Defining the Involvement of p38α MAPK in the Production of Anti- and Proinflammatory Cytokines Using an SB 203580-resistant Form of the Kinase*

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The stress-activated kinases of the p38 mitogen-activated protein kinase (p38 MAPK) family are serine-threonine kinases that are activated by environmental stresses like heat, UV irradiation, or osmotic stress (1, 2). In higher vertebrates, there are four related genes encoding p38α, p38β, p38γ, and p38δ (3–11). The p38 MAPK proteins exhibit a high degree of structural and functional evolutionary conservation (1, 2); the yeast ortholog, Hog1, is also activated in response to osmotic stress, and expression of the human protein in yeast lacking Hog1 restores their ability to grow in high osmolarity media (12).

Activation of p38 MAPK is triggered not only by physical stresses but also by the products of microorganisms such as endotoxin (1, 2) and proinflammatory cytokines such as IL-1 or TNFα (13, 14) indicating that p38 MAPK has multiple roles in innate immune responses. One of the groups that discovered mammalian p38 MAPK identified it through its ability to bind to a family of pyridinyl imidazole inhibitors (exemplified by the compound SB 203580), which were shown to inhibit the release of IL-1 or TNFα from endotoxin-stimulated monocytes (1). Subsequently it has been shown that, of the four isoforms of p38 MAPK, only p38α and p38β are inhibited by SB 203580 (1, 4, 8–10, 15–17). We have shown that p38 MAPK is also involved in physiological responses (18) by demonstrating its identity with a protein we had shown previously to be phosphorylated on tyrosine in response to the hemopoietic growth factors such as IL-3, granulocyte-macrophage colony-stimulating factor-1, colony-stimulating factor-1, and Steel locus factor (19). There is also evidence that p38 MAPK has multiple roles in adaptive immune responses in that it is activated by ligation of CD40 and Fas (20), the antigen receptors of T or B lymphocytes (20–23), and the receptor for the Fc fragment of immunoglobulins (18, 24).

The functional consequences of activation of p38 MAPK are complex and are still being elucidated. Ablation of the p38α gene is lethal due to the defective production of erythropoietin in response to hypoxia with consequent anemia (25). Thus, our knowledge of the function of p38 MAPK is still based mainly on the use of pharmacological inhibitors and the expression of dominant-negative mutants of p38 MAPK or components of its signaling pathway. These studies have suggested roles for p38 MAPKs in the production of inflammatory factors, such as TNFα, IL-1 (1, 26, 27), IL-6 (28), IFN-γ (23, 29, 30), IL-12 (30, 31), and, somewhat paradoxically, the anti-inflammatory factor IL-10 (30, 32, 33), as well as a role in thymic differentiation (34) and negative selection (35).

The interpretation of experiments based on the use of either pharmacological inhibitors or dominant-negative mutants is problematic in that in both cases it is difficult to conclude unequivocally that the observed effects are caused by inhibition of p38 MAPK activity rather than by interference with other processes. The compound SB 203580 has been used in over 300 published studies to investigate the roles of p38 MAPK in a variety of physiological and pathological processes. The specificity of the compound for p38 MAPK has been tested by Cuenda and colleagues (15) who showed that it failed to inhibit 12 other kinases at 100 μM. However, it clearly has an ability to inhibit c-Jun NH₂-terminal kinase, Lck, Raf-1, and the type I transforming growth factor-β receptor (36–38). SB 203580 was reported to inhibit the activation of extracellular signal-regulated kinase in neutrophils (39) and induced a paradoxical response.
increase in activation of Raf-1 in vivo (40). Lali et al. (41) showed that SB 203580 could block protein kinase B phosphorylation at a concentration that was only about 10-fold higher than that required to inhibit p38 MAPK activity. Moreover the possibility that a particular compound might affect the function of other proteins, which might not even be kinases, cannot be formally excluded. For example, Cox1 and Cox2 activities were shown to be inhibited by SB 203580 (42). Clearly systematic testing of inhibitors of p38 MAPK for their effect on all other proteins and their variants is not feasible; not in the least because, even if a comprehensive collection of proteins were available, it would be a long time before we would understand all of their functions and could design appropriate assays.

Here we used a different strategy to identify effects of SB 203580 that were due to inhibition of p38 MAPK. This was based on determining whether a given effect of SB 203580 could be prevented by the expression of an SB 203580-resistant mutant form of p38α (SB-R-p38α) that fails to bind to SB 203580 but appears otherwise normal in its activation and activity (43). SB 203580 binds competitively to the ATP pocket of the kinase (16), and three amino acids (Thr-106, His-107, and Leu-108) within the ATP pocket have been shown to be critical for SB 203580 binding. Substitution of these three amino acids with Met, Pro, and Phe, respectively, is sufficient to abolish the ability of p38α to bind to SB 203580, thus abrogating its sensitivity to inhibition by SB 203580 (43). The demonstration that expression of SBR-p38α abrogates a phenotype observed when parental cells are treated with SB 203580 formally establishes that the phenotype was due to inhibition of p38 MAPK activity. In cell types such as macrophages, which only express p38α and not the other SB 203580-sensitive isoform, p38β (44), it can also be concluded that the critical activity in the phenomenon under study was that of p38α. Expression of an SB 203580-resistant mutant of p38 MAPK was used to demonstrate that the inhibitory effect of SB 203580 on activation of MAPKAP kinase-2 and mitogen- and stress-activated protein kinase-1 involved inhibition of p38 MAPK but not the other SB 203580-sensitive isoform, p38β (44), and three amino acids (Thr-106, His-107, and Leu-108) within the ATP pocket have been shown to be critical for SB 203580 binding. Substitution of these three amino acids with Met, Pro, and Phe, respectively, is sufficient to abolish the ability of p38α to bind to SB 203580, thus abrogating its sensitivity to inhibition by SB 203580 (43). The demonstration that expression of SBR-p38α abrogates a phenotype observed when parental cells are treated with SB 203580 formally establishes that the phenotype was due to inhibition of p38 MAPK activity. In cell types such as macrophages, which only express p38α and not the other SB 203580-sensitive isoform, p38β (44), it can also be concluded that the critical activity in the phenomenon under study was that of p38α. Expression of an SB 203580-resistant mutant of p38 MAPK was used to demonstrate that the inhibitory effect of SB 203580 on activation of MAPKAP kinase-2 and mitogen- and stress-activated protein kinase-1 involved inhibition of p38 MAPK but that the ability of SB 203580 to activate Raf-1 did not (45).

We have expressed SBR-p38α in two cell lines, resembling, respectively, monocyte or mast cells, and in two types of primary cells, macrophages and T lymphocytes. By demonstrating that a monocytic cell line that expresses SBR-p38α was resistant to the inhibitory effect of SB 203580 on the LPS-stimulated production of IL-10, IL-1β, and IL-6, we have conclusively implicated p38α in the production of these cytokines. Likewise the inhibitory effects of SB 203580 on the production of IL-6 and TNFα in mast cell line MC9 stimulated by ligation of the Fc-γ receptor were abrogated by expression of SBR-p38α. Expression of SBR-p38α in primary bone marrow-derived macrophages abrogated the ability of SB 203580 to inhibit the LPS-stimulated production of TNFα; although not the production of IL-10. Expression of SBR-p38α in primary T lymphocytes completely abrogated the ability of SB 203580 to inhibit the production of IFN-γ that was induced by co-ligation of the T cell antigen receptor and CD28 but not that induced by IL-12. These results conclusively demonstrate the importance of p38α in regulating the production of IL-13, IL-6, IL-10, TNFα, and IFN-γ. Moreover the observed differences in the efficacy of expression of SBR-p38α in abrogating the inhibitory effect of SB 203580 on the production of the same cytokine (i.e. IFN-γ) in response to different stimuli indicate that different levels of p38α activity were required for optimal responses to different stimuli. Likewise there were indications that different levels of p38α activity were required for the production of the same cytokine by different cells.

### Experimental Procedures

#### Cell Lines and Cell Cultures—
Cell lines, monocyte WEHI 297.4 and macrophage WEHI 297.5 were derived from BALB/c mice. In RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 50 μg/ml streptomycin (Stem Cell Technologies, Vancouver, Canada), 10% heat-inactivated fetal calf serum, and 2% 10-fold concentrated WEHI-3B-conditioned medium as a source of IL-3. Splenocytes from BALB/c mice were stimulated with 5 μg/ml concanavalin A (Sigma) in RPMI 1640 medium at 5 × 10^6/ml for 24 h, and the resultant T lymphocytic blasts were expanded by culturing in 2% X063ml-2-conditioned medium as a source of IL-2 (46). The femurs and tibias from the same mice were harvested and flushed with 5 ml of phosphate-buffered saline. To obtain primary bone marrow-derived macrophages, hereafter referred to as BMMφ, bone marrow cells were collected in Dulbecco’s modified Eagle’s medium with 20% l-cell-conditioned medium as a source of colony-stimulating factor-1. Bosc23 cells, a human retroviral packaging cell line, were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum.

#### Transfections and Retroviral Infections—
For retroviral infections, cDNAs encoding hemagglutinin-tagged wild type p38α (WT-p38α) or the SBR p38α that were kindly provided by GlaxoSmithKline were cloned into the retroviral expression vector pMXsio, which contains an IRES (internal ribosomal entry site)-GFP expression cassette and a puromycin resistance gene. Bosc23 cells were transfected using LipofectAMINE (Invitrogen) as described by the manufacturer. 24 h after transfection, supernatants containing retrovirus were harvested and filtered (0.45 μm). For retroviral infection of cell lines, 2 × 10^5 cells were incubated with virus-containing supernatants supplemented with 10 μg/ml Polybrene (Sigma). After 16 h, puromycin at 2 μg/ml Sigma was added to select for transduced cells. For infection of primary macrophage progenitors, bone marrow cells were flushed from femurs and tibias and cultured for 24 h in a mixture of 2% 10-fold concentrated WEHI-3B-conditioned medium and 2% 10-fold concentrated WEHI-3B-conditioned medium as a source of IL-3, 15% stem cell factor-conditioned medium, and 10 ng/ml recombinant mIL-6 (Intergene, Purchase, NY). They were then co-cultured with transfected Bosc23 cells with 6 μg/ml Polybrene for 48 h. The medium was then changed with fresh medium supplemented with the same cytokines. After 48 h, bone marrow cells expressing high levels of GFP were enriched by fluorescence-activated cell sorting using a FACSVantage S.E. (BD Biosciences) and cultured in L-cell-conditioned medium to obtain BMMΦ. For infection of primary T cells, BALB/c splenocytes that had been stimulated with concanavalin A for 24 h were co-cultured with transfected Bosc23 cells for 48 h in RPMI 1640 medium supplemented with 2% X063ml-2-conditioned medium and 5 μg/ml Polybrene. After another 48 h of culture, T cells expressing high levels of GFP were collected by fluorescence-activated cell sorting.

Cell Stimulations and Capture ELISA—LPS (Escherichia coli strain 0111:B4, Difco) was used at 15 μg/ml; anti-Fc-γ antibody 2.4G2 (Pharmingen) was used at 5 μg/ml; IFN-γ (R&D Systems, Minneapolis, MN) was used at the concentrations indicated, and IL-12 (R&D Systems) was used at 100 ng/ml. Anti-CD8 and anti-CD4 (BD Biosciences) monoclonal antibodies (Pharmingen) were used at 2 μg/ml. Cells were incubated with MeSO solvent control or SB 203580 (Calbiochem) dissolved in MeSO for 30 min before stimulation. The supernatants were harvested 24 h later, and a capture ELISA was performed as described previously (30). Recombinant murine IL-1α, IL-10, IL-12, and TNFα from R&D Systems were used as standards.

Immunoprecipitation and Kinase Assay—Cells were lysed in lysis buffer as described above, and the concentration of proteins in the lysate was determined by BCA protein assay (Pierce). Lysates containing equivalent amounts of protein were subjected to immunoprecipitation, and MAPKAP kinase 2 (MK2) kinase activity was determined using an in vitro kinase assay with HSP27 as a substrate (18).

### Results

#### WEHI 297.4 Cells Expressing SBR-p38α Respond to Stimuli with Increases in SB 203580-resistant p38 MAPK Activity—
We first confirmed that, when expressed in cells, the mutant SBR-p38α, in which amino acid residues Thr-106, His-107, and Leu-108 had been changed, responded normally to activating stimuli and that the resultant increases in p38 MAPK activity were insensitive to inhibition by SB 203580. SBR-p38α or WT-p38α were expressed in a monocytic cell line, WEHI 297.4, using retroviral infection. Clones expressing high levels of wild type or mutated exogenous p38α were identified by immuno-blotting with anti-p38α MAPK antibody. As shown in Fig. 1,
the levels of exogenous SBR-p38α were high, being more than 3-fold greater than the levels of endogenous p38 MAPK. To examine the activation of SBR-p38α and its sensitivity to SB 203580, WEHI 274.3 cells expressing either WT-p38α or SBR-p38α were stimulated with LPS or hyperosmolar sodium chloride to induce activation of p38 MAPK and its in vivo substrate MK2. To assess the levels of activation of p38 MAPK present in vivo, MK2 was immunoprecipitated, and its activity was assayed in an in vitro kinase assay using HSP27 as the substrate. The degree of phosphorylation of HSP27 reflects the level of activation of MK2 and, thus indirectly, the in vivo activity of p38α MAPK. As shown in Fig. 1, the increases in in vivo activity of p38 MAPK in cells that were expressing WT-p38α and were stimulated with LPS or sodium chloride were significantly inhibited by SB 203580. In contrast, in cells expressing SBR-p38α similar increases in levels of p38 MAPK activity were observed, and these were not sensitive to inhibition by SB 203580.

Expression of SBR-p38α MAPK in a Monocytic Cell Line Abrogates the Ability of SB 203580 to Inhibit the Production of IL-10, IL-1β, and IL-6—To determine the involvement of p38α MAPK in the production of cytokines, WEHI 274.3 cells expressing exogenous WT-p38α or SBR-p38α MAPK were stimulated with LPS. Supernatants were collected after 24 h, and the levels of cytokines present were determined by ELISA. As expected, SB 203580 inhibited IL-10 production by WEHI 274.3 cells expressing WT-p38α MAPK in a dose-dependent manner with greater than 95% inhibition at 5 μM and with an IC50 of 0.1 μM. In contrast, in cells expressing SBR-p38α the production of IL-10 was no longer inhibited by SB 203580 (Fig. 2a). Likewise the production of IL-1β by cells expressing WT-p38α was very effectively inhibited by SB 203580 (Fig. 2b) with 95% inhibition at 5 μM SB 203580. Once again in cells expressing SBR-p38α, the ability of SB 203580 to inhibit the production of IL-1β was totally abrogated. The production of IL-6 was partially inhibited by SB 203580; thus at 5 μM, SB 203580 inhibited the production of IL-6 by 60%. In cells expressing SBR-p38α, SB 203580 no longer inhibited IL-6 production (Fig. 2c). These results clearly demonstrate that p38α MAPK plays an essential role in the production of IL-10 and IL-1β. The observation that the production of IL-6 from LPS-stimulated WEHI 274.3 cells was less effectively inhibited by SB 203580 than the production of IL-10 or IL-1β could mean either that p38 MAPK activity was essential for only a fraction of IL-6 production or that the amount of activity needed was smaller than that required for IL-10 or IL-1β production and was equal to or less than the activity remaining in the presence of SB 203580 (5 μM). In either case, the observation that expression of SBR-p38α abrogated the partial inhibition of IL-6 production by SB 203580 demonstrates that the effect of SB 203580, although partial and manifest at higher concentrations, was due to inhibition of p38 MAPK activity.

Expression of SBR-p38α in the Mast Cell Line MC/9 Abrogates SB 203580-mediated Inhibition of the Production of IL-6 and TNFα Stimulated by Ligation of FcyR II/III—To examine the importance of p38 MAPK in the production of cytokines in another cell type in response to a different stimulus, we expressed WT-p38α or SBR-p38α in the mast cell line MC/9. Mast cells produce multiple cytokines in response to cross-linking of
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Fig. 3. Expression of SBR-p38α MAPK abrogates the inhibitory effect of SB 203580 on the production of IL-6 and TNFα by the mast cell line MC/9 stimulated by anti-Fc-ε R II/III antibody 2.4G2. MC/9 cells expressing WT-p38α (WT) or SBR-p38α (SBR) were stimulated with the anti-Fc-ε R II/III antibody 2.4G2 at 5 μg/ml. 6 h after stimulation (a) IL-6 and TNFα (b) were assayed as described under “Experimental Procedures.” Data of one experiment are presented as mean ± S.D. and are representative of three independent experiments.

Fig. 4. Effects of expression of SBR-p38α MAPK in primary bone marrow-derived macrophages on the LPS-stimulated production of TNFα, IL-10, and IL-12/IL-23p40. With the use of an IRES-GFP vector, bone marrow cells expressing high levels of GFP, and thus exogenous WT-p38α (WT) or SBR-p38α (SBR), were purified by fluorescence-activated cell sorting, and equivalent numbers were seeded into a 96-well plate with a source of colony-stimulating factor-1 to induce macrophage differentiation as described under “Experimental Procedures.” After 5 days of culture in the presence of colony-stimulating factor-1, non-adherent cells were washed off, and the remaining adherent macrophages were stimulated with LPS at 15 μg/ml in the presence of the indicated concentrations of SB 203580 (3 or 5 μM) or of the solvent Me2SO alone (0 μM). 24 h after stimulation, supernatants were harvested and assayed for levels of TNFα (a), IL-10 (b), and p40 (c) by ELISA as described under “Experimental Procedures.” The results of one experiment that are representative of three independent experiments are presented as mean ± S.D.

Thus the net enhancement of TNFα production by SB 203580 in cells expressing SBR-p38α appeared to result from abrogation of the direct inhibitory effect of SB 203580 on TNFα production, but not of its indirect enhancing effect on TNFα production, mediated by suppression of IL-10 production. This is consistent with our observation that IL-10 production was more sensitive to inhibition by SB 2030580 than was TNFα production.

Expression of SBR-p38α Has No Effect on the Net Enhancement of IL-12/IL-23p40 by SB 203580 from BMMφ Stimulated with LPS—Macrophages are a major source of IL-12, an important immunomodulatory molecule comprised of two subunits, the inducible p40 and the constitutively expressed p35. IL-12p40 is also the subunit of a newly described cytokine, IL-23, which shares some activities with IL-12 but has distinct activities as well (47). We showed previously that SB 203580 enhanced the net production of IL-12 from LPS-stimulated peritoneal macrophages through a mechanism that, in part, involved inhibition of the production of IL-10 and suppression of its inhibitory effect on IL-12 production (30). To further investigate the role of p38α MAPK in p40 production, BMMφ expressing WT- or SBR-p38α MAPK were stimulated with LPS, and IL-12/IL-23p40 was assayed with ELISA. As shown in Fig. 4c, SB 203580 enhanced p40 production from BMMφ expressing either WT-p38α or SBR-p38α. Thus, the expression of SBR-p38α failed to abrogate the ability of SB 203580 to enhance the LPS-stimulated production of p40, although as shown in Fig. 4a, it abolished the ability of SB 203580 to inhibit TNFα production by the same cells. However, in that the expression of SBR-p38α failed to abrogate the ability of SB

Both Fc-ε R I and Fc-γ R. MC/9 cells expressing either WT- or SBR-p38α were stimulated with the rat anti-Fc-γ R II/III antibody 2.4G2. We observed that binding of 2.4G2 antibody alone, without cross-linking by a secondary anti-rat antibody, triggered the release of IL-6 and TNFα from MC/9 (Fig. 3). SB 203580 at 5 μM inhibited the production of IL-6 by parental MC/9 cells (data not shown) and by cells expressing WT-p38α by about 50% (Fig. 3a). The production of TNFα was also inhibited but to a lesser extent (Fig. 3b). As was the case with the monocytic cell line WEHI 274.3, expression of SBR-p38α in MC/9 cells resulted in the complete abrogation of the inhibitory effect of SB 203580 on the production of IL-6 and TNFα (Fig. 3, a and b). These data demonstrate that the production of IL-6 and TNFα by mast cells stimulated by ligation of the Fc-γ R was at least in part dependent on the activity of p38α MAPK.

Expression of SBR-p38α in Primary Macrophages Abrogates the Ability of SB 203580 to Inhibit the LPS-stimulated Production of TNFα—Next we examined the effects of SB 203580 on the production of cytokines from primary macrophages. We used retroviral vectors to express WT-p38α or SBR-p38α in bone marrow progenitor cells, which we induced to differentiate into BMMφ using colony-stimulating factor-1. Immunoblotting showed that the average levels of WT-p38α or SBR-p38α achieved in these polyclonal populations were some 10-fold lower than those achieved in the clones of cell lines and ranged from 30 to 50% of the levels of endogenous p38 MAPK (data not shown). We observed that SB 203580 inhibited the LPS-stimulated production of TNFα by polyclonal populations of BMMφ expressing WT-p38α MAPK with an IC50 of 1–3 μM, with 5 μM SB 203580 inhibiting levels of TNFα by 80%. IL-10 production was markedly more sensitive to inhibition by SB 203580 with an IC50 of 0.1 μM (data not shown). Expression of SBR-p38α in polyclonal populations of BMMφ completely abrogated the inhibitory effect of SB 203580 on the LPS-stimulated production of TNFα. Indeed, in BMMφ expressing SBR-p38α, SB 203580 reproducibly enhanced the level of LPS-stimulated TNFα production over that seen in control populations that were treated with the solvent control (Fig. 4a). A probable explanation for this enhancing effect of SB 203580 was suggested by the observation that the production of IL-10 by macrophages expressing SBR-p38α continued to be inhibited by SB 203580 (Fig. 4b).
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203580 to inhibit the production of IL-10 in these same cultures (Fig. 4b), these results are consistent with our previous observation that the ability of SB 203580 to enhance IL-12 production was due to its ability to inhibit IL-10 production (30).

Expression of SBR-p38α in Activated Primary T Cells Has Different Effects on SB 203580-mediated Inhibition of IFN-γ and IL-10 Production Depending on the Stimulus—We next investigated the effects of expression of SBR-p38α on the action of SB 203580 on cytokine production in primary T cells. T lymphocytes were activated with concanavalin A and were infected with retroviral vectors encoding WT-p38α or SBR-p38α and GFP under the control of an IRES. Cells expressing high levels of GFP, and thus high levels of either WT-p38α or SBR-p38α, were purified using fluorescence-activated cell sorting. The T lymphocytes were reactivated by stimulation with anti-CD3 and CD28. Thus the inhibitory effect of SB 203580 on the production of IL-10 by T cells expressing WT-p38α failed to abrogate the inhibitory effect of SB 203580 on the production of IL-10 by T cells expressing SBR-p38α, as shown in Fig. 5b. In contrast, the expression of SBR-p38α relieved only a fraction of the SB 203580-mediated inhibition of IL-10 production by the same cells (Fig. 5b).

We also studied the response of the same populations of cells to a different stimulus, IL-12, which in the presence of IL-2 induced previously activated T lymphocytes to produce IFN-γ and IL-10. We observed that SB 203580 (5 μM) inhibited the IL-12-induced production of both IFN-γ and IL-10. However, in contrast with the result when IFN-γ production was stimulated by co-ligation of CD3 and CD28, the expression of SBR-p38α failed to abrogate the SB 203580-mediated inhibition of the production of IFN-γ induced by IL-12 and IL-2 (Fig. 5c). The expression of SBR-p38α only partially reduced the inhibitory effect of SB 203580 on the IL-12-induced IL-10 production (Fig. 5d), resembling the results seen when these cell populations were stimulated by co-ligation of CD3 and CD28. Thus, the same population of T lymphocytes expressing SBR-p38α were resistant to the SB 203580-mediated inhibition of the production of IFN-γ in response to ligation of CD3 and CD28 but were only partially resistant to the SB 203580-mediated inhibition of the production of IFN-γ in response to IL-12 and IL-2. We interpret this difference as reflecting a requirement for higher levels of p38α activity for optimal production of IFN-γ in response to IL-12 and IL-2.

DISCUSSION

Not surprisingly, the widely used p38 MAPK inhibitor SB 203580 also inhibits other enzymes. The limitations of the specificity of pharmacological inhibitors and the lethality of p38α gene knockout make it difficult to unequivocally assign functions to p38α. Here we have exploited the ability of expression of an SB 203580-resistant form of p38α to abrogate those effects of SB 203580 that are due to inhibition of p38α to define its role in the production of both pro- and anti-inflammatory cytokines. These studies establish roles for p38α in the production of IFN-γ, TNFα, and IL-10 in both cell lines and two types of primary cells. There was an inverse correlation between the sensitivity of a response to inhibition by SB 203580 and the level of expression of SBR-p38α required to abrogate inhibition of that response by SB 203580. This suggests that there are differences in the level of p38α activity required for the production of different cytokines as well as for the same cytokine produced in response to different stimuli.

In the monocytic cell line WEHI 274.3, the production of both IL-10 and IL-1β was very sensitive to inhibition by SB 203580 (IC50, 0.1 μM), and the inhibitory effect of SB 203580 was completely abrogated by expression of SBR-p38α. The production of IL-6 from WEHI 274.3 was only partially inhibited by SB 203580, but this inhibition was completely abrogated by expression of SBR-p38α. Similar results were obtained in the mast cell line MC/9 where the production of IL-6 and TNFα in response to ligation of the Fc-γ receptor was inhibited, and the inhibitory effect of SB 203580 was completely abrogated by expression of SBR-p38α. These experiments demonstrate that the inhibitory effect of SB 203580 on the production of IL-1β, IL-6, IL-10, and TNFα was due to inhibition of p38α.

When primary bone marrow-derived macrophages were used, the results were somewhat different, but upon closer examination they yield similar conclusions. Thus we observed that, in contrast to the results obtained with WEHI 274.3 cells expressing high levels of SBR-p38α, expression of SBR-p38α in polyclonal populations of BMMφ failed to abrogate the inhibitory effect of SB 203580 on LPS-stimulated IL-10 production. We believe this reflects the fact that the production of IL-10 by LPS-stimulated BMMφ was very sensitive to inhibition by SB 203580 (IC50, 0.1 μM), which is consistent with similar results in human monocytes or murine peritoneal macrophages (30, 32). This very low IC50 compared with that for inhibition of the production of other cytokines presumably reflects the fact that the optimal production of IL-10 requires higher levels of p38α activity. The fact that expression of SBR-p38α in the monocytic cell line WEHI 274.3 at 10-fold higher levels than those achieved in the primary cells did abrogate the inhibitory effect of SB 203580 on LPS-induced IL-10 production (Fig. 2) is consistent with this notion. An alternative possibility is that p38 MAPK may not be critical for IL-10 synthesis in BMMφ and that SB 203580 inhibits IL-10 production through inhibition of another enzyme. However, our data would indicate that the IC50 of SB 203580 for inhibition of this enzyme would have to be at least as low (0.1 μM) as that for inhibition of LPS-stimulated IL-10 production in WEHI 274 monocytes where p38α was shown to be the target (Fig. 2). We have reported previously that SB 203580 enhanced the
production of IL-12/IL-23p40 by LPS-stimulated peritoneal macrophages through a mechanism that involved inhibition of IL-10 production (30) and possibly of another p38 MAPK-dependent pathway, such as prostaglandin E2 synthesis (48). Thus, given that overexpression of SBR-p38α was not sufficient to render BMM6 resistant to the inhibitory effect of SB 203580 on IL-10 production, it is not unexpected that overexpression of SBR-p38α did not reverse the enhancing effect of SB 203580 on p40 production (Fig. 4c).

Our observation that overexpression of SBR-p38α abrogated the inhibitory effect of SB 203580 on TNFα production (Fig. 4a) establishes a role for p38α in TNFα production. It is consistent with the report of Kotlyarov et al. (49) that the depletion of MK2, which is directly phosphorylated and activated by p38 MAPK, results in about a 90% reduction in TNFα production.

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