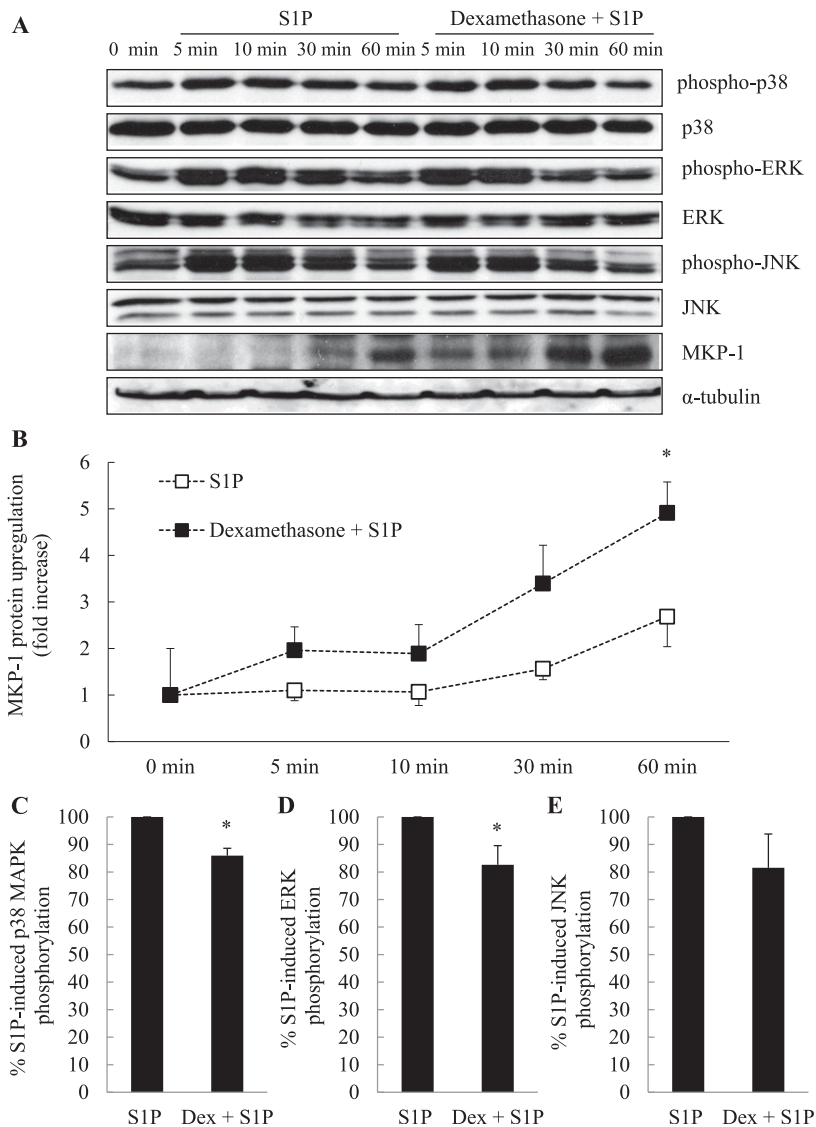


Figure 4. S1P-induced mitogen-activated protein kinase (MAPK) phosphorylation is inhibited by dexamethasone-induced mitogen-activated protein kinase phosphatase 1 (MKP-1). (A) Growth-arrested ASM cells pretreated for 1 hour with vehicle or 100 nM Dex were stimulated with S1P (1 μ M) for 0, 5, 10, 30, and 60 minutes. Cells were lysed and then analyzed by Western blotting using specific antibodies against phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total p38 MAPK, phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total ERK, phosphorylated (Thr¹⁸³/Tyr¹⁸⁵) and total c-Jun N-terminal kinase (JNK), and MKP-1. α -Tubulin was used as the loading control. (A) Representative Western blots. (B) Densitometric analysis of MKP-1 protein up-regulation expressed as fold increase over 0 minutes. (C–E) Effect of dexamethasone on S1P-induced p38 MAPK, ERK, and JNK phosphorylation (results expressed as % S1P-induced phosphorylation at 30 min). Statistical analysis was performed using two-way ANOVA and Bonferroni's post-test (B) or Student's unpaired *t* test (C–E). *Significant effect of dexamethasone ($P < 0.05$). Data are mean + SEM values from $n = 6$ primary ASM cell lines.

response to stimulation with S1P was greatly significantly reduced ($P < 0.05$) (Figure 6B).

In cell types apart from ASM, dexamethasone has been shown to repress inflammatory gene expression by altering the subcellular distribution of MSK1 and inducing translocation of MSK1 from the

nucleus to the cytoplasm (15). To ensure that this is not the underlying mechanism, we examined the nuclear translocation of MSK1 in ASM cells after dexamethasone pretreatment. MSK1 is found resident in the nucleus (16), as confirmed in the vehicle-treated cells shown in Figure 6C. S1P or

dexamethasone treatment alone did not alter MSK1 subcellular distribution. Moreover, the levels of MSK1 in the nucleus were unchanged by the pretreatment with dexamethasone before S1P stimulation.

Dexamethasone-Induced MKP-1 Inhibits S1P-Induced Activation of MSK1 and Induction of Histone H3 Phosphorylation

Because the molecular mechanism responsible for dexamethasone was not due to redistribution of nuclear MSK1 to the cytoplasm, we confirmed that the repressive effects of corticosteroids were due to up-regulation of the MAPK deactivator (MKP-1) and attenuation of p38 MAPK and ERK activation upstream of MSK1 phosphorylation. This was demonstrated by pretreating cells with triptolide, an inhibitor of MKP-1 up-regulation (14, 17, 18), and by observing the effect on downstream phosphoprotein signaling molecules responsible for cytokine secretion. Triptolide pretreatment completely abrogated MKP-1 protein induced by dexamethasone + S1P at 60 minutes (Figure 7A). In triptolide-treated cells 30 to 60 minutes after dexamethasone + S1P, where we had previously observed a decline in MAPK activity as MKP-1 up-regulation increased, we now demonstrate that S1P-induced MAPK signaling continues unabated in the absence of MKP-1. There are high levels of phosphorylated MSK1 (Thr⁵⁸¹) and histone H3 (Ser¹⁰) in the absence of MKP-1 at 60 minutes as confirmed by densitometry (Figures 7B and 7C). Taken together, our results suggest that corticosteroid-induced MKP-1 controls the extent and duration of S1P-induced MAPK-driven, MSK1/histone H3 proinflammatory signaling pathways to restrain cytokine secretion in ASM cells.

Discussion

S1P, a bioactive sphingolipid found in elevated amounts in the airways of individuals with asthma, has emerged as an important mediator responsible for airway inflammation and hyperresponsiveness in asthma. In this study, we uncover the molecular pathways underlying S1P-induced secretion of IL-6 in ASM cells and perform the first investigation examining

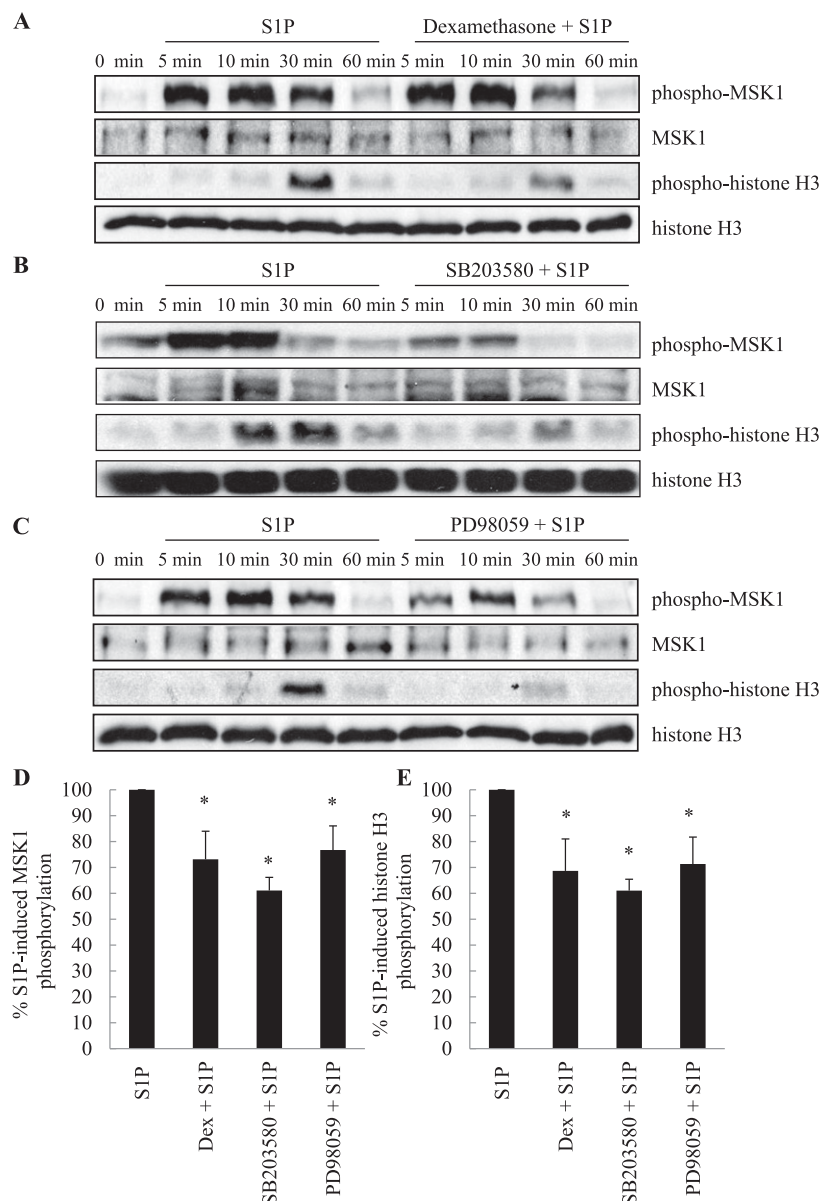


Figure 5. Dex inhibits S1P-induced activation of mitogen and stress-activated protein kinase 1 (MSK1) and induction of histone H3 phosphorylation. Growth-arrested ASM cells pretreated with vehicle or Dex (100 nM) for 1 hours (A), vehicle or SB203580 (1 μ M) for 30 minutes (B), or vehicle or PD98059 (10 μ M) (C) were stimulated with S1P (1 μ M) for 0, 5, 10, 30, and 60 minutes. Cells were lysed and analyzed by Western blotting using specific antibodies against phosphorylated (Thr⁵⁸¹) and total MSK1 and phosphorylated (Ser¹⁰) and total histone H3. (A–C) Representative Western blots. (D and E) Densitometric analysis of the effect of Dex, SB203580, or PD98059 on S1P-induced MSK1 (D) or histone H3 (E) phosphorylation (results expressed as % S1P-induced phosphorylation at 30 min). Statistical analysis was performed using Student's unpaired *t* test. *Significant inhibition ($P < 0.05$). Data are mean + SEM values from $n = 4$ primary ASM cell lines.

the inhibitory effects of corticosteroids on S1P-induced ASM synthetic function. Our results show that dexamethasone inhibits S1P-induced IL-6 mRNA expression and protein secretion from ASM cells by a repressive mechanism independent of the effects on cAMP/CREB/CRE-dependent

transcriptional pathway, IL-6 mRNA stability, and MSK1 nuclear relocation. Rather, our studies reveal that corticosteroids repress cytokine secretion in ASM cells via a mechanism dependent on MKP-1-mediated inhibition of MAPK-driven MSK1/histone H3 phosphorylation.

S1P is a product of sphingolipid metabolism (via the action of two sphingosine kinase [SphK] isoenzymes, SphK1 and SphK2) and is formed in multiple cell types in response to numerous stimuli. The pleiotropic actions of S1P are mediated via a family of G protein-coupled receptors, formerly known as EDG (Endothelial Differentiation Gene) receptors. Five members of the EDG family, S1P₁ (formerly EDG-1 [19]), S1P₂ (EDG-5 [20]), S1P₃ (EDG-3 [20]), S1P₄ (EDG-6 [21]), and S1P₅ (EDG-8 [22]), have been shown to be S1P receptors. S1P receptor expression is cell type and species specific, and most cell types express multiple receptors for S1P. We have shown that ASM cells express mRNA for S1P₁₋₄ (1) (S1P₅ was not examined). Because S1P receptors signal to multiple cellular signaling pathways via specific G proteins, myriad cellular processes are activated on activation with S1P as an extracellular ligand.

Our original observation in 2001 (1) demonstrated that S1P was found elevated in the airways of subjects with asthma 24 hours after segmental allergen challenge, correlated with concomitant increases in airway eosinophilia, protein influx, and fall in baseline lung function. The cellular source of S1P is the mast cell, whereby cross-linking of Fc ϵ RI activates SphK isozymes to phosphorylate sphingosine to result in the secretion of S1P (23). Over the past decade, substantial evidence has emerged to underscore the importance of S1P-stimulated pathways in the airway cell functions responsible for the development of asthma, such as ASM proliferation (1, 24), eosinophil chemotaxis (25), and contraction of ASM cells (1, 26), fibroblast cells (27), and guinea pig smooth muscle strips (28). These *in vitro* and *ex vivo* studies indicate a proasthmatic role for S1P, although in the course of asthma numerous other stimuli may activate other proinflammatory pathways. For this reason, recent evidence *in vivo* in murine models of asthma (2, 3) helps reinforce the important proasthmatic role played by S1P. An significant recent study (29) revealed that a specific inhibitor of SphK1 attenuates airway hyperresponsiveness and represses airway inflammation in a mast cell-dependent mouse model of allergic asthma; this underscores the important role played by S1P and highlights the promise of targeting the SphK1 isoform in asthma.

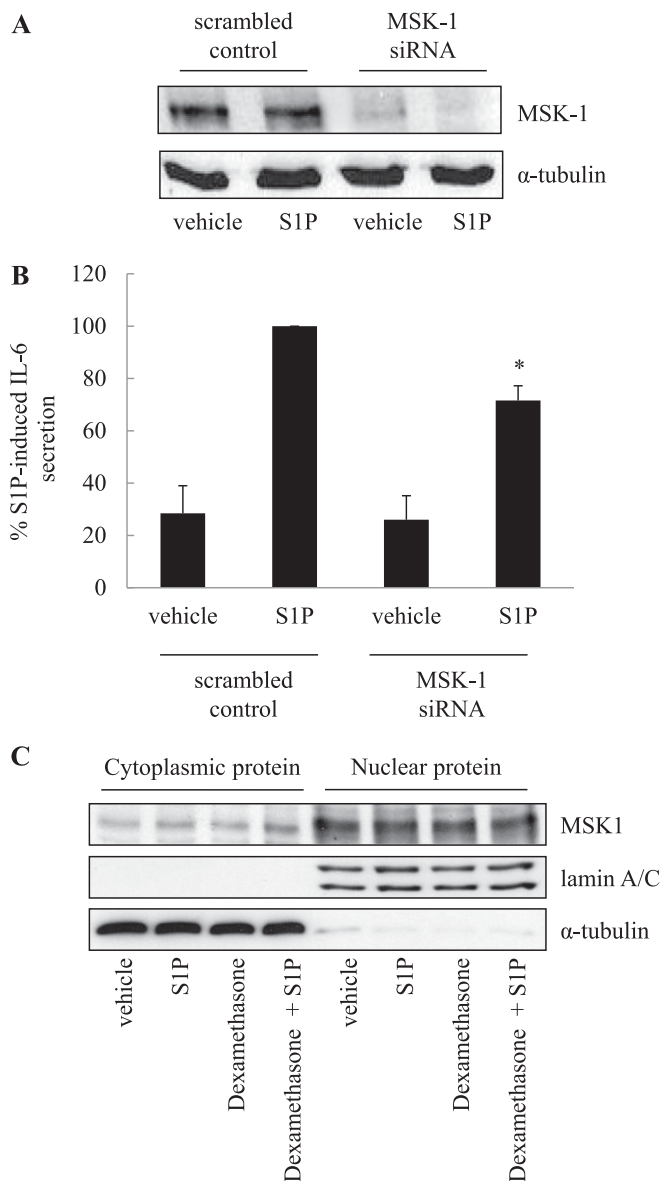


Figure 6. S1P induces IL-6 secretion via a MSK1-dependent pathway, but inhibition by dexamethasone is not due to altered subcellular distribution of MSK1. (A and B) ASM cells were transiently transfected using nucleofection with scrambled control or MSK1 siRNA, growth-arrested, and treated for 24 hours with vehicle or S1P (1 μ M). (A) To confirm that MSK1 siRNA reduces protein levels of MSK1, cells were lysed and immunoblotted for MSK1 using α -tubulin as the loading control. (B) To demonstrate that S1P induces IL-6 secretion via a MSK1-dependent pathway, supernatants were removed and IL-6 protein measured by ELISA. (A) Representative Western blot. (B) Results expressed as % S1P-induced IL-6 secretion in cells transfected with scrambled control siRNA (mean \pm SEM values from $n = 6$ primary ASM cell lines). Statistical analysis was performed using the Student's unpaired t test. *Significant decrease in % S1P-induced IL-6 secretion ($P < 0.05$). (C) Growth-arrested ASM cells pretreated for 1 hour with vehicle or dexamethasone (100 nM) and then treated with vehicle or S1P (1 μ M) for 30 minutes. Cytoplasmic and nuclear protein extracts were prepared and MSK1 subcellular distribution measured by Western blotting, compared with α -tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. Results are representative Western blots (from $n = 3$ primary ASM cell lines).

Thus, because S1P-mediated proinflammatory and hyperresponsive functions have been shown to be pivotal in

asthma, S1P and its receptors, as well as SphK1, have emerged as important candidates for the development of future

pharmacotherapeutic strategies in asthma (29–32). Another strategy to reduce the S1P-induced proinflammatory actions in asthma is to repress the cellular signaling pathways responsible for S1P-induced synthetic function. To this end, in the current study we demonstrate the repressive action of the first-line antiinflammatory medications used in asthma (i.e., corticosteroids). We show that the corticosteroid dexamethasone significantly inhibits S1P-induced IL-6 protein secretion and mRNA expression. Although many cytokines in addition to IL-6, including IL-13, IL-4, and IL-5, play a major role in airway inflammation and hyperreactivity in asthma, in this study we have chosen to focus on S1P-induced IL-6 because its secretion from ASM cells was first reported by us in 2001 (1). As such, IL-6 secretion can serve as a model system to delineate the molecular mechanisms underlying corticosteroid-mediated repression.

The molecular mechanisms underlying the repressive effects of corticosteroids on gene expression are multifactorial (reviewed in Refs. 33–35). Because an increased understanding of the molecular mechanisms by which corticosteroids suppress airway inflammation in asthma may identify novel targets for the development of corticosteroid-sparing pharmacotherapeutic strategies in the future, we performed further investigations to elucidate the mechanism responsible for the inhibition of S1P-induced IL-6 gene expression by dexamethasone. Our studies reveal that some of the more well-established mechanisms known to be responsible for corticosteroid action are not responsible for the inhibition of S1P-induced IL-6 gene expression. In cell types other than ASM, corticosteroids can produce a repressive effect by binding to the glucocorticoid receptor that then interacts with other transcription factors, such as CREB (36). In our study we show that dexamethasone pretreatment had no effect on S1P-induced CREB phosphorylation at Ser¹³³. In addition, we investigated the subcellular localization of MSK1 because corticosteroids have been shown to alter the distribution of this key downstream effector and repress inflammatory pathways (15); this possibility was also excluded.

Our data highlight another important way in which the corticosteroid-induced

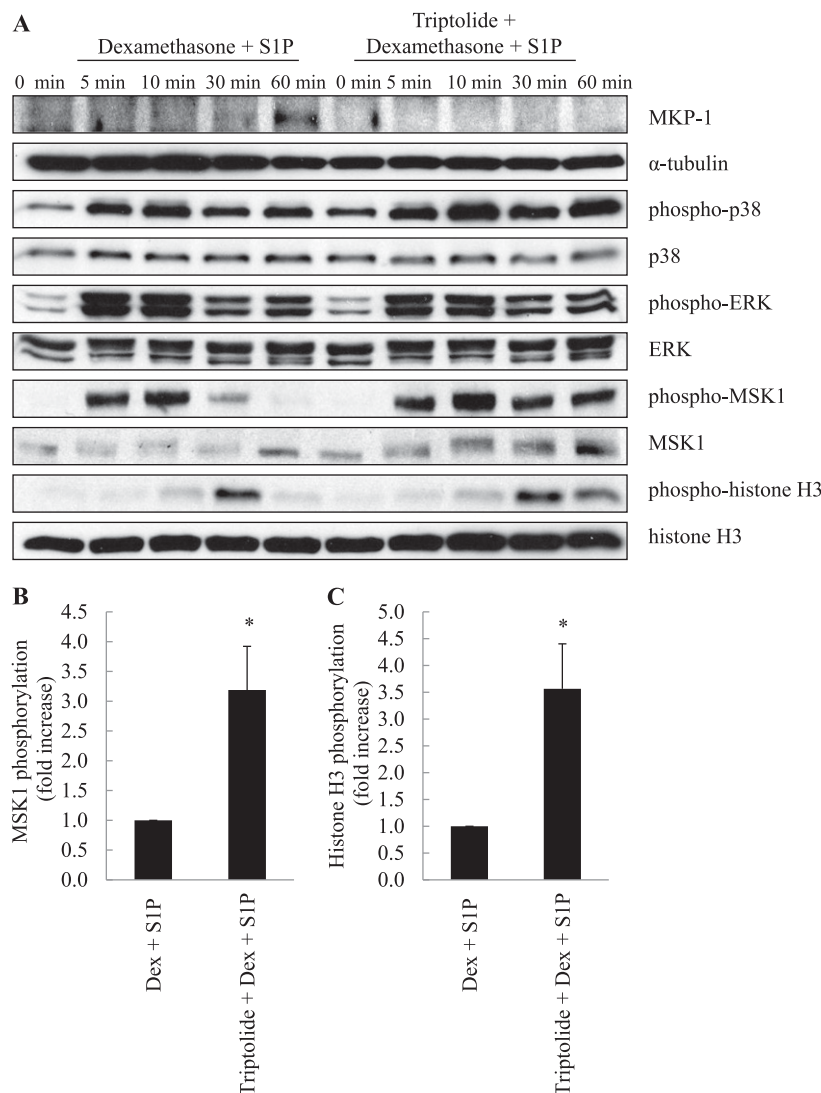


Figure 7. Dex-induced MKP-1 inhibits S1P-induced activation of MSK1 and induction of histone H3 phosphorylation. Growth-arrested ASM were pretreated with triptolide (1 μ M) for 30 minutes, followed by 1 hour pretreatment with vehicle or Dex (100 nM) and then stimulated with S1P (1 μ M) for 0, 5, 10, 30, and 60 minutes. Cells were lysed and then analyzed by Western blotting using specific antibodies against MKP-1 (α -tubulin was used as the loading control), phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total p38 MAPK, phosphorylated (Thr²⁰²/Tyr²⁰⁴) and total ERK, phosphorylated (Thr⁵⁸¹) and total MSK1, and phosphorylated (Ser¹⁰) and total histone H3. (A) Representative Western blots. (B and C) Densitometric analysis of the effect of triptolide on the repression of S1P-induced MSK1 (B) or histone H3 (C) phosphorylation by Dex (results expressed as fold increase in phosphorylation at 60 min). Statistical analysis was performed using Student's unpaired *t* test. *Significant inhibition (*P* < 0.05). Data are mean + SEM values from *n* = 3 primary ASM cell lines.

MKP-1 can repress airway inflammation. We and others have recently shown that many of the antiinflammatory actions of corticosteroids in ASM cells are mediated via the induction of the endogenous MAPK inhibitor, MKP-1. MKP-1 has multiple modes of repression: it acts as a critical negative feedback effector (4, 14), limiting the extent and duration of proinflammatory

cellular signaling; it can repress synthetic function of this immunomodulatory airway cell type by antiasthma drugs (37–39); and it can reduce p38 MAPK-mediated mRNA stability (10). In the current study, we demonstrate an additional mode of MKP-1-mediated repression (i.e., via inhibition of MAPK-driven MSK1/histone H3 phosphorylation). We confirm that S1P can

induce activation of a variety of MAPKs and that the increase in MKP-1 after corticosteroid treatment appeared to mirror the decrease in S1P-induced MAPK phosphorylation. These results add to the accumulating evidence in ASM cells, demonstrating that dexamethasone-induced MKP-1 shortens the temporal phosphorylation of MAPKs in response to TNF- α (10), IL-1 β (11), and now S1P. It is not an effect on S1P-induced IL-6 mRNA stability that is responsible for the repression of S1P-induced synthetic function by corticosteroids; herein we show that repression may be due to an effect on chromatin remodeling. We have shown that by shortening the temporal kinetics of MAPK-driven MSK1 phosphorylation, dexamethasone reduces phosphorylation of histone H3 at Ser¹⁰, a key regulatory site responsible for the promotion of a relaxed chromatin environment in transcription (12). The MKP-1 dependence was confirmed by treatment with triptolide, an inhibitor of MKP-1 up-regulation, where repressive effects of corticosteroids were reversed and the phosphorylation of MSK1/histone H3 was unrestrained.

S1P-induced IL-6 secretion is completely inhibited by corticosteroids. We speculate that this is due to the fact that S1P-induced IL-6 transcriptional regulation is NF- κ B independent (as demonstrated in our IL-6 luciferase constructs experiments) because NF- κ B appears refractory to inhibition by corticosteroids in ASM cells (40).

Collectively, our results confirm that S1P, a bioactive sphingolipid found elevated in asthma, induces synthetic function of the pivotal airway cell type—ASM. Although dexamethasone significantly inhibits IL-6 secretion via inhibition of gene expression at the transcriptional (rather than post-transcriptional) level, this repression was not due to inhibition of CREB activation, an effect of mRNA stability, or MSK1 translocation. Instead we show that by up-regulating the MAPK deactivator MKP-1, corticosteroids restrain MAPK-driven MSK1 activation and histone H3 phosphorylation, proinflammatory pathways known to control cytokine expression. Taken together, our study identifies molecular mechanisms underlying the antiinflammatory capacity of corticosteroids to repress pro-remodeling functions induced by the potent bioactive sphingolipid in the lung. ■

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