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FAST TRACK

Inhibition of the p38 Pathway Upregulates Macrophage JNK and ERK Activities, and the ERK, JNK, and p38 MAP Kinase Pathways Are Reprogrammed During Differentiation of the Murine Myeloid M1 Cell Line

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Abstract Mitogen-activated protein (MAP) kinases have been implicated as important mediators of the inflammatory response. Here we report that c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAP kinase activities are reprogrammed during the IL-6 induced macrophage-like differentiation of the murine myeloid M1 cell line. Moreover, p38 inhibition upregulates JNK and ERK activity in M1 cells and in thioglycollate-elicited peritoneal exudate macrophages. IL-6-induced M1 differentiation also induces expression of the anti-inflammatory cytokine IL-10, and p38 inhibition potentiates this increase in IL-10 expression in an ERK-dependent manner. Thus, we speculate that during inflammatory conditions in vivo macrophage p38 may regulate JNK and ERK activity and inhibit IL-10 expression. These data highlight the importance of p38 in the molecular mechanisms of macrophage function. J. Cell. Biochem. 86: 1–11, 2002. © 2002 Wiley-Liss, Inc.

Key words: cellular differentiation; inflammation; monocytes/macrophages; protein kinases; signal transduction

Mitogen-activated protein (MAP) kinase signal transduction pathways are evolutionarily conserved among eukaryotes and have been implicated in a variety of biological processes, including cell growth and differentiation, apoptosis, inflammation, and the responses to environmental stresses (e.g., osmotic shock, ultraviolet light) [Schaeffer and Weber, 1999]. These pathways modify the activities of a variety of cellular proteins to effect physiological responses appropriate to both the extent and the duration of the initiating stimuli, and the proper regulation of MAP kinase cascades is undoubtedly critical to the existence and integrity of many cell and tissue types.

Cellular differentiation is a fundamental aspect of many biological phenomena, such as mating and sporulation in yeast, tissue specification during the growth of metazoans, and the development of hematopoietic lineages in mammals. During differentiation, patterns of gene expression and cell cycle regulation are reprogrammed to produce a new cellular phenotype. Moreover, intracellular signaling pathways that coordinate responses to extracellular signals are presumably also reprogrammed; however, the precise details of these changes in stimulus perception remain largely unknown. The best example of a differentiation-dependent shift in MAP kinase pathway signaling is possibly found in the budding yeast *Saccharomyces cerevisiae*. In response to nitrogen starvation in the presence of a nonfermentable carbon source, diploid yeast cells exit the G1 phase of the cell cycle, transit through

Abbreviations used: ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAPKAPK-2, MAPK-activated protein kinase-2; MEK, MAPK/ERK kinase.

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meiosis I and II, and sporulate. The *SMK1* MAP kinase pathway is required for the expression of late sporulation-specific genes, spore wall morphogenesis, and the creation of four intact haploid ascospores [Krisak et al., 1994]. However, *SMK1* expression is developmentally controlled: *SMK1* is not expressed during vegetative growth, but *SMK1* expression is induced at least 200-fold at the time of expression of middle sporulation genes. This mechanism ensures that *SMK1* activity does not influence vegetative functions, and that the activity of the pathway is only utilized at the final stages of the cytodifferentiation process. A similar reprogramming mechanism has been observed during the differentiation of mammalian T lymphocytes. The mammalian MAP kinases are grouped according to structural and functional criteria, and the most prominent of these sub-families are the extracellular signal-regulated kinases (ERKs), the p38 MAP kinases, and the c-Jun NH_2-terminal kinases (JNKs). It has been demonstrated that JNKs are not expressed in peripheral $CD4^+$ T cells, and that JNK expression is dramatically induced during their activation and differentiation into effector cells [Rincon et al., 1997; Weiss et al., 2000]. This mechanism ensures that JNK activity does not interfere with the initial phases of antigen recognition (e.g., by inducing apoptosis of the reactive cells), and that JNK pathway signaling is only utilized at the later stages of peripheral $CD4^+$ T cell activation.

In the adult animal, macrophages develop from circulating monocytes and immature myeloid cells which are released from the bone marrow. In response to inflammatory stimuli, these cells emigrate to the peripheral tissues where they differentiate into activated, tissue-specific macrophages [Gordon, 1995; Takahashi et al., 1996]. This paradigm thus provides an opportunity to examine the influence of cellular differentiation on MAP kinase pathway activities within the context of the inflammatory response. Therefore, we investigated the influence of macrophage differentiation on ERK, p38, and JNK MAP kinase pathways using a murine myeloid cell line that acquires a macrophage-like phenotype in response to inflammatory stimuli. Basal p38 pathway activity was increased by the differentiation process, and differentiation altered the responses of ERK, p38, and JNK to stimulation with bacterial lipopolysaccharide (LPS). Further investigations revealed that the inhibition of p38 activity caused the potentiation of JNK and ERK activities both before and after differentiation, indicating that the p38 pathway represses JNK and ERK activities in this system. This inter-pathway repression was also observed in thioglycollate-elicited peritoneal exudate macrophages. Thus, cross-communication between MAP kinase pathways may be an integral component of signal transduction in macrophages.

MATERIALS AND METHODS

Materials

Lyophilized LPS (*Escherichia coli* serotype 0111:B4) (Sigma) was reconstituted in phosphate buffered saline, stored at 4°C for up to 1 month, and vortexed for 10 min before each use. SB203580, U0126, and U0124 were obtained from Calbiochem-Novabiochem. Murine recombinant IL-6 and IFN-γ and the Quantikine mouse IL-10 immunoassay kit were obtained from R&D Systems. Total RNA was isolated using the RNA/STAT-60 reagent (Tel-Test). The RiboQuant In Vitro Transcription and Ribonuclease Protection Assay kits, and the mCK-2b probe template set, were obtained from BD PharMingen. JNK, ERK, p38, MAPKAPK-2, and iNOS protein levels were assayed by immunoblot analysis using mouse-anti-JNK1/2 (BD PharMingen), rabbit-anti-ERK2, goat-anti-p38, goat-anti-MAPKAPK-2 (Santa Cruz Biotechnology), and mouse-anti-iNOS (Transduction Laboratories).

M1 Cell Culture

The mouse myeloid leukemia M1 cell line (American Type Culture Collection) was cultured in suspension at 37°C and 5% CO_2 in RPMI Medium 1640 (Life Technologies) supplemented with 10% FCS (Omega Scientific), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were seeded at 1.5×10^5 cells/ml and were not allowed to grow to more than 1×10^6 cells/mL. Log phase cultures were used at the start of all experiments. For time course experiments, a separate culture flask was prepared for each time point.

Peritoneal Macrophage Isolation and Culture

Female C57BL/6 mice (5–6 weeks of age) (The Jackson Laboratory) were injected i.p. with 1 mL of sterile 4% Brewer thioglycollate broth (Becton Dickinson), and 72 h later peritoneal

exudate cells were isolated by lavage using Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were washed several times by centrifugation at 1,000 rpm and plated at a density of 2.5×10^6 cells per 35-mm dish. After ≥ 5 h, nonadherent cells were removed by repeated washing, and the adherent macrophages were subjected to various treatments.

Kinase Assays

Cells were washed once with Krebs-Ringer-HEPES [50 mM HEPES (pH 7.4), 128 mM NaCl, 5 mM KCl, 2.45 mM $MgSO_4$, 1.3 mM $CaCl_2$], and lysed on ice in Triton lysis buffer [Hall and Davis, 2002]. JNK activity was analyzed by the solid phase protein kinase assay using GST-c-Jun (residues 1-79) [Hall and Davis, 2002]. ERK, p38, and MAPKAPK-2 activities were analyzed by the immune complex protein kinase assay using rabbit-anti-ERK2 (Santa Cruz Biotechnology), rabbit-anti-p38α [Raingeaud et al., 1995], and sheep-anti-MAPKAPK-2 (Upstate Biotechnology) as described [Hall and Davis, 2002]. Substrate proteins used were GST-Elk1 (residues 307-428) [Whitmarsh et al., 1995], GST-ATF2 (residues 1-109) [Gupta et al., 1995], and Hsp25 (StressGen Biotechnologies), respectively.

RESULTS

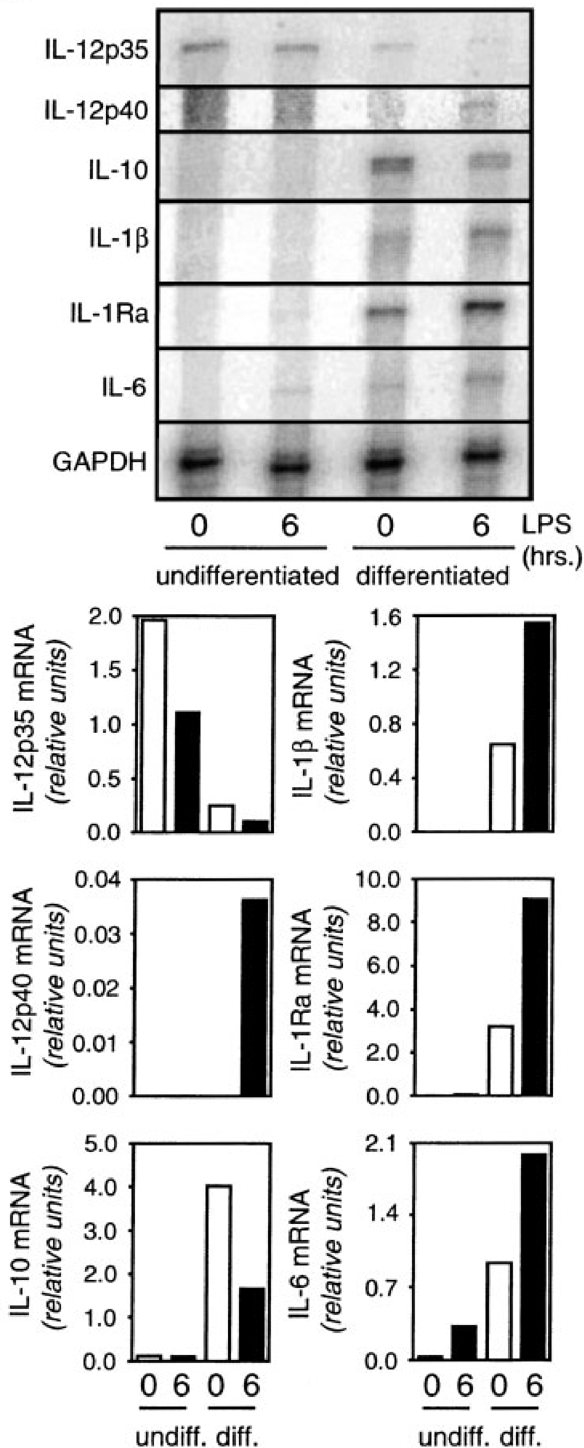
IL-6-Differentiated M1 Cells Express Inflammatory Markers Common to Differentiated Macrophages

M1 cells cultured for 72 h in the presence of 50 ng/ml recombinant murine interleukin-6 (IL-6) stop proliferating and acquire characteristics of terminally differentiated macrophages. For example, IL-6-induced differentiation produces a highly vacuolated cytoplasm and lobated nucleus, increases phagocytic potential, and induces the sequential expression of FcγRI, c-Fms, lysozyme, and Type IV collagenase [Fibach et al., 1973; Krystosek and Sachs, 1976; Sachs, 1978; Chiu and Lee, 1989; Pluznik et al., 1992; Ruhl and Pluznik, 1993; and data not shown]. In order to further characterize this macrophage-like phenotype, we examined the expression of several additional inflammatory markers in undifferentiated and IL-6-differentiated M1 cells (Fig. 1). IL-6-induced differentiation increased the basal expression levels of IL-10, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-6 mRNAs. Stimulation of IL-6-differentiated cultures with LPS increased the expression of IL-12p40, IL-1β, and IL-1Ra mRNAs; however, LPS stimulation did not augment the expression of these mRNAs in undifferentiated cultures. Stimulation of IL-6-differentiated cells with LPS also decreased IL-10 mRNA expression, and this may help to ensure a proper response to LPS given the anti-inflammatory properties of IL-10 protein [Lalani et al., 1997; Rennick et al., 1997; Stordeur and Goldman, 1998]. These results indicate that, consistent with their macrophage-like phenotype, IL-6-differentiated M1 cells exhibit increased basal and LPS stimulated expression of several inflammatory cytokine mRNAs.

The coordinated production and release of nitric oxide (NO) is an integral component of the bactericidal, cytotoxic, and cytostatic acitivities of macrophages [Nathan, 1992; Nathan and Xie, 1994; Gordon, 1995], and macrophage NO is generated by the inducible, Ca^{2+}-independent isoform of NO synthase (iNOS). It has been reported that iNOS mRNA is upregulated during IL-6-induced M1 differentiation [Nakajima et al., 1996; Sawada et al., 1997]; therefore, we examined the influence of IL-6-induced differentiation on iNOS protein expression (Fig. 1B). After 72 h of differentiation, M1 cells produced functional iNOS protein following stimulation with LPS plus IFN-γ; however, LPS/IFN-γ stimulation did not induce iNOS protein expression in M1 cultures differentiated for 0, 24, or 48 h. These data indicate that, unlike their undifferentiated counterparts, IL-6-differentiated M1 cells express the macrophage-specific inflammatory marker iNOS.

M1 Cell Differentiation Alters the Regulation of ERK, JNK, and p38 MAP Kinases

We next determined whether IL-6-induced M1 differentiation altered the regulation of basal or LPS stimulated ERK, JNK, and p38 MAP kinase activities (Fig. 2). Differentiation did not change basal ERK activity, and in undifferentiated cultures ERK was not activated in response to LPS. However, in differentiated cultures, ERK activity was induced ≥ 3-fold after 30 min of LPS treatment and returned to pre-stimulation levels by 2 h after LPS addition. These results demonstrate that IL-6-induced differentiation creates an M1 cell



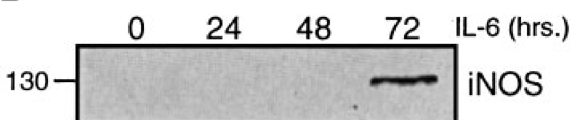
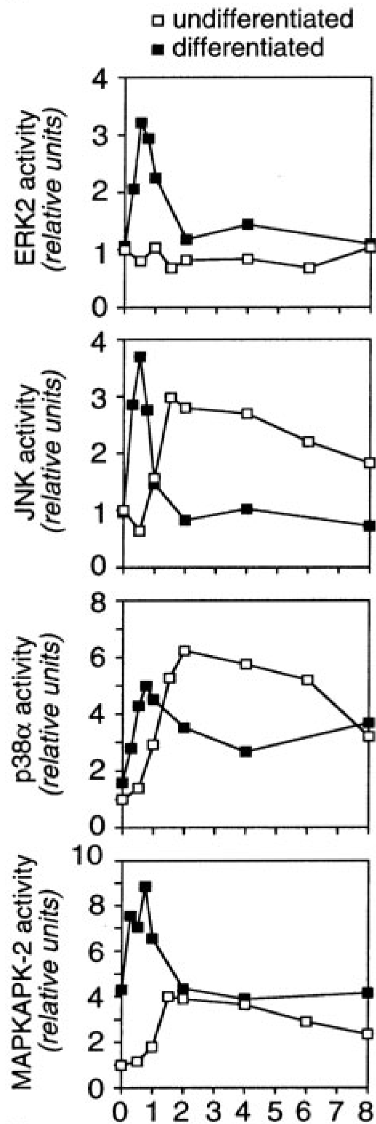


Fig. 1. The IL-6-induced differentiation of M1 cells increases expression of several molecular markers of macrophage activation. **A:** Cultures were stimulated for 0 or 6 h with 1μg/ml LPS (*undifferentiated*), and parallel cultures were treated with 50 ng/ml IL-6 for 72 h and then stimulated for 0 or 6 h with 1 μg/ml LPS (*differentiated*). Total RNA was isolated and subjected to RNase protection assays. Cytokine mRNA signals are shown as a percentage of the mRNA signal for the housekeeping gene *GAPDH*. **B:** Cultures were treated with 50 ng/ml IL-6 for 0, 24, 48, or 72 h, and then stimulated for 6 h with 1 μg/ml LPS plus 30 ng/ml IFNγ. Whole cell lysates were prepared and subjected to anti-iNOS immunoblot analysis. Molecular weight in kilodaltons is indicated at left. Analysis of the culture supernatants for NO production [Green et al., 1982] indicated the presence of functional iNOS proteins in the 72-h culture.

in which ERK can be activated in response to LPS, from an M1 cell in which ERK is nonresponsive to LPS treatment.

IL-6-induced differentiation also created an M1 cell in which JNK and p38 are more rapidly activated in response to LPS (Fig. 2). In undifferentiated cells, JNK activity was induced 3-fold by 1.5 h of LPS treatment and slowly returned to pre-stimulation levels over many hours. However, in differentiated cultures, JNK activity was induced $>$ 3-fold within 30 min and returned to pre-stimulation levels by 2 h after LPS addition. Similarly, p38 activity was maximally induced in undifferentiated cultures by 2 h of LPS treatment and slowly returned to pre-stimulation levels over the following 6 h; however, in differentiated cultures p38 activity was maximally induced within 1 h and decreased to a new and higher baseline value within 4 h of LPS addition.

Although IL-6-induced differentiation had no effect on basal JNK activity, basal p38 activity was increased approximately 50% by the differentiation process (Fig. 2). To confirm and extend this observation, MAPKAP kinase-2 activity was analyzed in undifferentiated and IL-6-differentiated cultures because it has been reported that MAPKAPK-2 is phosphorylated and activated by p38α both in vitro and in vivo [Stokoe et al., 1992; Allen et al., 2000]. Basal MAPKAPK-2 activity was increased $\geq$ 4-fold by the differentiation process, and the activation and recovery of MAPKAPK-2 in response to LPS was more rapid in the differentiated cultures than in the undifferentiated cultures. These results indicate that increased basal p38 and MAPKAPK-2 activity is coincident with IL-6-induced M1 differentiation.



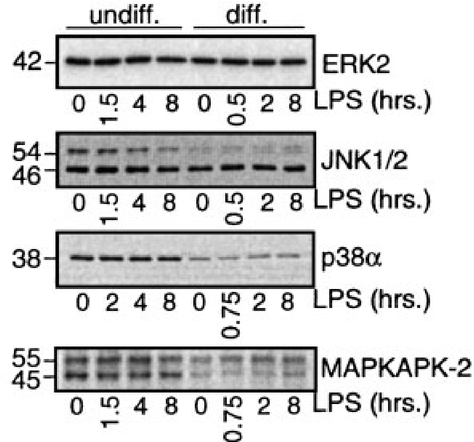


Fig. 2. The IL-6-induced differentiation of M1 cells changes ERK, JNK, and p38 MAP kinase activities. **A:** Cultures were stimulated for 0 through 8 h with 1 μg/ml LPS (open squares), and parallel cultures were treated with 50 ng/ml IL-6 for 72 h and then stimulated for 0 through 8 h with 1 μg/ml LPS (filled squares). Whole cell lysates were prepared at various times during LPS stimulation and assayed for JNK, ERK, p38, and MAPKAPK-2 activities. The activity profiles in each panel were standardized by dividing the value at each time point by the value in the undifferentiated condition at 0 h of LPS. **B:** As a control, the whole cell lysates were subjected to anti-JNK1/2, anti-ERK2, anti-p38, and anti-MAPKAPK-2 immunoblot analyses. Molecular weights in kilodaltons are indicated at left. By immunoblot analysis ERK1 protein was not detected in undifferentiated or IL-6-differentiated M1 cells.

p38 Inhibition Increases Basal and LPS Stimulated JNK and ERK Activities in Undifferentiated and IL-6-Differentiated M1 Cells

The IL-6-induced differentiation of M1 cells increased basal p38 and MAPKAPK-2 activity (Fig. 2). This raised the possibility that increased p38 activity might be required for the production of certain aspects of the macrophage-like phenotype. Therefore, having determined that IL-6-induced differentiation confers LPS-inducibility on the ERK MAP kinase, and that differentiation increases the rate of LPS-induced JNK activation (Fig. 2), we examined whether the inhibition of p38 during differentiation might influence these changes in these parallel MAP kinase pathways. To do this, we utilized the pyridinyl-imidazole derivative SB203580, which has been shown to bind and inhibit the p38α and p38β isoforms [Lee et al., 1994; Jiang et al., 1996; Stein et al., 1997]. M1 cultures were pre-treated for 1 h with 10 μM SB203580 or vehicle (DMSO) and then differentiated with IL-6 (*differentiated* in Fig. 3). Basal JNK and ERK activities in the differentiated, SB203580-treated cultures were approximately 2-fold greater than controls, and JNK and ERK activities in the SB203580-treated cultures remained potentiated during a subsequent stimulation with LPS. Indeed, after differentiation in the presence of SB203580, the LPS-inducibility of ERK was maintained and the increased rate of LPS-induced JNK activation was unaltered, but the SB203580 treatment did increase the overall magnitude of these responses. We conclude, therefore, that p38 activity regulates the amount of JNK and ERK activity in differentiated M1 cells.

SB203580 treatment also potentiated JNK and ERK activities in undifferentiated M1 cells.

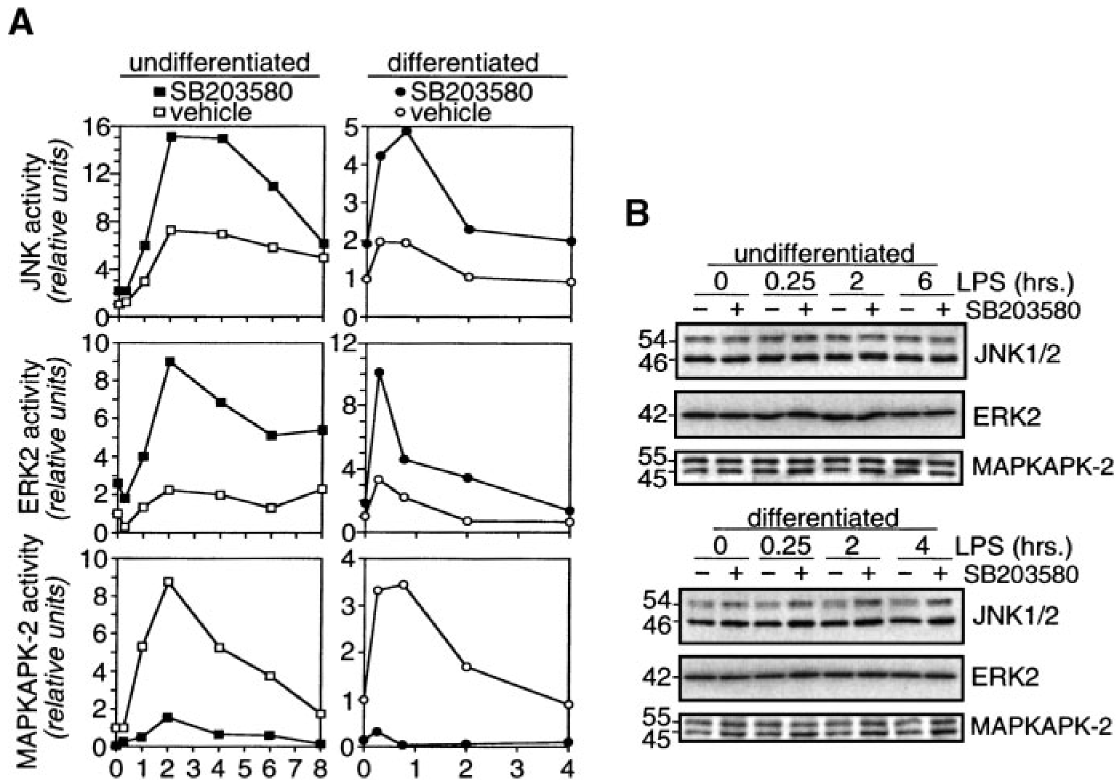


Fig. 3. Inhibition of p38 MAP kinase increases basal and LPS-stimulated JNK and ERK activities in undifferentiated and IL-6-differentiated M1 cells. **A**: Cultures were pre-treated for 1 h with 10 μM SB203580 (filled squares) or vehicle (open squares) and then stimulated with 1 μg/ml LPS for 0 through 8 h. In parallel, cultures were pre-treated for 1 h with 10 μM SB203580 (filled circles) or vehicle (open circles), treated with 50 ng/ml IL-6 for 72 h, and then stimulated with 1 μg/ml LPS for 0 through 4 h. Whole cell lysates were prepared at various times during LPS stimulation and assayed for JNK, ERK, and MAPKAPK-2 activities. The activity profiles in each panel were standardized by dividing the value at each time point by the value in the vehicle-treated condition at 0 h of LPS. In two additional experiments, ERK activity in the vehicle-treated undifferentiated cultures was not increased in response to LPS. **B**: As a control, the whole cell lysates were subjected to anti-JNK1/2, anti-ERK2, and anti-MAPKAPK-2 immunoblot analyses. Molecular weights in kilodaltons are indicated at left.

Naive cultures were treated for 1 h with SB203580 or vehicle-alone, and then stimulated with LPS for 8 h (*undifferentiated* in Fig. 3). At the end of the 1 h pre-treatment, JNK and ERK activities in the SB203580-treated cultures were $\geq$2-fold greater than controls, and JNK activity in the SB203580-treated cultures remained potentiated during the subsequent stimulation with LPS. Moreover, while ERK was not activated by LPS in undifferentiated M1 cultures, ERK was activated $>$3-fold in response to LPS in the SB203580-treated cultures, with maximal activation occuring 2 h after LPS addition. These results demonstrate that the inhibition of p38 in undifferentiated M1 cells increases JNK activity and converts endogenous ERK from an LPS-nonresponsive to an LPS-responsive state. Taken together, these data indicate that p38 regulates JNK and ERK in both undifferentiated and IL-6-differentiated M1 cells.

To confirm that p38 activity was repressed by SB203580 in these experiments, MAPKAPK-2 assays were performed (Fig. 3). SB203580 is known to bind p38 in a reversible manner in vivo; therefore, its inhibition of p38 activity cannot be measured by the anti-p38 immune complex kinase assay, and a more satisfactory approach is to measure the activity of the downstream p38 substrate MAPKAPK-2. In undifferentiated M1 cultures, MAPKAPK-2 activity was severely inhibited after treatment with SB203580. MAPKAPK-2 was also inhibited in differentiated cultures that had been pre-treated with SB203580, and this indicated that the inhibitory effect of SB203580 persisted throughout the 72-h differentiation process. These results demonstrate that under the

conditions utilized in these experiments, SB203580 inhibited p38 pathway signaling.

It should be noted that M1 differentiation in response to IL-6 involved a reduction in the levels of expression of the 54-kD forms of JNK1 and JNK2 (Fig. 2B). The expression of these 54-kD isoforms was slightly greater in some of the differentiated, LPS stimulated cultures in Figure 3 that had been pre-treated with SB203580. Therefore, it is possible that some of the increased JNK activity in these cultures may have resulted from the increased expression of these 54-kD isoforms. Nevertheless, these minor changes in isoform expression undoubtedly account for only a small fraction of the SB203580-dependent increases in JNK activity.

p38 Inhibition During IL-6-Induced M1 Cell Differentiation Increases IL-10 Expression in an ERK-Dependent Manner

The potentiation of JNK and ERK activities following p38 pathway inhibition suggested that SB203580 treatment may alter other aspects of M1 differentiation. We had determined that the expression of IL-10 mRNA is increased in M1 cells during IL-6-induced differentiation (Fig. 1). Subsequent investigation revealed that IL-10 mRNA and IL-10 protein were increased even further in IL-6-differentiated M1 cultures that had been pre-treated with SB203580 (Fig. 4A,B). Thus, pre-treatment of M1 cultures with SB203580 increases the amount of IL-10 expressed upon completion of the 72-h differentiation interval. This raises the intriguing possibility that at least some of the reported anti-inflammatory effects of SB203580 [Griswold et al., 1988; Lee et al., 1993; Reddy et al., 1994; Badger et al., 1996; Lee and Young, 1996] result from its ability to increase expression of the anti-inflammatory cytokine IL-10.

Inhibition of M1 cell p38 activity caused a concomitant increase in ERK activity (Fig. 3); therefore, we investigated the possibility that increased ERK activity was required for the increased IL-10 expression in differentiated cultures that had been pre-treated with SB203580. For these experiments, we utilized inhibitors of the MAP kinase kinases MEK1 and MEK2, the direct upstream activators of both ERK1 and ERK2 [Alessi et al., 1995; Favata et al., 1998]. We reasoned that by selectively inhibiting MEK/ERK activity, we might disclose a requirement for MEK/ERK activity in the SB203580-dependent mechanism of IL-10 induction. M1 cells were treated with vehicle, SB203580, or SB203580 plus increasing concentrations of the MEK1/2 inhibitor U0126, and then differentiated for 72 h with IL-6 (Fig. 4C). Increasing concentrations of U0126 caused a concomitant repression of both the SB203580-dependent increase in IL-10 mRNA and (as expected) the SB203580-dependent increase in ERK activity. Similar results were obtained when U0126 was added 24 h before completion of the 72-h differentiation interval, and when the less potent MEK1/2 inhibitor PD98059 was used (not shown). The negative control compound U0124 had no effect on the SB203580-dependent increase in IL-10 expression or ERK activity. These results indicate that an SB203580-dependent increase in ERK activity is required for the SB203580-dependent increase in IL-10 expression.

p38 Inhibition Increases LPS Stimulated JNK and ERK Activity in Primary Mouse Macrophages

JNK and ERK activities were potentiated in undifferentiated and IL-6-differentiated M1 cells following inhibition of p38 MAP kinase (Figs. 3 and 4). This allowed us to predict that JNK and ERK might be similarly regulated in primary mouse macrophages, following inhibition of p38 MAP kinase. Therefore, we examined the effect of p38 inhibition on JNK and ERK activities in thioglycollate-elicited peritoneal exudate macrophages. Peritoneal macrophage cultures were treated for 1 h with SB203580 or vehicle-alone, and then stimulated with 100 ng/ml LPS for 3 h (Fig. 5). MAPKAPK-2 activity was inhibited in all SB203580-treated cultures. JNK, ERK, and MAPKAPK-2 activities were maximally induced by 30 min of stimulation with 100 ng/ml LPS, and peak JNK and ERK activation levels were unaffected by the SB203580 pre-treatment. However, between 60 and 180 min after the addition of 100ng/ml LPS, JNK and ERK activities were potentiated in cultures that were pre-treated with SB203580. Thus, following maximal activation with 100 ng/ml LPS, JNK and ERK activities are potentiated by the inhibition of p38 pathway signaling.

Given that 100 ng/ml may represent a supraphysiological dose of LPS treatment [Fenton and Golenbock, 1998], we also tested the effect of SB203580 on JNK and ERK activities in primary mouse macrophages during stimulation with 1 ng/ml LPS (Fig. 5). Macrophage

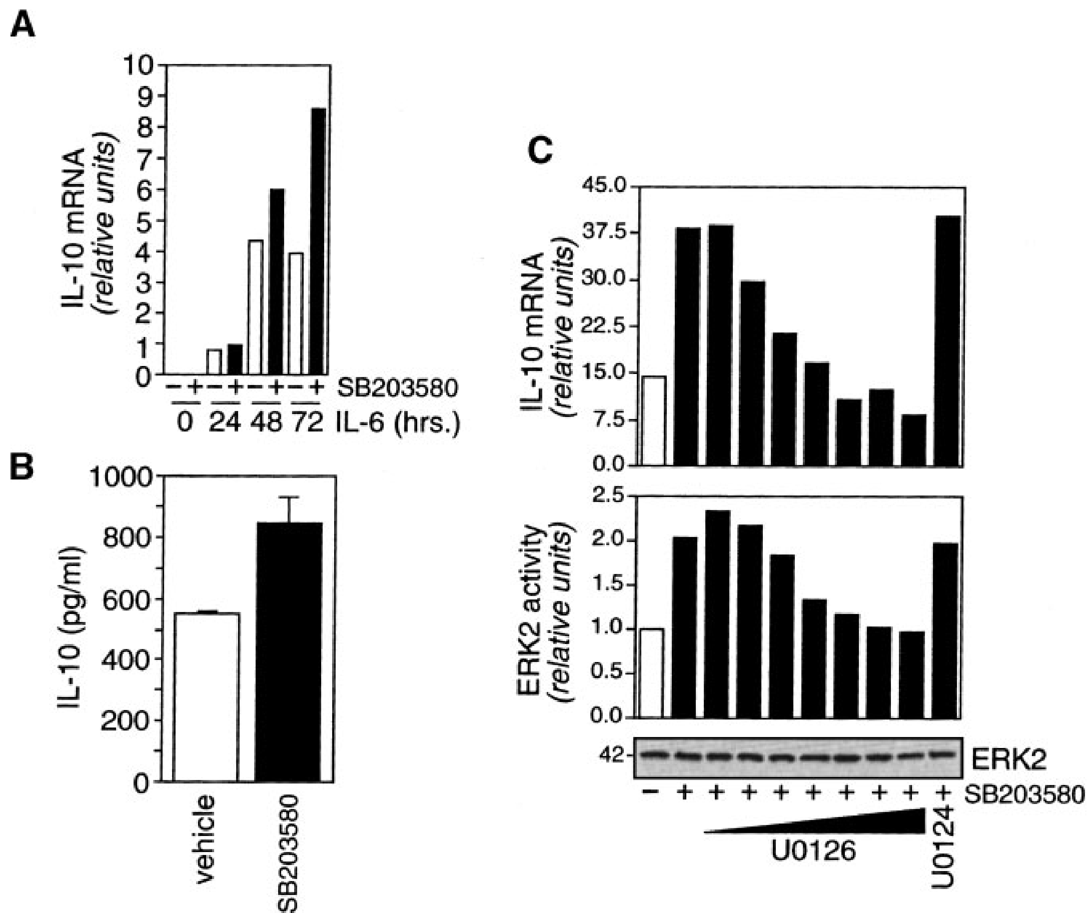


Fig. 4. Inhibition of p38 MAP kinase during IL-6-induced M1 differentiation increases IL-10 expression in an ERK-dependent manner. **A**: Cultures were pre-treated for 1 h with 10 μM SB203580 (filled bars) or vehicle (open bars), and then treated with 50 ng/ml IL-6 for 0, 24, 48, or 72 h. Total RNA was isolated and subjected to an RNase protection assay to examine IL-10 mRNA expression. IL-10 mRNA signals are shown as a percentage of the mRNA signal for the housekeeping gene *L32*. **B**: Cultures were pre-treated for 1 h with 10 μM SB203580 or vehicle, and then treated with 50 ng/ml IL-6 for 72 h. Culture supernatants were harvested and secreted IL-10 protein was measured by ELISA. The mean and standard error are shown for six replicate samples from each condition. **C**: Cultures were pre-treated for 1 h with vehicle (open bars), 10 μM SB203580 (filled bars), 10 μM SB203580 plus 0.01, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 μM U0126, or 10 μM SB203580 plus 10 μM U0124 (negative control), and then treated with 50 ng/ml IL-6 for 72 h. **Top panel**: Total RNA from each culture was analyzed for IL-10 mRNA expression by RNase protection assay. IL-10 mRNA signals are shown as a percentage of the mRNA signal for *GAPDH*. **Middle panel**: Whole cell lysates from each culture were analyzed for ERK activity, and activity values were standardized by dividing by the value in the vehicle-treated condition. **Bottom panel**: As a control, the whole cell lysates were subjected to anti-ERK2 immunoblot analysis. Molecular weight in kilodaltons is indicated at left.

cultures were treated for 1 h with SB203580 or vehicle-alone, and then stimulated with 1 ng/ml LPS for 3 h. JNK, ERK, and MAPKAPK-2 activities were maximally induced by 60 min of stimulation with 1 ng/ml LPS. Maximal JNK activity was greater in the SB203580-treated cultures compared to vehicle-treated controls, and JNK activity remained potentiated in the SB203580-treated cultures between 60 and 180 min of LPS treatment. Similarly, at 60 min of 1 ng/ml LPS ERK activity was ~3-fold greater in SB203580-treated cultures compared to vehicle-treated controls, and ERK activity remained potentiated in the SB203580-treated cultures between 60 and 180 min of LPS treatment. The maximal level of ERK activity in the SB203580-treated cultures was almost identical to the amount of ERK activity in vehicle-treated control cultures following a 30-min stimulation with 100 ng/ml LPS. Thus, SB203580 pre-treatment caused a maximal induction of ERK activity in response to an

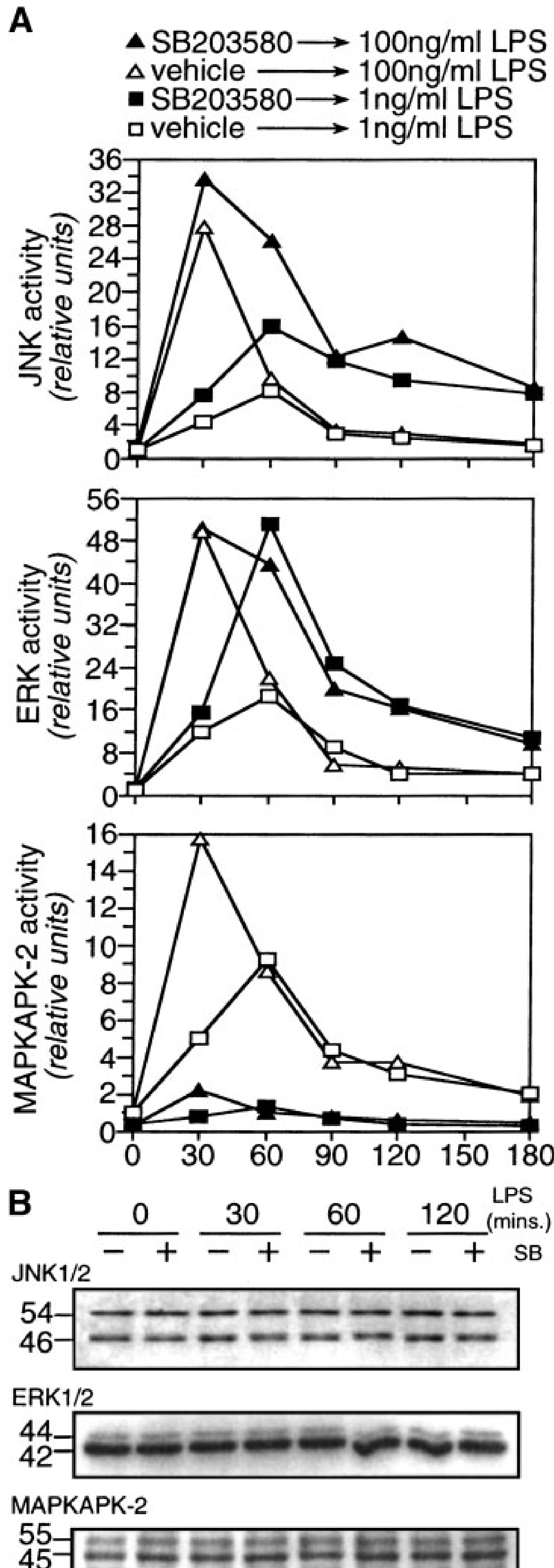


Fig. 5. Inhibition of p38 MAP kinase increases LPS stimulated JNK and ERK activities in primary mouse macrophages. **A**: Peritoneal exudate macrophages were isolated from C57BL/6 females, pre-treated for 1 h with 10 μM SB203580 (filled symbols) or vehicle (open symbols), and then stimulated for 0 through 180 min with 100 ng/ml (triangles) or 1 ng/ml (squares) LPS. Whole cell lysates were prepared at various times and assayed for JNK, ERK, and MAPKAPK-2 activities. The activity profiles in each panel were standardized by dividing the value at each time point by the value in the vehicle-treated condition at 0 h of LPS. Similar results were obtained in two additional experiments. **B**: As a control, the whole cell lysates were subjected to anti-JNK1/2, anti-ERK2, and anti-MAPKAPK-2 immunoblot analyses. Molecular weights in kilodaltons are indicated at left. The upper band in each lane of the anti-ERK2 blot is a band of cross-reactivity with ERK1 migrating at the expected size of 44 kD.

LPS concentration that is at least one order of magnitude lower than the concentration normally required for this response. Taken together, these results demonstrate that in primary mouse macrophages JNK and ERK are potentiated by the inhibition of p38 pathway signaling.

DISCUSSION

In the present work, we demonstrate that JNK, ERK, and p38 MAP kinase activities are reprogrammed during the IL-6-induced, macrophage-like differentiation of the murine myeloid M1 cell line, and that p38 represses JNK and ERK activity in both M1 cells and in LPS stimulated peritoneal exudate macrophages. First, IL-6-induced M1 differentiation increased the rate of LPS stimulated JNK and p38 activation, converted ERK activity from an LPS-nonresponsive to an LPS-responsive state, and increased basal p38 pathway signaling (Fig. 2). We conclude, therefore, that these MAP kinase pathways may be similarly regulated during the inflammation-induced differentiation of myeloid cells in vivo. Second, the p38 inhibitor SB203580 upregulated JNK and ERK activity in both M1 cells (Fig. 3) and LPS stimulated murine thioglycollate-elicited peritoneal exudate macrophages (Fig. 5). JNK activity was also potentiated in LPS stimulated peritoneal exudate macrophages that harbor a homozygous deletion of the p38 activator MKK3 [Lu et al., 1999] compared to $Mkk3^{+/+}$ controls [J.P. Hall, unpublished observations]. These data indicate that p38 represses JNK and ERK activities in both M1 cells and peritoneal exudate macrophages, and we propose, therefore,

that JNK and ERK activities may be regulated by p38 in various macrophage lineages throughout the body.

SB203580 was originally developed from a series of bicyclic imidazoles that potently inhibit cyclooxygenase (COX) and lipoxygenase (LO) activities in cell extracts, isolated inflammatory cells, and animal models of inflammation [Lee et al., 1993]. Therefore, we tested whether the COX and LO inhibitors indomethacin and MK-886 also de-repress JNK and ERK activities in M1 cells and peritoneal exudate macrophages, and whether they can synergize with SB203580 in producing this effect. The ability of SB203580 to upregulate JNK and ERK in these cells was not influenced by these COX and LO inhibitors, and these compounds did not synergize with SB203580 [J.P. Hall, unpublished observations]. We conclude, therefore, that the potentiation of macrophage JNK and ERK activities by SB203580 is caused by an inhibition of p38 activity.

Treatment of M1 cultures with SB203580 before IL-6-induced differentiation caused a potentiation of IL-10 expression upon completion of the 72-h differentiation interval (Fig. 4). This increase required a concomitant increase in ERK activity (Fig. 4C), and COX and/or LO inhibition was not involved in this SB203580-dependent increase in IL-10 expression [J.P. Hall, unpublished observations]. Thus, the inhibition of p38 by SB203580 causes an ERK-dependent increase in IL-10 expression during IL-6-induced M1 differentiation. These data raise the profound possibility that in inflammatory microenvironments in vivo macrophage p38 activity may inhibit expression of the anti-inflammatory cytokine IL-10.

In the budding yeast *S. cerevisiae*, the osmoregulatory pathway MAP kinase Hog1p (the orthologue of mammalian p38 MAP kinase) negatively regulates the mating and pseudohyphal response pathways which are mediated by the MAP kinases Fus3p and Kss1p, respectively [Hall et al., 1996; Madhani et al., 1997; O'Rourke and Herskowitz, 1998]. The MAPKKK Ste11p is shared by all of these pathways. It has been suggested that as part of a feedback control mechanism Hog1p represses the osmoregulatory pathway above the level of this shared upstream component [O'Rourke and Herskowitz, 1998], and that the mating and pseudohyphal pathways are downregulated following the Hog1p-dependent diminution of Ste11p activity. A similar phenomenon may be occuring in the macrophage systems described here. For example, p38 may downregulate its own activators in a negative feedback loop. If the JNK, ERK, and p38 pathways all share a common upstream component (and the fact that LPS activates all three pathways in macrophages may substantiate this possibility), and if this feedback regulation by p38 occurs above this shared component, then this could account for the de-repression of JNK and ERK activity following p38 inhibition. A second, and equally likely explanation, relies on the action of a MAP kinase-specific phosphatase, several of which have been shown to be activated by their MAP kinase targets. It is possible that in macrophages, a p38-regulated phosphatase also inactivates JNK and ERK, and that following the inhibition of p38, the activation of this phosphatase is diminished and JNK and ERK are concomitantly de-repressed. Future studies will need to address these possibilities in detail.

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