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# C3a Enhances Nerve Growth Factor-Induced NFAT Activation and Chemokine Production in a Human Mast Cell Line, HMC-1

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## Abstract

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# C3a Enhances Nerve Growth Factor-Induced NFAT Activation and Chemokine Production in a Human Mast Cell Line, HMC-1[1]

**Jasimuddin Ahamed,[2] Rampura T. Venkatesha, E. Berla Thangam, and Hydar Ali[3]**

**Activation of cell surface G protein-coupled receptors leads to transphosphorylation and activation of a number of receptor tyrosine kinases. Human mast cells express G protein-coupled receptors for the complement component C3a (C3aR) and high affinity nerve growth factor (NGF) receptor tyrosine kinase, TrkA. To determine whether C3a cross-regulates TrkA signaling and biological responses, we used a human mast cell-line, HMC-1, that natively expresses both receptors. We found that NGF caused tyrosine phosphorylation of TrkA, resulting in a sustained $Ca^{2+}$ mobilization, NFAT activation, extracellular-signal regulated kinase (ERK) phosphorylation, and chemokine, macrophage inflammatory protein-1$\beta$ (MIP-1$\beta$) production. In contrast, C3a induced a transient $Ca^{2+}$ mobilization and ERK phosphorylation but failed to stimulate TrkA phosphorylation, NFAT activation, or MIP-1$\beta$ production. Surprisingly, C3a significantly enhanced NGF-induced NFAT activation, ERK phosphorylation, and MIP-1$\beta$ production. Pertussis toxin, a $G_{i/o}$ inhibitor, selectively blocked priming by C3a but had no effect on NGF-induced responses. Mitogen-activated protein/ERK kinase inhibitor U0126 caused ~30% inhibition of NGF-induced MIP-1$\beta$ production but had no effect on priming by C3a. However, cyclosporin A, an inhibitor of calcineurin-mediated NFAT activation, caused substantial inhibition of NGF-induced MIP-1$\beta$ production both in the absence and presence of C3a. These data demonstrate that NGF caused tyrosine phosphorylation of TrkA to induce chemokine production in HMC-1 cells via a pathway that mainly depends on sustained $Ca^{2+}$ mobilization and NFAT activation. Furthermore, C3a enhances NGF-induced transcription factor activation and chemokine production via a G protein-mediated pathway that does not involve TrkA phosphorylation.** ***The Journal of Immunology,*** **2004, 172: 6961–6968.**

Nerve growth factor (NGF)[4] was originally described as a neurotrophic factor that plays a critical role in the development and maintenance of sensory and sympathetic neurons (1–3). There is now a substantial body of evidence that demonstrates an important role of NGF in allergic and inflammatory diseases. For example, activation of mast cells via the cross-linking of cell surface Fc$\epsilon$RI results in the synthesis and release of NGF (4). Furthermore, increased levels of NGF have been demonstrated in plasma, and nasal and bronchoalveolar lavage fluids obtained from allergic patients (5–7). Several lines of evidence suggest that NGF released from mast cells mediates autocrine/paracrine effects on mast cell development and activation. NGF causes mast cell hypertrophy and hyperplasia in many tissues and organs (8). NGF is a mast cell chemoattractant and it also induces histamine release from these cells (9–12). NGF has recently been shown to stimulate the expression of IL-6 in bone marrow-derived mast cells (13). Mast cells are a rich source of chemokines, and these proinflammatory cytokines play important roles in allergic and inflammatory diseases (14–18). However, whether NGF stimulates chemokine production in mast cells has not been determined.

The biological effects of NGF are mediated via two types of cell surface receptors, p75 and TrkA (19). Mast cells express TrkA but not p75 (11, 20, 21). TrkA-mediated signaling pathways have been studied most extensively in neuronal cell lines. In this system, NGF causes tyrosine phosphorylation of TrkA leading to the activation of two main signaling pathways. Phosphorylation of TrkA at $Tyr^{490}$ leads to the binding of Shc, resulting in transient activation of the Ras/mitogen-activated protein (MAP) kinase signaling cascade (22, 23). In contrast, phosphorylation of TrkA at the other major phosphorylation site, $Tyr^{785}$, mediates docking and sustained activation of phospholipase C$\gamma$ (PLC$\gamma$) (23, 24). Whereas the transient Ras/MAP kinase pathway mediates neuronal cell survival (25, 26), the sustained PLC$\gamma$ activation leads to the expression of $Na^+$ channel gene (27). NFAT are a family of transcription factors expressed in a wide variety of cell types (28–31). Ag stimulation of T cells and mast cells leads to tyrosine phosphorylation of PLC$\gamma$, resulting in a sustained $Ca^{2+}$ mobilization. The subsequent activation of NFAT regulates the expression of cytokine and chemokine genes (28, 32). Furthermore, NGF-induced outgrowth of embryonic axons requires NFAT activation (29). Whether NGF stimulates NFAT activation in mast cells and what biological response it induces is not known.

In addition to NGF, the complement component C3a plays an important role in the pathogenesis of allergic and inflammatory diseases (33–35). In leukocytes, C3a mediates its biological effects via the activation of a cell surface G protein-coupled receptor (GPCR) (18, 36). Cross-regulation of receptor tyrosine kinases by GPCRs has been studied quite extensively. In most cases, GPCR

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[4] Abbreviations used in this paper: NGF, nerve growth factor; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; GAIP, $G_\alpha$-interacting protein; GIPC, GAIP-interacting protein C terminus; GPCR, G protein-coupled receptor; HMC, human mast cell; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein-1; MIP-1$\beta$, macrophage inflammatory protein-1$\beta$; PDZ, PSD-95/Dig/ZO-1; PLC$\gamma$, phospholipase C$\gamma$; PTX, pertussis toxin; PC12, pheochromocytoma 12.



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activation leads to phosphorylation of receptor tyrosine kinases, resulting in their activation (37). Indeed, in pheochromocytoma 12 (PC12) cells, adenosine and neuropeptides use their respective cell surface GPCRs to cause tyrosine phosphorylation of TrkA, resulting in the activation of cell survival signaling pathway (38–40). Whether C3a also cross-regulates TrkA-mediated responses in mast cells has not been determined. To address this issue, we have used a human mast cell line (HMC-1), that natively expresses both C3aR and TrkA (18, 20, 41). In this study, we show that NGF stimulates chemokine production in HMC-1 cells via TrkA phosphorylation, sustained $Ca^{2+}$ mobilization, and NFAT activation. Surprisingly, we found that C3a enhanced NGF-induced $Ca^{2+}$ mobilization, NFAT activation, and chemokine production without causing TrkA phosphorylation.

## Materials and Methods

### *Materials*

NGF (2.5S) was obtained from Upstate Biotechnology (Lake Placid, NY). Purified C3a was obtained from Advance Research Technologies (San Diego, CA). Recombinant C5a was purchased from Sigma-Aldrich (St. Louis, MO). U0126, K252a, PMA, $Ca^{2+}$-ionophore A23187, cyclosporin A (CsA), and recombinant *Escherichia coli* LPS were purchased from Calbiochem (La Jolla, CA). Pertussis toxin (PTX) and all tissue culture reagents were purchased from Invitrogen (Gaithersburg, MD). Indo-1 AM and pluronic F-127 were from Molecular Probes (Eugene, OR). ECL Western blotting analysis kits were purchased from Amersham (Arlington, IL). Dual Luciferase Assay kit was obtained from Promega (Madison, WI). TrkA, phospho-TrkA, and extracellular signal-regulated kinase (ERK) Abs were purchased from Cell Signaling Technology (Beverly, MA). Capture Abs to macrophage inflammatory protein-1$\beta$ (MIP-1$\beta$; MAB 271), monocyte chemoattractant protein-1 (MCP-1; MAB 679), and IL-8 (DY 208), and polyclonal biotinylated Abs to MIP-1$\beta$, MCP-1, and IL-8 were purchased from R&D Systems (Minneapolis, MN).

### *Calcium measurement*

$Ca^{2+}$ mobilization was determined as described previously (18, 42). Briefly, HMC-1 cells ($1 \times 10^6$) were loaded with 1 $\mu$M Indo-1 AM in the presence of 1 $\mu$M pluronic F-127 for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES-buffered saline. $Ca^{2+}$ mobilization was measured in a Hitachi F-2500 spectrophotometer (San Jose, CA) with an excitation wavelength of 355 nM and an emission wavelength of 410 nM (18).

### *TrkA phosphorylation*

HMC-1 cells ($5 \times 10^6$) were stimulated with C3a (10 nM), NGF (30 ng/ml), and C3a plus NGF at 37°C for 5 min. Reaction was stopped by the addition of 3 vol of ice-cold PBS containing 1 mM sodium orthovanadate. Cells were lysed with 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 10 $\mu$g/ml PMSF) for 1 h at 4°C, and centrifuged at $14,000 \times g$ for 10 min; supernatants were transferred to 1.5-ml tube and incubated with 2 $\mu$g/ml TrkA Ab for 1 h on a rotating wheel, followed by incubation with 20 $\mu$l of protein G agarose beads to immunoprecipitate TrkA receptor. SDS-sample buffer (50 $\mu$l) was added to the pellet and heated to 90°C for 10 min. Proteins were separated on a 7.5% SDS-polyacrylamide gel and Western blotting was performed with phosphotyrosine-specific Ab, and TrkA phosphorylation was detected by ECL.

### *Transient transfection of HMC-1 cells and NFAT/NF-κB luciferase activity*

HMC-1 cells ($2 \times 10^7$) were mixed with 10 $\mu$g of pNFAT or pNF-$\kappa$B luciferase reporter construct (Stratagene, La Jolla, CA) in a total volume of 200 $\mu$l of transfection medium. To standardize the expression level of the transfected constructs, 0.5 $\mu$g of pRL-SV40 *Renilla* luciferase construct (Promega) was added to the transfection mixture. Cells were then electroporated with a single pulse using a Bio-Rad gene pulser (Hercules, CA) as described previously (43). Cells ($0.2 \times 10^6$) were seeded to each well of a 48-well plate. The following day, cells were incubated in serum-free medium and stimulated with C3a, NGF, or C3a plus NGF for 6 h at 37°C, unless otherwise indicated. More than 80% of the cells were viable at the time of the assay as determined by trypan blue staining. Cells were lysed and assayed for luciferase activity with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. The *Firefly* luciferase activity was normalized on the basis of *Renilla* luciferase activity.

### *Phosphorylation of ERK*

Cells were stimulated with C3a, NGF, or C3a plus NGF for 5 min and the reactions were stopped by the addition of a 3-fold excess ice-cold PBS containing 1 mM sodium orthovanadate. The cells were lysed with 2× SDS sample buffer, and ERK phosphorylation was determined by Western blotting with a phospho-ERK1/2 Ab. The membrane was stripped and reprobed with an Ab that reacts with unphosphorylated ERK1/2, as described previously (18).

### *Assay of chemokine protein production by ELISA*

HMC-1 cells ($0.2 \times 10^6$/well) were cultured in complete growth medium overnight. Cells were serum starved and stimulated with C3a, NGF, or C3a plus NGF for 6 h, unless otherwise stated. Chemokine protein levels (MIP-1$\beta$, MCP-1, and IL-8) were quantified by sandwich ELISA using matched Ab pairs as described previously (18).

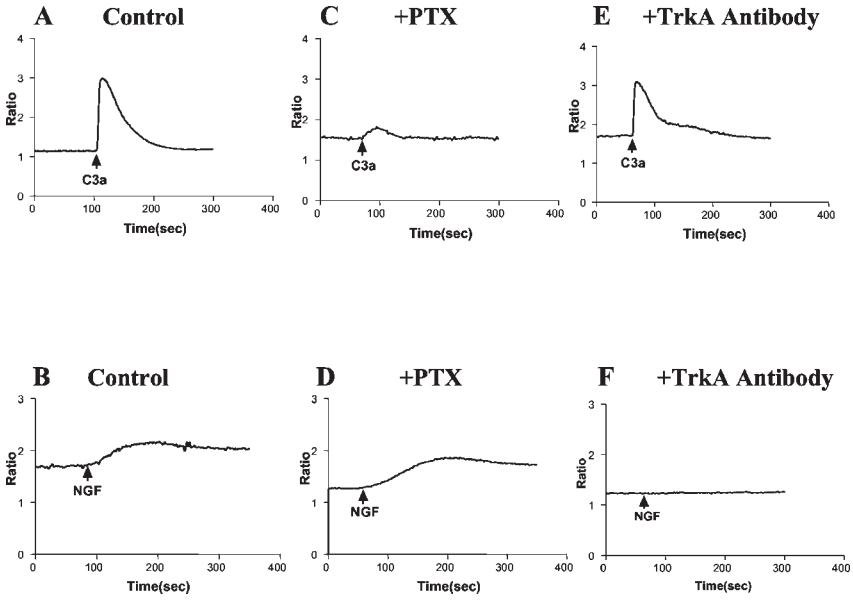


**FIGURE 1.** C3a- and NGF-induced $Ca^{2+}$ mobilization in HMC-1 cells. Cells were cultured overnight in normal medium (Control; *A* and *B*), medium supplemented with PTX (100 ng/ml; *C* and *D*), or TrkA-specific Ab (2 $\mu$g/ml; *E* and *F*). Cells were then washed and loaded with Indo-1 AM, stimulated with C3a (10 nM; *A*, *C*, and *E*) or NGF (30 ng/ml; *B*, *D*, and *F*), and intracellular $Ca^{2+}$ mobilization was determined. The data shown are representative of three similar experiments.

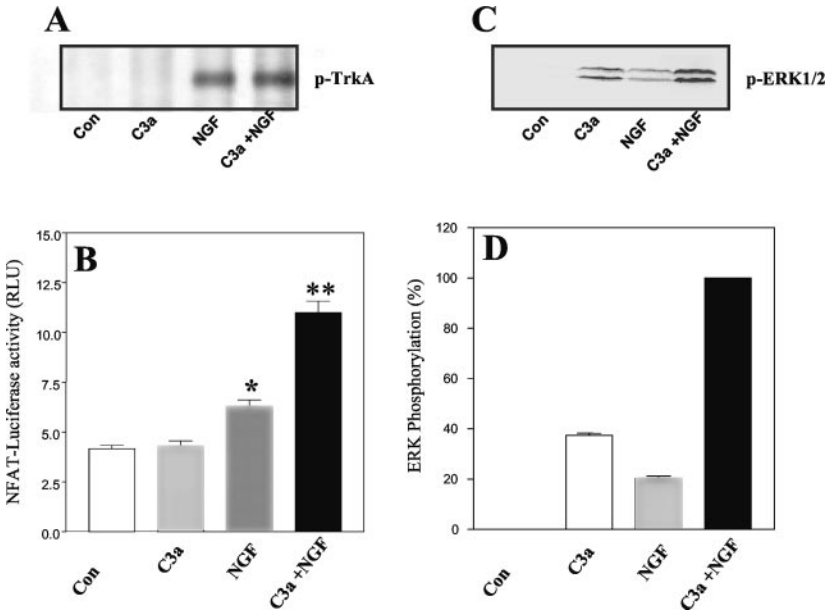


**FIGURE 2.** C3a does not cause TrkA phosphorylation but enhances NGF-induced NFAT activation and ERK phosphorylation. *A*, HMC-1 cells were exposed to buffer (Con), C3a (10 nM), NGF (30 ng/ml), or C3a + NGF for 5 min. Cell lysates were immunoprecipitated with anti-TrkA Ab, and receptor phosphorylation was determined by Western blotting with a phosphotyrosine-specific Ab (p-TrkA). *B*, HMC-1 cells were cotransfected with NFAT-dependent luciferase reporter and *Renilla* luciferase plasmids. Cells were stimulated for 6 h with C3a, NGF, or C3a + NGF. The NFAT luciferase activity was measured from cell lysate. Data presented are relative luciferase activity, normalized to *Renilla* luciferase activity as relative light units (RLU) ($n = 3$). *C*, Cells were stimulated with C3a, NGF, or C3a + NGF for 5 min. ERK activation was determined by Western blotting with phospho-specific ERK1/2 Ab (p-ERK1/2). *D*, Bar graph represents summary of ERK phosphorylation from four independent experiments, where C3a + NGF stimulated cells (100%). The data shown in *B* are mean ± SEM of five experiments. *, $p < 0.05$ in the absence or presence of NGF and **, $p < 0.01$ NGF in the absence and presence of C3a.

## Results

### *Regulation of C3aR and TrkA in HMC-1 cells*

We initially used a $Ca^{2+}$ mobilization assay to characterize endogenous C3aR and TrkA in HMC-1. As shown in Fig. 1*A*, C3a (10 nM) stimulated a rapid and transient $Ca^{2+}$ spike that returned to basal within 2 min. In contrast, NGF (30 ng/ml) induced a slower response that remained elevated for a longer time period (Fig. 1*B*). To test the role of G protein signaling on C3a and NGF responses, cells were treated with PTX (100 ng/ml, overnight) and $Ca^{2+}$ mobilization was determined. PTX caused a substantial inhibition of C3a-induced $Ca^{2+}$ mobilization (Fig. 1*C*) but had no effect on NGF response (Fig. 1*D*). In contrast, anti-TrkA Ab did not inhibit C3a-induced $Ca^{2+}$ mobilization but completely blocked the response to NGF (Fig. 1, *E* and *F*). The inhibitory effect of anti-TrkA Ab was specific for TrkA, as an isotype-matched Ab had no effect on the NGF response (data not shown). These data suggest that C3aR and TrkA use G protein and tyrosine kinase signaling pathways, respectively, to induce $Ca^{2+}$ mobilization in HMC-1 cells.

### *C3a does not cause TrkA phosphorylation but enhances NGF-induced NFAT activation and ERK phosphorylation*

Lee and Chao (38) recently showed that adenosine receptor activation resulted in $Ca^{2+}$ mobilization in PC12 cells. Furthermore, adenosine caused transphosphorylation of TrkA via a $Ca^{2+}$-dependent pathway. Given that C3a stimulated $Ca^{2+}$ mobilization in HMC-1 cells (Fig. 1), we hypothesized that C3a could also cause TrkA phosphorylation in HMC-1 cells. To test this hypothesis, cells were stimulated with C3a for 5 min and cell lysate was immunoprecipitated with anti-TrkA Ab. Tyrosine phosphorylation of TrkA was determined by Western blotting using a phosphotyrosine-specific Ab. As shown in Fig. 2*A*, C3a did not cause phosphorylation of TrkA in HMC-1 cells. TrkA phosphorylation was not evident even 1 h after C3a stimulation (data not shown). In contrast, NGF (30 ng/ml, 5 min) induced a robust tyrosine phosphorylation of TrkA. To determine whether C3a could enhance NGF-induced response, we stimulated HMC-1 cells simultaneously with C3a and NGF. We found that C3a had little or no effect on NGF-induced TrkA phosphorylation (Fig. 2*A*).

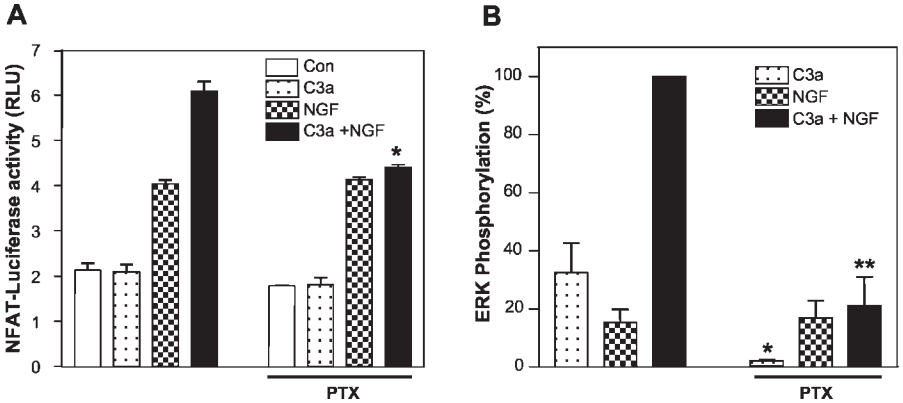


**FIGURE 3.** Effects of PTX on C3a- and NGF-induced NFAT activation and ERK phosphorylation. *A*, HMC-1 cells transiently expressing NFAT luciferase and *Renilla* constructs were incubated with buffer or PTX (100 ng/ml, overnight). Cells were stimulated with C3a (10 nM), NGF (30 ng/ml), or C3a + NGF for 6 h and NFAT luciferase activity (normalized to *Renilla* activity as relative light units (RLU)) was measured from the cell lysate. *B*, PTX-treated or untreated HMC-1 cells were stimulated with C3a, NGF, or C3a + NGF for 5 min and ERK1/2 activation was determined by Western blotting using phospho-ERK1/2 Ab. Bands were quantified by densitometry and expressed as percentage vs C3a plus NGF-stimulated cells (100%). The data shown are mean ± SEM of four experiments. *, $p < 0.05$ and **, $p < 0.01$ in the absence and presence of PTX treatment.

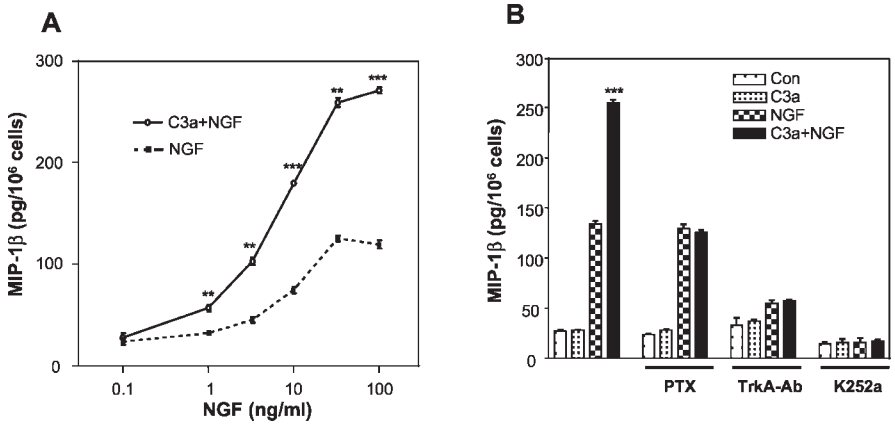


**FIGURE 4.** C3a enhances NGF-induced MIP-1$\beta$ production in HMC-1 cells. *A*, Cells were stimulated with different concentrations of NGF for 6 h in the absence and presence of C3a (10 nM). MIP-1$\beta$ production was determined by sandwich ELISA. *B*, HMC-1 cells were preincubated with buffer or PTX (100 ng/ml, overnight), TrkA Ab (2 $\mu$g/ml, overnight), or K252a (200 nM, 60 min). Cells were then exposed to C3a (10 nM), NGF (30 ng/ml), or C3a + NGF for 6 h, and MIP-1$\beta$ production was determined. The data shown are mean ± SEM of three experiments. **, $p < 0.01$ and ***, $p < 0.001$ in the absence and presence of C3a.

Using an EMSA, we have recently shown that a sustained $Ca^{2+}$ mobilization is associated with the activation of transcription factor NFAT in HMC-1 cells (18). As NGF stimulated a sustained $Ca^{2+}$ mobilization (Fig. 1), we hypothesized that NGF could stimulate NFAT activation. To test this possibility, we generated transient transfectants in HMC-1 cells expressing NFAT linked to a luciferase reporter construct and determined the effects of NGF on NFAT-luciferase reporter activity. As shown in Fig. 2*B*, NGF caused a significant increase in NFAT-luciferase reporter activity. In agreement with our previous studies using EMSA (18), we found that C3a did not stimulate NFAT reporter activity. Interestingly, C3a caused significant enhancement of the response to NGF (Fig. 2*B*).

NGF has been shown to activate ERK phosphorylation in HMC-1 cells (20). We therefore sought to determine whether C3a could also enhance NGF-induced ERK phosphorylation. For these experiments, cells were stimulated with C3a, NGF, or C3a plus NGF for 5 min, and ERK phosphorylation was determined. As shown in Fig. 2, *C* and *D*, C3a and NGF caused ERK phosphorylation in HMC-1 cells. However, the response to C3a was ~50% greater than that of NGF. Interestingly, when C3a and NGF were added together, there was a synergistic activation of ERK phosphorylation. These findings demonstrate that although C3a did not stimulate TrkA phosphorylation, it enhanced NGF-induced downstream signaling such as NFAT activation and ERK phosphorylation.

In PC12 cells, NGF-induced ERK phosphorylation is mediated, at least in part, by a $G_{\alpha i/o}$ family of G proteins (44, 45). To determine the role of G proteins on NGF-induced signaling in HMC-1 cells, we tested the effects of PTX on C3a- and NGF-induced responses. PTX had no effects on NGF-induced NFAT activation (Fig. 3*A*) or ERK phosphorylation (Fig. 3*B*). However, PTX inhibited C3a-induced ERK phosphorylation and priming of NFAT activation and ERK phosphorylation by C3a (Fig. 3, *A* and *B*).

### *C3a enhances NGF-induced MIP-1β and MCP-1 production in HMC-1 cells*

Our next goal was to determine the functional consequence of the cross-regulation between C3aR and TrkA for NFAT activation and ERK phosphorylation in HMC-1 cells. We have previously shown that MIP-1$\beta$ and MCP-1 are up-regulated both at the levels of mRNA and protein by a variety of stimulants in HMC-1 cells (18). We decided to initially examine the effects of NGF and C3a plus NGF on MIP-1$\beta$ production. As shown in Fig. 4*A*, NGF caused a dose-dependent increase in MIP-1$\beta$ production in HMC-1 cells. The $EC_{50}$ value for NGF was ~10 ng/ml and the maximal response was obtained at a concentration of ~30 ng/ml. We have

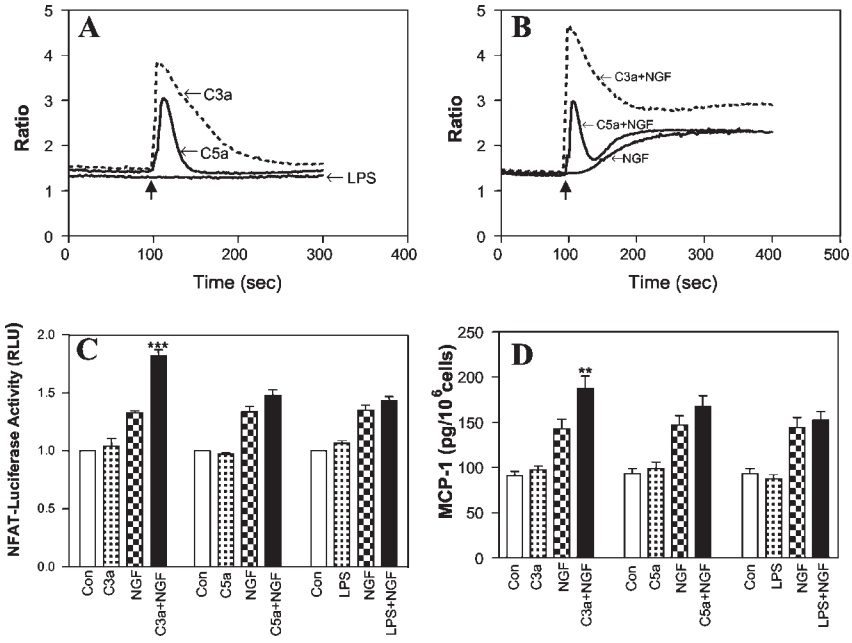


**FIGURE 5.** C3a but not C5a or LPS enhances NGF-induced $Ca^{2+}$ mobilization, NFAT activation, and MCP-1 production in HMC-1 cells. *A*, Cells were loaded with Indo-1 AM and the effects of C3a (10 nM), C5a (10 nM), and LPS (100 ng/ml) on $Ca^{2+}$ mobilization were determined. *B*, Cells were stimulated with NGF, C3a + NGF, or C5a + NGF, and $Ca^{2+}$ mobilization was determined. *C*, Transient transfectants were generated in HMC-1 cells coexpressing NFAT luciferase and *Renilla* plasmids. Cells were then stimulated with C3a (10 nM), C5a (10 nM), or LPS (100 ng/ml) in the absence and presence of NGF (30 ng/ml). NFAT luciferase activity was determined in cell lysate. The data are expressed as relative NFAT luciferase activity. *D*, MCP-1 production was measured by ELISA from culture supernatants at 6 h after stimulation. The data shown are mean ± SEM of three to four experiments performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$ in the absence and presence of C3a, C5a, or LPS.

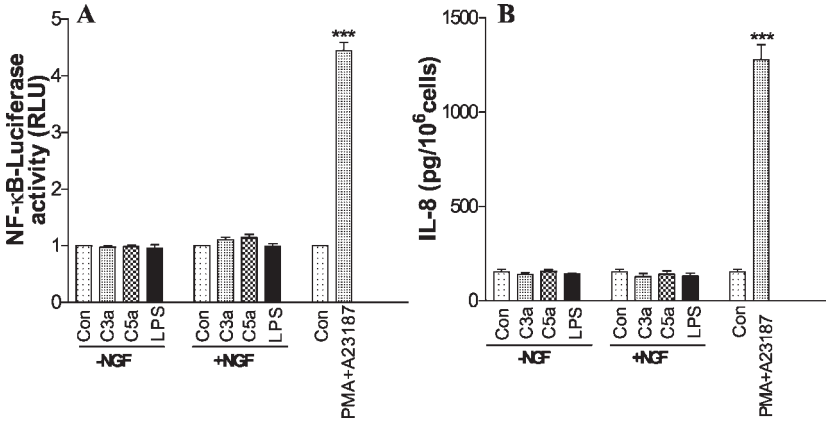


**FIGURE 6.** NGF does not cause NF-κB activation or IL-8 production in HMC-1 cells. Transient transfectants were generated in HMC-1 cells coexpressing NF-κB luciferase and *Renilla* plasmids. Cells were then stimulated with C3a (10 nM), C5a (10 nM), or LPS (100 ng/ml) in the absence and presence of NGF (30 ng/ml). Cells were also stimulated with PMA (100 nM) + A23187 (100 nM). *A*, Relative NF-κB luciferase activity was determined in cell lysate. The data are expressed as fold increase over control. *B*, IL-8 production was measured by ELISA from culture supernatants at 6 h after stimulation. The data shown are mean ± SEM of three to four experiments performed in triplicate. ***, $p < 0.001$ in the absence and presence of PMA + A23187.

previously shown that transient $Ca^{2+}$ mobilization induced by C3a did not provide sufficient signal for NFAT activation or MIP-1β production in HMC-1 cells (18). However, as shown in Fig. 4*A*, C3a (10 nM) enhanced NGF-induced MIP-1β production by 2- to 3-fold. Chemokine production in response to C3a/NGF required protein synthesis, as treatment of cells with cycloheximide completely blocked the response (data not shown).

To determine the role of the $G_i$ family of G proteins on chemokine production, cells were treated with PTX and its effects on NGF and C3a plus NGF-induced MIP-1β production were determined. PTX selectively blocked the priming by C3a but had no effect on the response to NGF (Fig. 4*B*). In contrast, a TrkA-specific Ab or an inhibitor for TrkA signaling, K252a, blocked NGF-induced MIP-1β production both in the absence and presence of C3a. These data suggest that NGF-induced MIP-1β production is mediated via the activation of TrkA, whereas the enhanced response by C3a requires the activation of PTX-sensitive G protein.

To determine whether C3a selectively enhances NGF-induced responses in HMC-1 cells, we tested the effects of C5a and LPS on NGF-induced responses in HMC-1 cells. We found that C5a caused a transient $Ca^{2+}$ mobilization in HMC-1 cells whereas LPS had no effect (Fig. 5*A*). Interestingly, only C3a but not C5a or LPS enhanced NGF-induced $Ca^{2+}$ mobilization (Fig. 5*B*), NFAT activation (Fig. 5*C*), MCP-1 production (Fig. 5*D*), and MIP-1β (data not shown) in HMC-1 cells.

### *Effects of C3a/NGF on MIP-1β and MCP-1 production in HMC-1 cells does not require NF-κB activation*

NF-κB is a crucial transcription factor that regulates the expression of many proinflammatory cytokines and immunoregulatory molecules (46–48). Although NGF causes NFAT activation in neuronal tissues (29, 49) and HMC-1 cells (Fig. 2*B*), whether it also stimulates NF-κB activation has not been determined. We therefore generated transient transfectants in HMC-1 cells expressing NF-κB luciferase construct and tested the effect of NGF on NF-κB reporter activity. As shown in Fig. 6*A*, NGF alone or in combination with C3a, C5a, and LPS did not stimulate NF-κB luciferase activity. In contrast, PMA plus $Ca^{2+}$ ionophore A23187 resulted in ~5-fold increase in NF-κB reporter activity (Fig. 6*A*). NF-κB activation is required for chemokine IL-8 production in a variety of cell types (50–53). As shown in Fig. 6*B*, NGF alone or in combination with C3a, C5a, or LPS did not stimulate IL-8 production. In contrast, PMA/A23187 caused a substantial generation of this chemokine in HMC-1 cells. These data suggest that C3a selectively enhances NGF-induced MIP-1β and MCP-1 production in HMC-1 cells via the activation of NFAT but not NF-κB.

### *Effects of ERK phosphorylation and NFAT activation on MIP-1β production in HMC-1 cells*

As C3a enhanced NGF-induced ERK phosphorylation in HMC-1 cells (Fig. 2), we tested the role of this pathway on the cross-regulation of MIP-1β production. U0126 (MAP/ERK-1/2 inhibitor) completely blocked NGF-induced ERK phosphorylation both in the absence and presence of C3a (Fig. 7*A*). However, it inhibited NGF-induced chemokine production by 38 ± 1.04% and blocked C3a- plus NGF-induced response by 31 ± 12% (Fig. 7*B*). These data suggest that ERK phosphorylation pathway plays a minor role in NGF-induced chemokine production and has little or no effect on C3a-induced priming.

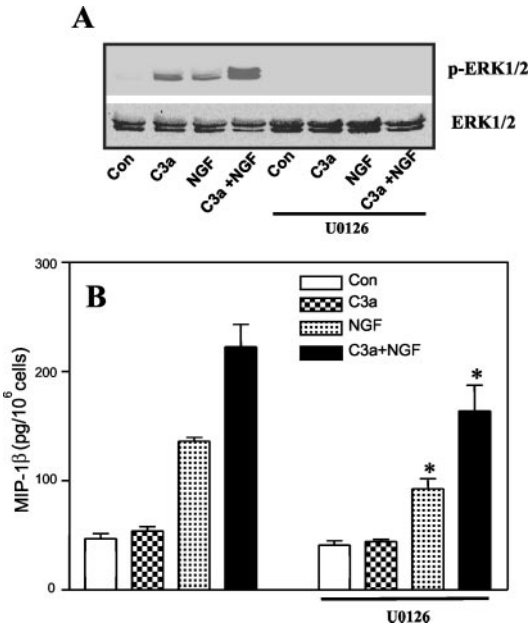


**FIGURE 7.** Role of ERK phosphorylation on C3a- and NGF-induced MIP-1β production in HMC-1 cells. *A*, Cells were preincubated with buffer or U0126 (10 μM, 30 min) and stimulated with C3a (10 nM), NGF (30 ng/ml), or C3a + NGF for 5 min, and ERK phosphorylation was determined by Western blotting using phospho-ERK1/2 Ab (p-ERK1/2, *upper panel*). The membrane was stripped and reprobed with ERK1/2 Ab (*lower panel*). *B*, Cells were preincubated with buffer or U0126 (10 μM, 30 min) and stimulated with C3a, NGF, or C3a + NGF for 6 h, and MIP-1β production was determined. Data presented are mean ± SEM of five experiments. *, $p < 0.05$ in the absence and presence of U0126.

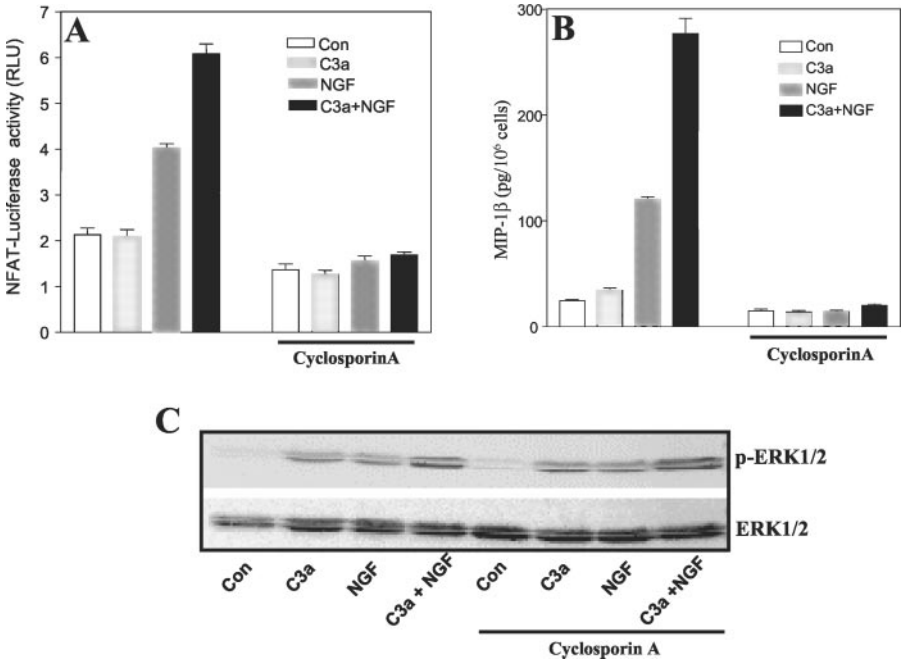


**FIGURE 8.** NFAT activation is required for C3a- and NGF-induced MIP-1$\beta$ production in HMC-1 cells. Transient transfectants were generated in HMC-1 cells coexpressing NFAT luciferase and *Renilla* plasmids. Cells were then preincubated with or without CsA, (100 nM, 30 min) and stimulated with C3a (10 nM), NGF (30 ng/ml), or C3a + NGF. NFAT luciferase activity from the lysate (*A*) and MIP-1$\beta$ production (*B*) was measured from culture supernatants 6 h after stimulation. *C*, Cells were preincubated with CsA and stimulated with C3a, NGF, or C3a + NGF for 5 min, and ERK phosphorylation was determined by Western blotting using a phospho-ERK specific Ab (p-ERK1/2, *upper panel*). Protein loading was confirmed by reprobing the membrane with ERK1/2 Ab (*lower panel*).

Given that C3a enhanced NGF-induced NFAT activation (Fig. 2 and 3*A*), we hypothesized that the ability of C3a to prime NGF-induced MIP-1$\beta$ production could be mediated via the activation of NFAT. To test this possibility, we used CsA, a potent inhibitor of calcineurin-mediated NFAT activation. CsA (100 nM) completely inhibited both NGF-induced NFAT activation (Fig. 8*A*) and MIP-1$\beta$ production (Fig. 8*B*) in the presence and absence of C3a. The inhibitory effects of CsA were specific for NFAT, as it had no effect on C3a-, NGF-, or C3a- plus NGF-induced ERK phosphorylation (Fig. 8*C*).

## Discussion

Although NGF exerts its biological effects predominantly on neurons, it can also influence the development and activation of many hemopoietic cell types. The mast cell was the first nonneuronal cell type identified as a target for NGF (8). In addition to TrkA, mast cells express GPCRs for C3a (18, 36). Given that both C3aR and TrkA play important roles in allergic and inflammatory diseases, we hypothesized that these receptors could cross-regulate each other to enhance signaling and cell activation. In the present study, we used HMC-1 that natively expresses both C3aR and TrkA, and demonstrated a novel cross-talk between these receptors for $Ca^{2+}$ mobilization, NFAT activation, and chemokine MIP-1$\beta$ production. Interestingly, this type of cross-regulation is specific for C3aR and TrkA receptors as C5a, which mediates many biological responses similar to C3a, failed to enhance NGF-induced responses in HMC-1 cells.

The biological effects of NGF are mediated via two types of cell surface receptors, p75 and TrkA (19). Previous studies have shown that mast cells express TrkA but not p75 (11, 20, 21). In the present study, we demonstrated that anti-TrkA Ab and a specific Trk antagonist, K252a, blocked NGF-induced $Ca^{2+}$ mobilization and MIP-1$\beta$ production. These data are consistent with the notion that the biological effects of NGF on mast cells are mediated via the activation of TrkA. Despite extensive reports on NGF-induced responses in mast cells, very little information is available on TrkA-mediated signaling pathways in these cells. In the present study, we have shown that NGF caused ERK phosphorylation in HMC-1 cells. However, U0126, which completely blocked NGF-induced ERK phosphorylation, had little effect on MIP-1$\beta$ production. These data suggest that NGF-induced chemokine production in HMC-1 cells requires the activation of additional signaling pathways.

The data presented herein demonstrate that $Ca^{2+}$ mobilization and the subsequent activation of the transcription factor NFAT provides an important mechanism for NGF-induced chemokine production in HMC-1 cells. NFAT is a cytosolic transcription factor that was originally shown to regulate the expression of cytokine genes in Ag-stimulated mast cells and T cells (28, 54). The mechanism of NFAT activation involves a $Ca^{2+}$-dependent activation of the phosphatase calcineurin, which dephosphorylates NFAT resulting in its translocation to the nucleus. The immunosuppressive drug CsA inhibits NFAT activation by blocking calcineurin activation. We have recently shown fMLP, via the activation of a cell surface GPCR, stimulated a sustained $Ca^{2+}$ mobilization and chemokine MIP-1$\beta$ production in HMC-1 cells (18). We also demonstrated that the ability of fMLP to induce chemokine production was dependent on NFAT activation. The findings in the present study that NGF also stimulated a sustained $Ca^{2+}$ mobilization and NFAT luciferase activity suggest that this transcription factor could also be involved in NGF-induced MIP-1$\beta$ production. This contention is supported by the observations that both NGF-induced NFAT activation and chemokine production were inhibited by CsA.

A novel finding of the present study was that C3a, which caused a transient $Ca^{2+}$ mobilization, did not stimulate MIP-1$\beta$ production by itself but significantly enhanced the response to NGF in HMC-1 cells. The studies on the cross-regulation of C3aR and TrkA provided additional support for the notion that chemokine production in HMC-1 cells depends mainly on NFAT activation. This contention is supported by the following observations. First, although C3a enhanced both ERK phosphorylation and NFAT activation, blocking ERK phosphorylation had no effect on C3a-induced priming of chemokine production. Second, CsA blocked both NGF-induced NFAT activation and chemokine production, but not ERK phosphorylation in the absence or presence of C3a.

The molecular mechanism by which C3a enhances NGF-induced NFAT activation and MIP-1$\beta$ production is not known. In PC12 cells, adenosine, lysophosphatidic acid, and neuropeptides

use their respective GPCRs to cause transactivation of TrkA (38–40, 55). We therefore hypothesized that the ability of C3a to enhance NGF-induced NFAT activation and chemokine production in mast cells could involve the tyrosine phosphorylation of TrkA. However, this possibility is unlikely, as C3a did not cause TrkA phosphorylation in HMC-1 cells. Furthermore, although NGF caused tyrosine phosphorylation of TrkA, C3a had no effect on this response. These findings suggest that the locus for priming by C3a is downstream of TrkA phosphorylation.

An interesting finding of the present study was that although C3a and C5a activate HMC-1 cells via their respective GPCRs, only C3a enhanced NGF-induced signaling and chemokine production. These findings raise the intriguing possibility that C3a could regulate TrkA signaling via the formation of a signaling complex downstream of TrkA phosphorylation. $G_\alpha$-interacting protein (GAIP)-interacting protein C terminus (GIPC) is a PSD-95/Dig/ZO-1 (PDZ)-binding protein that is linked to GPCR signaling pathway via its interaction with GAIP, a regulator of G protein signaling. It has recently been shown that GIPC binds to both phosphorylated TrkA and GAIP to form a macromolecular signaling complex in PC12 cells (56). Hu et al. (57) recently showed that $\beta_1$-adrenergic receptor interacts with GIPC and that this interaction requires the presence of serine residue in the PDZ motif T/SXV. It is interesting to note that only C3aR but not C5aR (CD88) possesses the PDZ motif T/SXV at its carboxyl terminus. Thus, the possibility that C3aR interacts with GIPC or other adapter molecules (58, 59) and that this interaction leads to the formation of a macromolecular signaling complex with phosphorylated TrkA resulting in enhanced $Ca^{2+}$ mobilization, NFAT activation, and chemokine production remains to be determined.

Cross-regulation between GPCRs and receptor tyrosine kinases has been studied quite extensively. It has been shown that epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor receptors become tyrosine phosphorylated after GPCR activation leading to MAP kinase activation (37, 60–64). Furthermore, adenosine and neuropeptides, via their respective GPCRs, cause transphosphorylation of TrkA resulting in the enhancement of both Akt phosphorylation and cell survival (39, 40). In the present study, we described a novel finding that C3a enhances NGF-induced transcription factor NFAT activation and chemokine production via a pathway that does not require transphosphorylation of a receptor tyrosine kinase, TrkA. Although HMC-1 is an immature mast cell line and does not display all properties of primary mast cells, it is widely used as a model to study mast cell signaling and biology in vitro (65–73). It is important to note that human eosinophils, which express both C3a and TrkA, respond to NGF for cytokine production via a pathway that depends on NFAT activation (74, 75). Furthermore, NGF-induced outgrowth of embryonic axons requires NFAT activation (29). A large number of other immune and neuronal cell types that express C3aR and other GPCRs also possess TrkA and NFAT (29, 74, 76, 77). Thus, the type of cross-regulation described in the present study between C3aR and TrkA in HMC-1 cells is likely to be a general phenomenon.

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