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Interferon-γ and Transforming Growth Factor-β Modulate the Activation of Mitogen-Activated Protein Kinases and Tumor Necrosis Factor-α Production Induced by Fcγ-Receptor Stimulation in Murine Macrophages

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Engagement of receptors for the Fc region of IgG (FcγR) can activate a variety of biological responses in macrophages, and these responses can be modulated either positively or negatively by co-stimulation with a variety of agents including cytokines such as interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β). We have previously demonstrated that FcγR crosslinking activates the mitogen-activated protein kinase (MAPK) family members p42^{MAPK}, p38, and JNK. Herein, we examined the modulatory effect of IFN-γ, TGF-β, and platelet-activating factor (PAF) on FcγR-induced MAPK activation in murine macrophages. FcγR-induced activation of p42^{MAPK} and JNK was augmented nearly two-fold by pretreatment with IFN-γ. Conversely, TGF-β pretreatment suppressed FcγR-induced activation of p42^{MAPK}, JNK, and p38. These modulatory effects of IFN-γ and TGF-β on MAPK activation correlated with changes in FcγR-stimulated TNF-α production by these two cytokines.

Receptors for the Fc region of IgG (FcγR) are found on numerous cells types of immunologic importance, and crosslinking these receptors can induce such responses as phagocytosis and the generation of mediators including the pleiotropic cytokine tumor necrosis factor-α (TNF-α) (1). In addition, a variety of other factors can modulate FcγR-dependent responses. In macrophages, interferon-γ (IFN-γ) augments FcγR-stimulated TNF-α synthesis (2,3), while TGF-β suppresses such expression (3). The mechanisms by which these factors modulate this response are unclear. One possiblity is that these modulating agents may qualitatively or quantitatively alter the signaling events stimulated by FcγR-engagement.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases which are activated after the engagement of a variety of cell surface receptors (4,5). The three most well characterized members of this family in mammalian cells are extracellular signal-regulated kinases (ERKs, with 2 isoforms p42^{MAPK} and p44^{MAPK}), c-Jun NH_2-terminal kinases (JNKs), and p38 (mammalian homolog of yeast HOG-1). The activation of these kinases are thought to play an important role in linking extracellular stimuli to cellular responses such as cell growth, death, and differentiation (4,5). In macrophages, FcγR-engagement has been found to activate p42^{MAPK} (6,7), JNK (7), and p38 (7). Moreover, we (7) and others (8) have implicated the activation of p42^{MAPK} in FcγR-stimulated TNF-α synthesis. Herein, we questioned the modulating effect of IFN-γ, TGF-β, and platelet-activating factor (PAF) on FcγR-stimulated activation of p42^{MAPK}, JNK and p38 as well as TNF-α synthesis in murine macrophages. We found that pretreatment of macrophages with IFN-γ augmented FcγR-stimulated p42^{MAPK} and JNK activation, while TGF-β suppressed FcγR-stimulated activation of p42^{MAPK}, JNK, and p38. The modulation in MAPK activation caused by IFN-γ and TGF-β correlated with changes in FcγR-stimulated TNF-α production induced by these cytokines, suggesting that modulation in these MAPK signaling pathways may mediate the modulation in TNF-α expression by FcγR-crosslinking.

MATERIALS AND METHODS

Macrophages. Murine bone marrow-derived macrophages were obtained as previously described (9). Briefly, bone marrow cells were flushed from tibias, femurs, and pelvises of C3H/HeJ mice and grown in DMEM containing 2mM glutamine, 100U/ml penicillin, 100μg/ml

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streptomycin, 10% fetal bovine serum, and 10% L929 cell-conditioned medium as a source of M-CSF. Cells were cultured at 37 °C with 10% CO_2 for 5 to 7 days before use.

Reagents. Monoclonal antibody 2.4G2 against murine FcγRII/III was purchased from Pharmingen (San Diego, CA). Affinity-purified $F(ab')_2$ fragment goat anti-rat IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Polyclonal anti-rat MAP (anti-p42/p44) was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). Polyclonal anti-ERK2/p42, anti-p38, and anti-p46 JNK1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase linked $F(ab')_2$ sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from Amersham Life Sciences (Arlington Heights, IL). For immunoprecipitation, polyclonal anti-p38 antiserum (a generous gift from Dr. G.L. Johnson) was generated in rabbits using the COOH-terminus of p38 (CFVPPPLDQEEMES) conjugated to KLH as antigen. Recombinant murine IFN-γ and purified human TGF-β1 were purchased from Genzyme Corp (Cambridge MA). PAF was obtained from Avanti Polar Lipids (Birmingham, AL). The MEK-1 inhibitor PD 098059 was purchased from New England Biolabs (Beverly, MA).

Immunoprecipitation and kinase activity assay for $p42^{MAPK}$ and p38. After stimulation, macrophage monolayers were washed with ice-cold HEPES buffer (pH 7.4) and immediately lysed with 500μl of ice-cold RIPA lysis buffer (50mM Tris pH 7.2, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton-X-100, 10mM sodium pyrophosphate and 2.1μg/ml aprotinin). The cell lysate was then precleared with 15μl of protein A-Sepharose beads. Immunoprecipitations were carried out in the cold for 2 hours using 0.9μg of polyclonal anti-$p42^{MAPK}$ antibody or 2μl of anti-p38 anti-serum and 15μl of protein A-Sepharose beads. The beads were then washed twice with RIPA buffer and twice with PAN buffer (10mM Pipes pH 7.0, 100mM NaCl) with 21μg/ml aprotinin. *In vitro* kinase assays were conducted in a buffer containing 20mM HEPES pH 7.6, 200mM $MgCl_2$, 20μM ATP, 2 nM DTT, 100μM sodium orthovanadate, and 25 μM β-glycerophosphate with 20μCi [γ-^{32}P]ATP using a recombinant fragment of ATF-2 (amino acids 1-110) as substrate. The reaction was terminated by adding an equal volume of 2× Laemmli sample buffer and boiling for 5 minutes. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. Phosphorylated proteins were visualized by autoradiography and quantified by gamma counting in a Beckman LS 500TD counter.

JNK/SAPK activity assay. JNK/SAPK activity was measured using a solid phase system with recombinant c-Jun (1-79) GST fusion protein bound to glutathione-Sepharose beads as both ligand and substrate. Macrophages were lysed with a buffer containing 50mM Tris pH8.0, 137mM NaCl, 10% glycerol, 1% NP-40, 1mM NaF, 1mM PMSF, 2mM sodium orthovanadate, 10μg/ml leupeptin, and 10μg/ml aprotinin. Fifteen microliters of c-Jun-Sepharose beads were added to postnuclear cell lysate and incubated for 2 hours at 4 °C. Beads were washed twice with lysis buffer and twice with a c-Jun kinase buffer (20mM HEPES pH 7.6, 30μM β-glycerophosphate, 10mM p-nitrophenylphosphate, 10mM $MgCl_2$, 0.5mM sodium orthovanadate, and 0.5mM DTT). After washing, beads were resuspended in kinase buffer containing 90μCi/ml of [γ^{32}P]ATP and incubated at 30 °C for 30 minutes. Reactions were terminated by adding an equal volume of 2× Laemmli sample buffer and boiling for 5 minutes. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. Phosphorylated c-Jun was visualized by autoradiography and quantified by gamma counting.

Immunoblotting. Nitrocellulose membranes were blocked with 5% BSA in TTBS (137mM NaCl, 25mM Tris pH7.5, 0.1% Tween 20). Membranes were incubated with either anti-$p42/p44^{MAPK}$, anti-p38, or anti-p46/JNK1 antibodies for 2 hours at room temperature and then with HRP-conjugated donkey anti-rabbit IgG. Immunocomplexes were detected using the ECL system (Amersham Corp).

Quantification of TNF-α. Macrophage culture supernatants were collected at various times after FcγR cross-linking and stored at −20 °C until assayed. TNF-α protein was quantified using a commercially available ELISA kit (Genzyme Corp, Cambridge, MA) according to manufacturer's instructions. Values are reported as picograms of TNF-α per 10^6 cells. Cell counts were determined by lysing adherent cells with Zapoglobin and quantifying nuclei with a ZM Coulter counter (Coulter Instruments, Hialeah, FL).

RESULTS AND DISCUSSION

We have previously demonstrated that crosslinking FcγR with the monoclonal antibody 2.4G2 results in the activation of $p42^{MAPK}$, JNK, and p38 in murine bone marrow-derived macrophages as measured by *in vitro* kinase assays (7). As compared to control cells, FcγR crosslinking resulted in a 10-fold increase in the phosphorylation of ATF-2, as exogenous substrate, in assays performed with immunoprecipitated $p42/p44^{MAPK}$ (Figure 1). Peak activity occurred 5 minutes after receptor crosslinking (7). While the anti-ERK antibody immunoprecipitated significant amounts of both $p42^{MAPK}$ and $p44^{MAPK}$ (Figure 1), we have previously shown that only the $p42^{MAPK}$ isoform is tyrosine phosphorylated upon FcγR engagement suggesting that $p42^{MAPK}$ is selectively activated by FcγR crosslinking (7). In a solid phase *in vitro* kinase assay for JNK activity, FcγR crosslinking induced a 4-5 fold increase in c-Jun phosphorylation (Figure 2), which peaked approximately 20 minutes after receptor stimulation (7). There was a three-fold increase in p38 MAPK activity following FcγR crosslinking (Figure 3), which peaked approximately 12 minutes after stimulation (7). To investigate the modulatory effects of IFN-γ, TGF-β, and PAF on FcγR-induced MAPK activation, macrophages were treated for 18 hours with these agents prior to FcγR crosslinking and kinase assays. None of these agents altered the low basal activation state of $p42^{MAPK}$ or JNK (Figures 1and 2), but all three agents suppressed the more marked basal level of p38 activity (Figure 3). Pretreatment of macrophages with IFN-γ followed by FcγR-crosslinking resulted in a near two-fold increase in $p42^{MAPK}$ and JNK activation as compared to FcγR-crosslinking alone. In contrast, IFN-γ pretreatment modestly suppressed FcγR-induced p38 activation as compared with non-pretreated cells (Figure 3). On the other hand, pretreatment of macrophages with TGF-β resulted in a near two-fold suppression in FcγR-induced JNK (Figure 2) and p38 (Figure 3) activation and to a lesser degree suppression of $p42^{MAPK}$ activation (Figure 1). Pretreatment of macrophages with PAF had a much less marked effect on FcγR-induced MAPK activation. PAF slightly augmented $p42^{MAPK}$ activation while JNK and p38 activities were modestly suppressed.

How IFN-γ and TGF-β may be modulating FcγR-induced activation of MAPKs is not entirely clear. As shown in figures 1 and 3, neither agent altered the amount of $p42^{MAPK}$, $p44^{MAPK}$, or p38 immunoprecipitated from cell lysates as measured by Western blot-

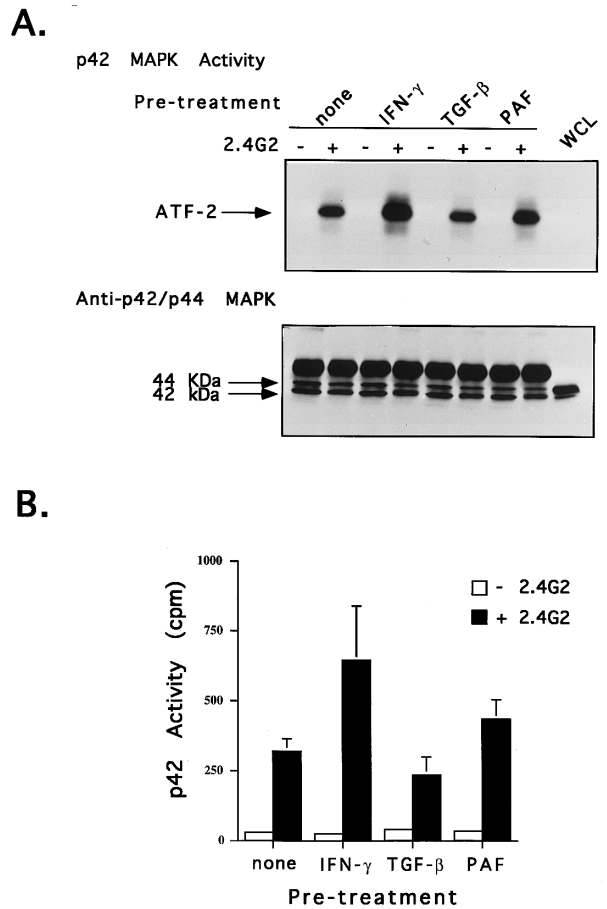

FIG. 1. Modulation of FcγR stimulated p42^{MAPK} activation in murine macrophages. Murine bone marrow-derived macrophages were treated with either IFN-γ (10U/ml), TGF-β (10 ng/ml), or PAF (1μM) for 18 hours prior to FcγR crosslinking with mAb 2.4G2 (10μg/ml) against FcγRII/III and F(ab′)2 goat anti-rat IgG (5μg/ml) at 37 °C for 5 minutes. The p42^{MAPK} was immunoprecipitated from cell lysates, and assayed for activity using a fragment of ATF-2 as substrate by *in vitro* kinase assay. Reaction mixtures were separated by SDS-PAGE and electroblotted onto nitrocellulose. Panel A (top) is an autoradiograph from an *in vitro* kinase assay showing phosphorylation of ATF-2 by p42^{MAPK} activated by FcγR crosslinking. Bottom of panel A is a p42/p44^{MAPK} immunoblot showing equivalent immunoprecipitation for each condition. Staining of whole cell lysate (WCL) is shown as reference. The results shown are representative of three separate experiments. Panel B shows the quantification of ATF-2 phosphorylation from p42^{MAPK} *in vitro* kinase assays. Regions on nitrocellulose membranes corresponding to the location of ATF-2 fragment were cut out, and [^{32}P]-incorporation quantified by gamma counting. Results are the means±SEM of three experiments.

ting. This would suggest that changes in substrate phosphorylation were not due to changes in overall kinase levels. Another potential mechanism through which IFN-γ and TGF-β may modulate FcγR-induced MAPK activation includes changes in the number or type of FcγRs expressed on the macrophage cell surface, but differential effects on different MAPKs makes this less likely. Alternatively, the level or activation of signaling components in the activation/deactivation cascades leading to activation/deactivation of the MAPKs could be modulated by IFN-γ and TGF-β. Recently, p42^{MAPK} has been found to associate with IFN-α/β receptors, and IFN-β stimulation results in the activation p42^{MAPK}, providing evidence for "cross talk" between the interferon-stimulated JAK-STAT signaling cascade and the MAPK pathway (10).

In this study, we also examined the modulatory effects of IFN-γ, TGF-β, and PAF on FcγR-induced macrophage TNF-α synthesis. TNF-α protein released into

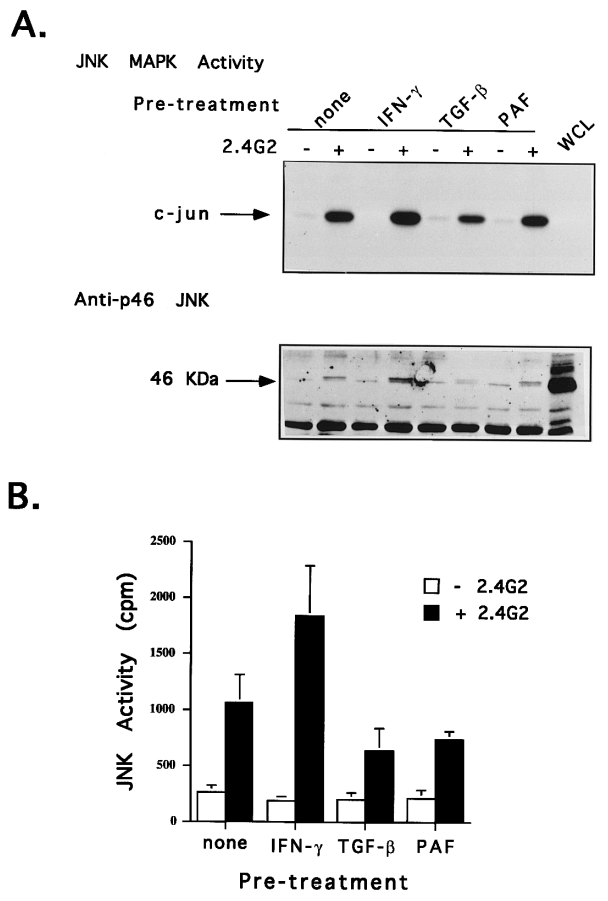

FIG. 2. Modulation of FcγR stimulated JNK activation in murine macrophages. Murine bone marrow-derived macrophages were treated with either IFN-γ (10 U/ml), TGF-β (10ng/ml), or PAF (1μM) for 18 hours prior to FcγR crosslinking with mAb 2.4G2 (10μg/ml) against FcγRII/III and F(ab′)2 goat anti-rat IgG (5μg/ml) at 37 °C for 20 minutes. JNK activity was measured in a solid-phase *in vitro* kinase assay with c-Jun-GST as substrate. Reaction mixtures were separated by SDS-PAGE and electroblotted onto nitrocellulose. Panel A (top) is an autoradiograph from an *in vitro* kinase assay showing phosphorylation of c-Jun-GST by JNK activated by FcγR crosslinking. Bottom of panel A is a JNK1 (p46) immunoblot showing gel shift in p46 after FcγR crosslinking. Staining of whole cell lysate (WCL) is shown as reference. The results shown are representative of three separate experiments. Panel B shows the quantification of c-Jun-GST phosphorylation from JNK *in vitro* kinase assays. Regions on nitrocellulose membranes corresponding to the location of c-Jun-GST fragment were cut out, and [^{32}P]-incorporation quantified by gamma counting. Results are the means±SEM of three experiments.

the cell culture supernatant was measured by ELISA. Over a twenty-four hour period, FcγR cross linking alone with the monoclonal antibody 2.4G2 induced a marked increase in macrophage TNF-α production (Figure 4). Pretreatment of macrophages with IFN-γ for 18 hours augmented FcγR-induced TNF-α synthesis by 2-fold. In addition, the rate of TNF-α synthesis induced by FcγR crosslinking was also augmented by IFN-γ. In contrast, TGF-β largely suppressed FcγR-induced TNF-α synthesis. The opposing modulatory effects of IFN-γ and TGF-β on macrophage TNF-α production have also been reported with LPS as the triggering stimulus (11). Pretreatment with PAF did not significantly alter the level or time course of FcγR induced TNF-α production. PAF is, however, an effective stimulus for these macrophages. PAF stimulated a transient increase in intracellular Ca^{++} (data not shown), and primed for expression of phosphatidylserine receptors (receptors mediating the uptake of apoptotic cells) in response to co-stimulation by FcγR-crosslinking (data not shown) or phagocytic particles (12). Collectively, these results suggest that modulating agents such as IFN-γ, TGF-β, or PAF can very specifically alter macrophage responses to a common triggering stimulus such as FcγR-crosslinking.

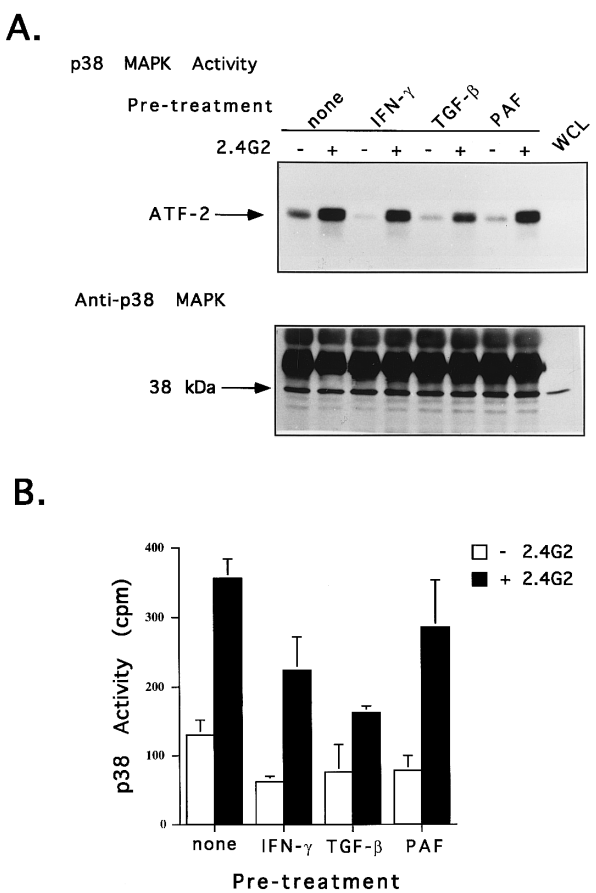

FIG. 3. Modulation of FcγR stimulated p38 activation in murine macrophages. Murine bone marrow-derived macrophages were treated with either IFN-γ (10U/ml), TGF-β (10ng/ml), or PAF (1μM) for 18 hours prior to FcγR crosslinking with mAb 2.4G2 (10μg/ml) against FcγRII/III and F(ab′)2 goat anti-rat IgG (5μg/ml) at 37 °C for 12 minutes. The p38 was immunoprecipitated from cell lysates, and assayed for activity using a fragment of ATF-2 as substrate by *in vitro* kinase assay. Reaction mixtures were separated by SDS-PAGE and electroblotted onto nitrocellulose. Panel A (top) is an autoradiograph from an *in vitro* kinase assay showing phosphorylation of ATF-2 by p38 activated by FcγR crosslinking. Bottom of panel A is a p38 immunoblot showing equivalent immunoprecipitation for each condition. Staining of whole cell lysate (WCL) is shown as reference. The results shown are representative of three separate experiments. Panel B shows the quantification of ATF-2 phosphorylation from p38 *in vitro* kinase assays. Regions on nitrocellulose membranes corresponding to the location of ATF-2 fragment were cut out, and [^{32}P]-incorporation quantified by gamma counting. Results are the means±SEM of three experiments.

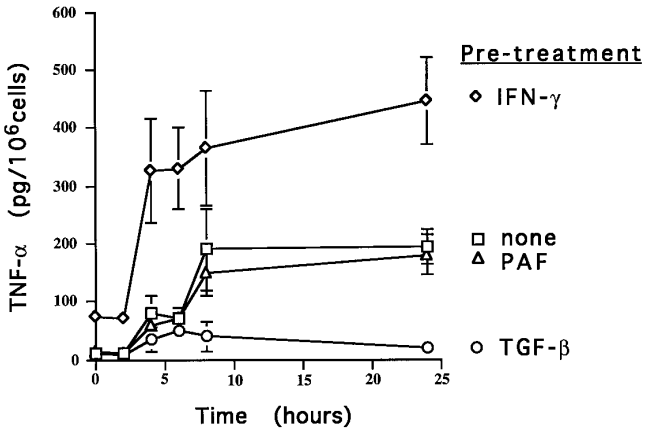

FIG. 4. Modulation of FcγR stimulated TNF-α synthesis. Murine bone marrow-derived macrophages were treated with IFN-γ (10U/ml), TGF-β (10ng/ml), or PAF (1 μM) for 18 hours prior to FcγR crosslinking with mAb 2.4G2 (10μg/ml) against FcγRII/III and F(ab′)2 goat anti-rat IgG (5μg/ml). At various times post-stimulation, culture supernatants were removed, and TNF-α protein levels were quantified by ELISA. Values shown are the means±SEM of three or more experiments.

We have previously reported that FcγR-stimulated TNF-α synthesis was inhibited by the MEK-1 inhibitor PD 098059 (7). This suggests an important role for $p42^{MAPK}$ signaling in FcγR-induced TNF-α synthesis. Given the capacity of IFN-γ to modulate both FcγR-stimulated $p42^{MAPK}$ activation and TNF-α synthesis, we questioned the effect of IFN-γ on the PD 098059 inhibition of FcγR-stimulated TNF-α synthesis. In non-pretreated macrophages, PD 098059 effectively inhibited FcγR-stimulated TNF-α synthesis with an IC_{50} of approximately 0.1μM (Figure 5). At 30μM, PD 098059 inhibited 95% of the induced TNF-α synthesis. Treating macrophages with IFN-γ for 18 hours prior to FcγR cross liking resulted in a rightward shift in the PD 098059 dose response curve for the inhibition of TNF-α synthesis (Figure 5). The IC_{50} of PD 098059 in IFN-γ treated cells was 10-fold higher (1μM) as compared

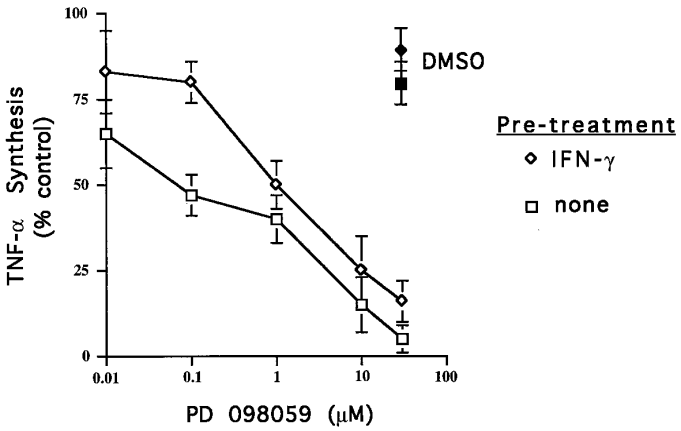

FIG. 5. IFN-γ alters the inhibition of FcγR-stimulated TNF-α production by the MEK-1 inhibitor PD 098059. Murine bone marrow-derived macrophages were treated with or without IFN-γ (10 U/ml) for 18 hours, followed by the MEK-1 inhibitor PD 098059 or DMSO control (0.15% v/v) for 1 hour before FcγR crosslinking with mAb 2.4G2 (10μg/ml) against FcγRII/III and F(ab′)2 goat anti-rat IgG (5μg/ml). Culture supernatants were collected 12 hours after FcγR crosslinking, and TNF-α protein measured by ELISA. Values are expressed as a percentage of non-PD 098059 treated cell values and are the means±SEM of three or more experiments.

to non-pretreated cells. At higher concentrations, PD 098059 was equally effective at inhibiting FcγR-stimulated TNF-α synthesis in both non-pretreated and IFN-γ-pretreated macrophages. The mechanism for the shift in the PD 098059 IC_{50} for inhibition of FcγR-induced TNF-α synthesis in IFN-γ treated cells is not known. PD 098059 is reported to be a noncompetitive inhibitor of MEK-1 (the upstream kinase of $p42^{MAPK}$); however, PD 098059 does not completely inhibit the *in vivo* activation of $p42^{MAPK}$ in cells treated with high concentrations of potent MEK activators (13). Consequently, IFN-γ may act by augmenting FcγR-induced MEK-1 activation. Our findings that 1) IFN-γ augmented FcγR-induced activation of $p42^{MAPK}$ and TNF-α expression, and 2) in the presence of IFN-γ, the IC_{50} of PD 098059 for inhibition of FcγR-induced TNF-α expression was significantly increased would lend credance to our hypothesis that IFN-γ is influencing the $p42^{MAPK}$ signaling pathway activated by FcγR-crosslinking.

The link between the activation of MAPKs and TNF-α production in mononuclear phagocytic cells is just beginning to be elucidated. Using the MEK-1 inhibitor PD 098059, we (7) and Trotta and colleagues (8) have implicated $p42^{MAPK}$ as necessary for FcγR-induced TNF-α expression. In addition to $p42^{MAPK}$, the activation of p38 has been implicated in the regulation of TNF-α synthesis. A series of pyridinyl-imidazole compounds have been reported to inhibit LPS-induced p38 activation and TNF-α synthesis in monocytes (14,15). The correlation between the modulatory changes in FcγR-stimulated activation of MAPKs and TNF-α production suggests that one means by which the cytokines IFN-γ and TGF-β may modulate FcγR-induced TNF-α synthesis is through quantitative changes in the activation of MAPKs.

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