

Roles of phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase in the regulation of protein kinase C- α activation in interferon- γ -stimulated macrophages

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Summary

Members of the protein kinase C (PKC) family are activated by interferon- γ (IFN- γ) and modulate IFN- γ -induced cellular responses by regulating the activity of transcription factors. We previously reported that PKC- α enhances the ability of IFN regulatory factor-1 to transactivate the class II transactivator (*CIITA*) promoter IV in IFN- γ -stimulated macrophages. In addition, we showed that IFN- γ induces the nuclear translocation of PKC- α but the mechanisms for this remain to be elucidated. In this study, we sought to identify signalling pathways involved in IFN- γ -induced activation of PKC- α and to characterize their potential roles in modulating IFN- γ -induced responses in macrophages. IFN- γ -mediated nuclear translocation of PKC- α was a Janus activated kinase 2 (JAK2)-independent process, which required phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK). However, PKC- α phosphorylation was independent of PI3K and p38 MAPK, indicating that IFN- γ -induced phosphorylation and nuclear translocation of PKC- α are mediated by distinct mechanisms. In addition, inhibition of PI3K, but not of p38 MAPK, strongly impaired IFN- γ -induced *CIITA* and *MHC II* gene expression. Finally, PKC- α associated with signal transducer and activator of transcription 1 (STAT1) and was required for the phosphorylation of STAT1 on serine 727 in IFN- γ -stimulated macrophages. Taken together, our data indicate that PI3K and p38 MAPK modulate IFN- γ -stimulated PKC- α nuclear translocation independently of JAK2 activity and that both PI3K and PKC- α are required for type IV *CIITA* and *MHC II* gene expression in IFN- γ -stimulated macrophages.

Keywords: interferon- γ ; macrophages; nuclear translocation; phosphatidylinositol 3-kinase; protein kinase C

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Introduction

Interferon- γ (IFN- γ) is a potent macrophage activator that plays a key role in host defense. Following binding to its multisubunit receptor, IFN- γ exerts its action by triggering phosphorylation on tyrosine residues of the IFN- γ receptor-associated Janus-activated kinases (JAKs) JAK1 and JAK2. These events are followed by tyrosine and serine phosphorylation, homodimerization and nuclear translocation of the transcription factor signal transducer and activator of transcription 1 (STAT1).¹ Binding of STAT1 to specific sequences present in the promoter region of early IFN- γ -responsive genes leads to their rapid expression.²

Among those, the IFN regulatory factor-1 (IRF-1), in synergy with STAT1, activates the transcription of several genes, including the class II transactivator (*CIITA*), a non-DNA-binding protein essential for IFN- γ -induced expression of major histocompatibility complex (MHC) class II in macrophages.^{3–5} In addition to the JAK-STAT1 pathway, IFN- γ induces gene expression through STAT1-independent pathways^{6,7} and modulates various signalling pathways, including phosphatidylinositol 3-kinase (PI3K), MyD88/p38 mitogen-activated protein kinase (MAPK), extracellular-signal regulated kinases 1/2 (ERK1/2) and Jun N-terminal kinase, as well as members of the protein kinase C (PKC) family.^{8–20}

PKC is a family of structurally related serine/threonine-specific protein kinases comprising at least 10 members with distinct requirements for activity.^{21,22} There is evidence that PKC isoenzymes modulate cellular responses to IFN- γ by regulating the activity of various transcription factors.^{14–18,20} Hence, full STAT1 transcriptional activity requires serine phosphorylation²³, which implicates, among others, the activity of PKC isoenzymes. Depending on the cell type, IFN- γ -induced phosphorylation of STAT1 on serine 727 requires the activity of either PKC- δ or PKC- ϵ .^{14,17,24} In addition, IFN- γ stimulates PU.1 phosphorylation through the activation of PKC- α and/or PKC- β I, leading to the association of PU.1 with the promoter of gp91^{phox} and to increased gp91^{phox} expression in human monocytes.¹⁶ PKC- α also contributes to the expression of *CIITA* by enhancing the ability of IRF-1 to transactivate the *CIITA* promoter IV in IFN- γ -stimulated RAW 264.7 cells.²⁰

Whereas the JAK–STAT1 pathway received a great deal of attention, little is known concerning the mechanisms by which IFN- γ activates PKC isoenzymes. PI3K appears to play an important role in the activation of PKC- ϵ and PKC- δ in IFN- γ -stimulated mesangial cells and in promyelocytic leukemia cells, respectively.^{14,17,25} In epithelial cells, IFN- γ -induced intercellular adhesion molecule-1 (ICAM-1) gene expression is mediated by the activation of phosphatidylinositol phospholipase C- γ 2, which is required for the activation of PKC- α , followed by those of c-Src and STAT1.¹⁵ In macrophages and monocytes, IFN- γ induces the nuclear translocation of PKC- α and PKC- β I, where they can interact with transcription factors.^{16,20} In contrast to the majority of nuclear proteins, PKC isoenzymes lack a canonical nuclear localization signal, indicating that other mechanisms are involved in the transport process. In the present study, we sought to identify signaling pathways involved in IFN- γ -induced activation and nuclear translocation of PKC- α in macrophages, and to characterize their roles in IFN- γ -induced gene expression.

Materials and methods

Macrophages

Bone marrow-derived macrophages (BMDM) were obtained by growing bone marrow cells from female BALB/c mice at 37° in 5% CO₂ in complete medium [Dulbecco's Modified Eagle's minimal essential medium with glutamine (Life Technologies Inc., Burlington, ON, Canada), containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 10 mM HEPES, pH 7.4, and antibiotics] for 7 days in the presence of 15% (v/v) L929 cell-conditioned medium, a source of colony-stimulating factor-1 (CSF-1).²⁶ BMDM were made quiescent by culturing them in the absence of CSF-1 for 18 hr prior to use. The murine macrophage cell line RAW 264.7 was grown in complete medium.

Reagents

Recombinant mouse IFN- γ (R&D Systems, Minneapolis, MN) was used at a final concentration of 100 U/ml, and lipopolysaccharide (LPS) from *Escherichia coli* serotype O127:B8 (Sigma, St Louis, MO) was used at a final concentration of 100 ng/ml. The inhibitors and their final concentrations used were: tyrphostin AG-490 (250 μ M), Gö-6976 (1 μ M), SB203580 (10 μ M), PD98059 (30 μ M), Ly294002 (100 μ M), U-73122 (50 μ M), U-73343 (50 μ M), PP2 (50 μ M) and PP3 (50 μ M) (Calbiochem, San Diego, CA). The inhibitors were prepared following the manufacturer's instructions. BpV(phen) (kindly provided by Dr Martin Olivier, McGill University, Montreal, QC, Canada) was prepared in phosphate-buffered saline (PBS) and used at a final concentration of 10 μ M. Those inhibitors are respectively selective for JAK2, conventional PKC, p38 MAPK, ERK1/2, PI3K, phospholipase-C, U-73122 inactive analog, Src-family protein tyrosine kinases, the negative control for PP2 and protein tyrosine phosphatases.

Reverse transcription–polymerase chain reaction

Total RNA was prepared and reverse transcribed as described previously.^{20,27} For the polymerase chain reaction (PCR), samples were amplified under the following conditions: 30 seconds at 94°, 1 min at 56° and 1 min at 72° (30 cycles). The PCR products were migrated on a 1.3% (w/v) agarose gel and the pictures were acquired using an AlphaImager 3400 (Alpha Innotech Corporation, San Leandro, CA). The primers used for hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) were AD-55 (forward) 5'-GTTGGATACAGGCCAGACTTTG TTG-3' and AD-56 (reverse) 5'-GATTCAACTTG CGCTCATCTTAGGC-3'; the primers used for type IV *CIITA* were AD-267 (forward) 5'-ACAGCCACAGCCGC GACCATA-3' and AD-268 (reverse) 5'-CTCTGCTCC AATGTGCTCCTA-3'; and the primers used for *MHC II* were AD-70 (forward) 5'-GGAATTCTGGGAATCTCAGG TTCCCAGTG-3' and AD-71 (reverse) 5'-GGAATTCTGA ACACCATGCTCAGCCTCTG-3'.²⁰

Signalling pathway analyses

BMDM were seeded (3×10^6 cells/well) in six-well plates for 18 hr and incubated with or without selective inhibitors for 1 hr prior to stimulation with 100 U/ml of IFN- γ or 100 ng/ml of LPS for the indicated periods of time.

Western blot analyses

Cells were lysed and Western blot analyses were performed as described previously.²⁸ Rabbit polyclonal antisera against IRF-1 was from Santa Cruz Biotechnology

(Santa Cruz, CA), antibody against PKC- α was from BD Transduction Laboratories (Oakville, ON, Canada), antibodies against STAT1 and phospho-STAT1 (Ser727) were from Upstate Laboratories (Lake Placid, NY) and antibodies against p38 MAPK, phospho-p38 (Thr180/Tyr182), Akt, phospho-Akt (Ser473), phospho-PKC- α (Thr638) and phospho-STAT1 (Tyr 701) were from Cell Signaling Technology, Beverly, MA.

Translocation of the PKC- α -green fluorescent protein construct and confocal microscopy

Adherent RAW 264.7 cells were transfected with 0.3 μ g of the PKC- α -green fluorescent protein (GFP) construct²⁹ (kindly provided by C. Quittau-Prévostel, INSERM, France) using Eugene-6 (Roche Diagnostics, Laval, QC, Canada). Briefly, cells were seeded (10^5 cells/well) in 24-well plates containing microscope coverslips for 18 hr and transfected with 100 μ l of Eugene-6/DNA mix for 24 hr. Cells were incubated for 1 hr in the presence or the absence of signalling inhibitors and then stimulated for 2 hr with 100 U/ml of IFN- γ . Cells were fixed with 2% formaldehyde, permeabilized for 15 min at 21° with PBS containing 0.1% Triton-X-100 and 1% bovine serum albumin (BSA) and then incubated with PBS containing 1% Alexa Fluor 568 phalloidin (Molecular Probes, Oregon, OR, USA) and 0.2% DRAQ5 (BioStatus Ltd., Shephed, UK). Coverslips were mounted with Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL). Nuclear translocation of PKC- α was quantified using an oil immersion Nikon Plan Apo 100 \times objective mounted on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) equipped with a BioRad Radianc 2000 confocal imaging system (BioRad Laboratories, Hercules, CA), and data were analyzed using the LaserSharp 2000 software (BioRad, Hercules, CA). At least 30 transfected cells were counted for each experiment and the results presented are representative of three independent experiments. Percentage of nuclear translocation represents the number of transfected cells with nuclear translocation of human PKC- α -GFP on the total number of transfected cells observed.

Immunofluorescence

Adherent macrophages in 24-well plates containing microscope coverslips (Fisher Scientific, Ottawa, ON, Canada) (10^5 cells/well for RAW 264.7 cells or 2×10^5 cells/well for BMDM) were stimulated for 2 hr with 100 U/ml of IFN- γ and fixed with 2% formaldehyde in PBS. Cells were permeabilized with 0.1% Triton-X-100 and blocked with 1% BSA and 20% goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cells were then marked with mouse monoclonal anti-PKC- α (1 : 300 dilution) (Cell Signaling Technology) and

stained with PBS containing the anti-mouse immunoglobulin coupled to Alexa-488 (1 : 500 dilution) (Molecular Probes, Burlington, ON, Canada), 1% Alexa Fluor 568 phalloidin (Molecular Probes) and 0.2% DRAQ5 (BioStatus Ltd). Coverslips were mounted and the nuclear localization of PKC- α was assessed using confocal microscopy.

Co-immunoprecipitations and Western blot analyses

Adherent BMDM were incubated in the absence or in the presence of 100 U/ml of IFN- γ for the indicated periods of time. Cells were then washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1% Nonidet P-40; 150 mM NaCl; 0.25% deoxycholate) containing proteases and phosphatase inhibitors. PKC- α was immunoprecipitated using a rabbit anti-PKC- α (1 : 500 dilution; Transduction Laboratories, San Jose, CA), and immunocomplexes were resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE; 8% gel) and electroblotted onto Hybond-Enhanced Chemiluminescence (ECL) membranes (Amersham Biosciences, Piscataway, NJ). Immunodetection of STAT1 was achieved by enhanced chemiluminescence using a rabbit anti-STAT1 serum (1 : 1000 dilution; Upstate Laboratories). To detect PKC- α , we used a mouse anti-PKC- α immunoglobulin (IgG2b) (1 : 1000 dilution; Transduction Laboratories).

Results

PI3K and p38 MAPK are involved in the IFN- γ -induced nuclear translocation of PKC- α

In this study, we used PKC- α -GFP as a reporter to investigate the mechanisms by which IFN- γ stimulates the nuclear translocation of PKC- α .²⁰ As shown in Fig. 1, similarly to endogenous PKC- α in BMDM (Fig. 1b,d), the PKC- α -GFP fusion protein translocated to the nucleus of RAW 264.7 cells following stimulation with IFN- γ (Fig. 1a,c). To identify signalling pathways involved in IFN- γ -induced PKC- α nuclear translocation, we incubated RAW264.7 cells, transiently expressing PKC- α -GFP, in the absence or presence of the indicated pharmacological inhibitors prior to stimulation with IFN- γ . As shown in Fig. 2, inhibition of PI3K (with Ly294002) and p38 MAPK (with SB203580) reduced IFN- γ -induced nuclear translocation of PKC- α -GFP to the levels observed in unstimulated cells. By contrast, inhibition of JAK2 (with AG-490), PKC- α/β 1 (with Gö-6976), ERK1/2 (with PD98059), protein tyrosine phosphatases [with BpV(phen)], phospholipase C (with U-73122) and Src-family tyrosine kinases (with PP2) had no significant impact on the nuclear translocation of PKC- α -GFP. These results indicate that IFN- γ -induced nuclear translocation of PKC- α is a process requiring PI3K and p38

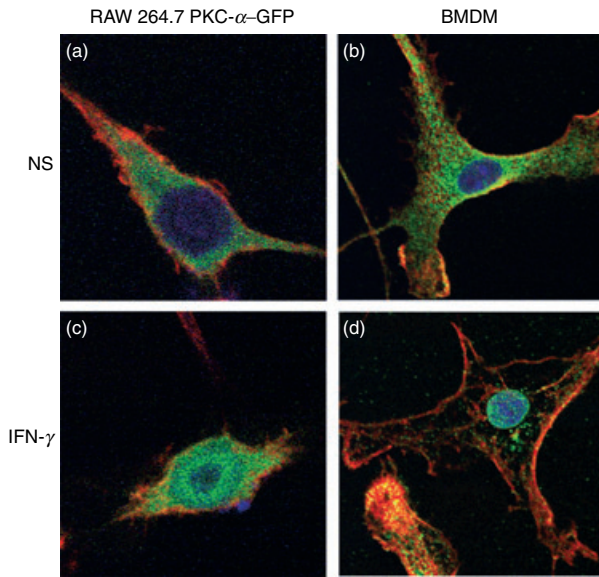


Figure 1. Interferon- γ (IFN- γ) induces protein kinase C- α (PKC- α) nuclear translocation in RAW264.7 cells and in bone marrow-derived macrophages (BMDM). RAW 264.7 cells transfected with PKC- α -green fluorescent protein (GFP) conjugates (a and c) or BMDM (b and d) were incubated in the absence (a and b) or presence (c and d) of 100 U/ml of IFN- γ for 2 hr and prepared for immunofluorescence analyses as described in the *Materials and methods* (red, F-actin; green, PKC- α ; blue, DNA). NS, non-stimulated.

MAPK, independently of JAK2 activity. Of note, under the conditions used, the JAK2 inhibitor AG-490 completely abrogated IFN- γ -induced STAT1 tyrosine phos-

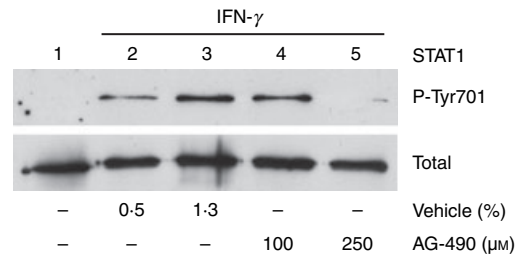


Figure 3. Interferon- γ (IFN- γ)-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) on Tyr701 requires Janus activated kinase 2 (JAK2) activity. Adherent bone marrow-derived macrophages (BMDM) were incubated for 30 min in the absence or the presence of 100 or 250 μ M AG-490 before the addition of 100 U/ml of IFN- γ for 15 min. Vehicle was 100% dimethyl sulphoxide (DMSO). Phosphorylation of STAT1 on Tyr701 (P-Tyr701) was determined by Western blotting, as described in the *Materials and methods*. Similar results were obtained in two independent experiments.

phorylation (Fig. 3). Phosphorylation of PKC on conserved serine and threonine residues of the catalytic domain is necessary for catalytic competence and proper intracellular localization.^{30,31} We thus determined whether PI3K and p38 MAPK are involved in the phosphorylation of PKC- α by IFN- γ . BMDM were incubated in the absence or in the presence of inhibitors for PI3K (Ly294002), p38 MAPK (SB203580), or JAK2 (AG-490) prior to the addition of 100 U/ml IFN- γ for 30 min, and PKC- α phosphorylation was assessed using Western blot analysis. As shown in Fig. 4, none of the inhibitors tested

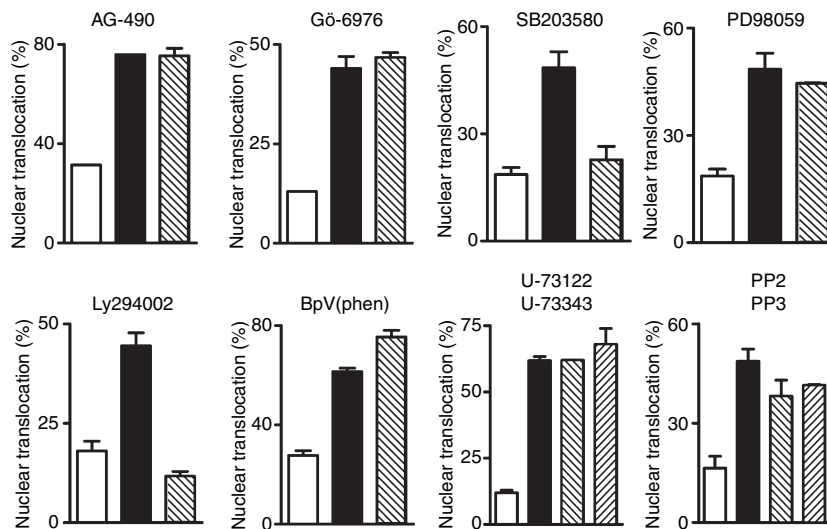


Figure 2. Effect of pharmacological inhibitors on the interferon- γ (IFN- γ)-induced nuclear translocation of protein kinase C- α (PKC- α). Adherent RAW 264.7 cells were transfected with PKC- α -green fluorescent protein (GFP) conjugate, incubated with the various pharmacological inhibitors at the concentrations indicated in the *Materials and methods* for 1 hr, stimulated with 100 U/ml of IFN- γ for 2 hr and then prepared for confocal microscopy analysis. At least 30 PKC- α -GFP transfected cells were counted for each inhibitor tested and the results are representative of three independent experiments. The percentage of nuclear translocation represents the ratio of nuclear PKC- α -positive cells/total transfected cells. White bars, unstimulated control; black bars, IFN- γ -stimulated cells; hatched bars, cells treated with inhibitors prior to IFN- γ stimulation.

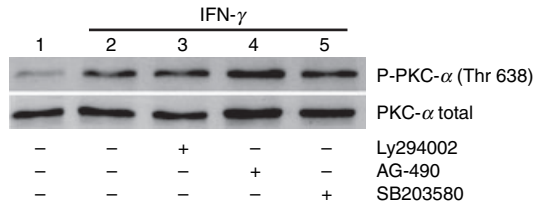


Figure 4. Interferon- γ (IFN- γ)-induced phosphorylation of protein kinase C- α (PKC- α) is independent of Janus activated kinase 2 (JAK2), phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK). Adherent bone marrow-derived macrophages (BMDM) were incubated in the absence or the presence of AG-490 (250 μ M), Ly294002 (100 μ M), or SB203580 (10 μ M) for 1 hr prior to the addition of 100 U/ml of IFN- γ for 30 min. The presence of phosphorylated PKC- α (P- PKC- α ; top panel) and total PKC- α (PKC- α total; bottom panel) in cell extracts was detected by Western blotting, as described in the *Materials and methods*. Similar results were obtained in two independent experiments.

affected PKC- α phosphorylation in IFN- γ -stimulated BMDM. These results indicate that similarly to its IFN- γ -induced nuclear translocation, PKC- α phosphorylation is independent of JAK2 activity. However, PKC- α phosphorylation was also independent of PI3K and p38 MAPK, suggesting that IFN- γ -induced phosphorylation and nuclear translocation of PKC- α are mediated by distinct mechanisms.

Activation of PI3K and p38 MAPK by IFN- γ in BMDM

As IFN- γ -induced PKC- α nuclear translocation involves PI3K and p38 MAPK, we further characterized the mechanisms by which these kinases are activated by IFN- γ in

BMDM. As shown in Fig. 5a, p38 MAPK phosphorylation was not significantly affected by the presence of inhibitors of either JAK2 (AG-490) or PI3K (Ly294002), indicating that these kinases are not involved in p38 MAPK activation by IFN- γ . To assess PI3K activation, we determined the phosphorylation status of Akt on serine 473 using Western blotting. Expectedly, IFN- γ -stimulated phosphorylation of Akt on serine 473 was inhibited by the PI3K inhibitor, Ly294002, but it was not affected by the presence either of the JAK2 inhibitor, AG-490, or of the p38 MAPK inhibitor, SB203580 (Fig. 5b). Collectively, our results indicate that in BMDM, activation of PI3K and p38 MAPK by IFN- γ occurs through distinct mechanisms, independently of JAK2 activity.

IFN- γ -induced type IV *CIITA* and *MHC II* gene expression require PI3K but not p38 MAPK

We previously reported that PKC- α activity regulates IFN- γ -induced *CIITA* and *MHC II* gene expression by modulating the ability of IRF-1 to transactivate the *CIITA* promoter IV.²⁰ Consistent with these observations, IFN- γ -stimulated type IV *CIITA* and *MHC II* (I-A) gene expression were strongly inhibited by the PKC- α inhibitor, Gö-6976 (Fig. 6a). We next determined whether PI3K and p38 MAPK contribute to the expression of type IV *CIITA* and *MHC II* in IFN- γ -stimulated BMDM. To this end, we compared the effect of the PI3K inhibitor, Ly294002, and the p38 MAPK inhibitor, SB203580, on IFN- γ -induced type IV *CIITA* and *MHC II* gene expression. We included the JAK2 inhibitor, AG-490, as a control. As expected, inhibition of JAK2 completely abrogated the expression of both type IV *CIITA* and *MHC II*. Similar results were obtained with the PI3K inhibitor, Ly294002, indicating

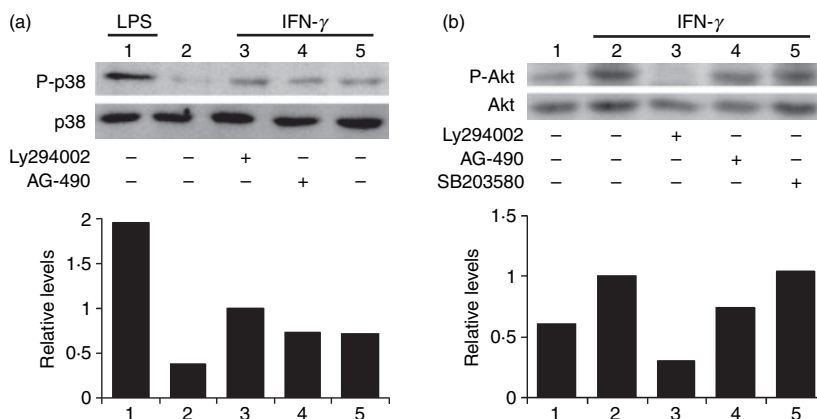


Figure 5. Interferon- γ (IFN- γ) induces p38 mitogen-activated protein kinase (MAPK) and Akt phosphorylation in bone marrow-derived macrophages (BMDM). Adherent BMDM were incubated in the absence or the presence of AG-490 (250 μ M), Ly294002 (100 μ M), or SB203580 (10 μ M) for 1 hr before the addition of 100 U/ml of IFN- γ for 5 min (a) or 30 min (b). Controls consisted of BMDM incubated for 15 min with 100 ng/ml of lipopolysaccharide (LPS). Cell extracts were analyzed by Western blotting for the presence of (a) phosphorylated (P-p38; top panel) and total p38 MAPK (bottom panel) and (b) phosphorylated (P-Akt; top panel) and total Akt (bottom panel). Similar results were obtained in two independent experiments.

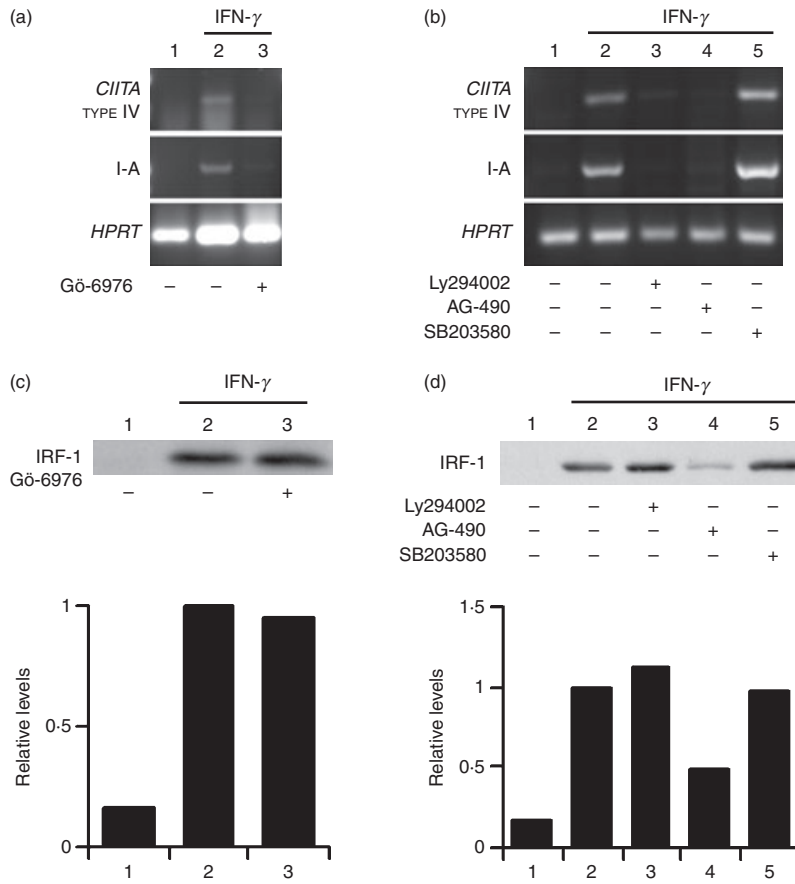


Figure 6. Interferon- γ (IFN- γ)-induced class II transactivator (*CIITA*) and major histocompatibility complex (*MHC*) class II (*I-A*) gene expression require phosphatidylinositol 3-kinase (PI3K), but not p38 mitogen-activated protein kinase (MAPK) (a and b). Bone marrow-derived macrophages (BMDM) were incubated for 1 hr in the absence or the presence of G \ddot{o} -6976 (1 μ M) (a), Ly294002 (100 μ M), AG-490 (250 μ M) or SB203580 (10 μ M) (b), and stimulated for 6 hr with 100 U/ml of IFN- γ . Total RNA was extracted and subjected to reverse transcription-polymerase chain reaction (RT-PCR) using specific primers for type IV *CIITA*, *MHC II* (*I-A*) and *Hypoxanthine-guanine phosphoribosyl transferase* (*HPRT*) (c and d). IFN- γ -induced IFN regulatory factor-1 (IRF-1) protein expression is independent of protein kinase C (PKC), PI3K and p38 MAPK. Adherent BMDM were incubated for 1 h with or without G \ddot{o} -6976 (1 μ M) (c), Ly294002 (100 μ M), AG-490 (250 μ M) or SB203580 (10 μ M) (d), and stimulated for 4 hr with IFN- γ . IRF-1 expression was assessed by Western blotting, as described in the Materials and methods. Similar results were obtained in two independent experiments.

that PI3K plays an important role in the regulation of IFN- γ -induced type IV *CIITA* and *MHC II* gene expression (Fig. 6b). By contrast, as previously reported,¹⁹ the p38 MAPK inhibitor, SB203580, had no significant effect on type IV *CIITA* and *MHC II* gene expression.

Together with STAT1, IRF-1 plays an essential role in the regulation of IFN- γ -induced type IV *CIITA* expression.^{32,33} It was thus of interest to determine whether inhibition of type IV *CIITA* expression by the PKC- α and the PI3K inhibitors was the consequence of defective IRF-1 expression. Consistent with its dependence on the JAK-STAT pathway,^{13,34} IRF-1 expression was inhibited by the JAK2 inhibitor, AG-490 (Fig. 6d). However, our results indicate that PKC- α , PI3K and p38 MAPK are not involved in the regulation of IFN- γ -induced IRF-1 expression (Fig. 6c,d).

IFN- γ induces the phosphorylation of STAT1 on serine 727 and its association with PKC- α

In addition to tyrosine phosphorylation, the complete transcriptional activity of STAT1 requires the phosphorylation of serine 727, and members of the PKC family have been implicated in this response.^{10,14,25} To verify the possible requirement for PKC- α , we assessed, using Western blotting, the IFN- γ -induced phosphorylation of STAT1 on serine 727 in the presence or the absence of the PKC- α inhibitor, G \ddot{o} -6976. We also included the PI3K inhibitor, Ly294002, the p38 MAPK inhibitor, SB203580, and the JAK2 inhibitor, AG-490. As shown in Fig. 7, IFN- γ -induced phosphorylation of STAT1 on serine 727 in BMDM was impaired by the PKC- α inhibitor G \ddot{o} -6976 but remained unaffected by the inhibitors of PI3K, p38

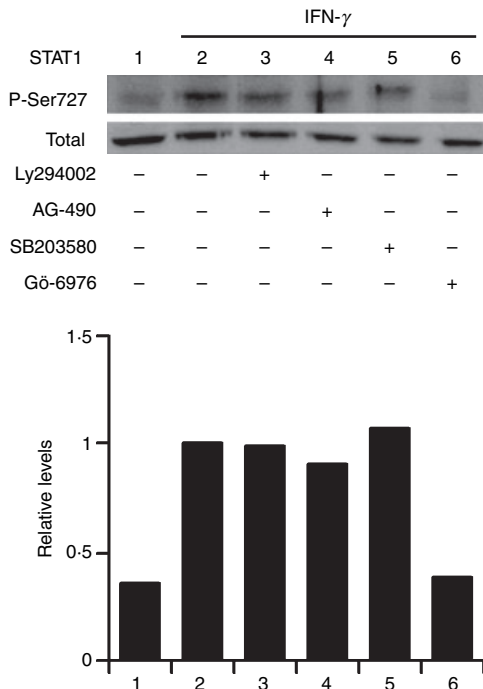


Figure 7. Interferon- γ (IFN- γ)-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) on serine 727 requires the activity of protein kinase C- α (PKC- α) but remains unaffected by the inhibition of phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK). Adherent bone marrow-derived macrophages (BMDM) were incubated for 30 min in the absence or the presence of Ly294002 (100 μ M), AG-490 (250 μ M), SB203580 (10 μ M), or Gö-6976 (1 μ M) before the addition of 100 U/ml of IFN- γ for 30 min. Phosphorylation of STAT1 on serine 727 was determined by Western blotting, as described in the Materials and methods. Similar results were obtained in two independent experiments.

MAPK and JAK2. The finding that Gö-6976 inhibited the IFN- γ -stimulated phosphorylation of STAT1 on serine 727 led us to determine whether PKC- α associates with STAT1 in response to IFN- γ . BMDM were stimulated with IFN- γ and, at various time-points, cells lysates were immunoprecipitated with an antiserum against PKC- α . Immunoprecipitated proteins were separated by SDS-PAGE and the presence of STAT1 was assessed by Western blotting. As shown in Fig. 8, PKC- α and STAT1 associated in an IFN- γ -dependent manner in BMDM.

Discussion

Macrophage responses to IFN- γ are mediated, to a large extent, by the JAK-STAT pathway.^{6,7} Nevertheless, the fact that IFN- γ regulates the expression of a large number of genes in cells lacking STAT1, and activates PI3K and ERK1/2 in cells deficient in either JAK1 or JAK2,^{7,10} is consistent with the notion that additional signalling pathways contribute to the modulation of cellular responses

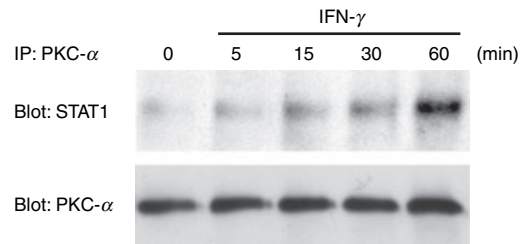


Figure 8. Interferon- γ (IFN- γ) induces the association of protein kinase C- α (PKC- α) with signal transducer and activator of transcription 1 (STAT1). Adherent bone marrow-derived macrophages (BMDM) were incubated in the absence or presence of 100 U/ml of IFN- γ for the indicated periods of time. PKC- α was immunoprecipitated and the presence of STAT1 in immunoprecipitates was determined by Western blot analysis, as described in the Materials and methods. The blot was stripped and reprobed with an anti-PKC- α immunoglobulin (IgG2b) to ensure that equal amounts of PKC- α were immunoprecipitated in each sample. Similar results were obtained in two independent experiments.

by IFN- γ .^{10,35} The participation of PKC isoenzymes in the regulation of IFN- γ -induced responses has been reported in various systems.^{14–18,20} In macrophages, we previously showed that PKC- α translocates to the nucleus in response to IFN- γ , and that PKC- α activity regulates IFN- γ -induced *MHC II* expression by modulating the ability of IRF-1 to transactivate the *CIITA* promoter IV.²⁰ In this study, we found that both PI3K and p38 MAPK are involved in the activation of PKC- α in macrophages stimulated with IFN- γ , and we further characterized the roles of these kinases in the regulation of IFN- γ -induced responses in macrophages.

Activation of PI3K by both type I and type II IFNs has been reported in several cell types.^{8,10,14,17,36,37} One of the main consequences of PI3K activation is the phosphorylation of STAT1 on serine 727, which has been shown to be mediated by either PKC- δ in human acute promyelocytic leukemia cells¹⁴ or PKC- ϵ in rat mesangial cells.¹⁷ In BMDM, we found that IFN- γ -induced STAT1 serine 727 phosphorylation required PKC- α but was independent of PI3K. This is consistent with the finding that PI3K had no effect on the phosphorylation of PKC- α . The finding that PI3K was required for IFN- γ -stimulated nuclear translocation of PKC- α suggests the existence of at least two distinct steps in the regulation of PKC- α activation in IFN- γ -stimulated BMDM. Hence, the initial step of PKC- α maturation (phosphorylation)³⁰ is regulated by a yet-unidentified pathway(s), whereas the targeting step (nuclear localization) requires PI3K.

Although both type I and type II IFNs activate p38 MAPK, the role of this kinase in the modulation of IFN responses remains poorly understood. Whereas p38 MAPK is required for STAT1-driven gene expression in response to IFN- α and IFN- β , it plays an important role in the regulation of the expression of chemokines and

cytokines in IFN- γ -stimulated macrophages.^{11,19} Consistent with previous reports,^{19,38,39} we found that, in BMDM, p38 MAPK is not required for IFN- γ -induced phosphorylation of STAT1 on serine 727 or for the expression of IRF-1, *CIITA* and *MHC II*. These events are thus clearly distinct from IFN- γ -induced chemokine and cytokine expression. As p38 MAPK was involved in the nuclear translocation of PKC- α , it is tempting to speculate that PKC- α may participate in the regulation of chemokine and cytokine expression. As p38 MAPK was not involved in the initial step of PKC- α activation (phosphorylation) by IFN- γ , the mechanism(s) by which p38 MAPK modulates PKC- α nuclear translocation remains to be identified.

We previously reported that PKC- α activity regulates IFN- γ -induced *MHC II* expression by modulating the ability of IRF-1 to transactivate the *CIITA* promoter IV.²⁰ Consistent with these previous findings, IFN- γ -stimulated type IV *CIITA* and *MHC II* expression was strongly inhibited by Gö-6976. Our data also indicated that whereas PI3K and p38 MAPK regulated IFN- γ -induced nuclear translocation of PKC- α , they had no significant impact on IFN- γ -stimulated PKC- α phosphorylation and on STAT1-mediated IRF-1 expression. In the light of these observations, it was of interest to determine the contribution of these pathways to the expression of type IV *CIITA* and *MHC II* in IFN- γ -stimulated BMDM. As recently shown,¹⁹ inhibition of p38 MAPK had no significant effect on the gene expression of either *CIITA* or *MHC II*. By contrast, inhibition of PI3K strongly impaired the expression of type IV *CIITA* and *MHC II* genes, indicating an important role for PI3K in the modulation of IFN- γ -induced responses in BMDM. Taken together, these data suggest that although PKC- α activity is essential, its nuclear translocation is not required for IFN- γ -stimulated type IV *CIITA* and *MHC II* gene expression, and point to the existence of yet-unidentified regulatory mechanisms involving PI3K. Clearly, additional investigation will be required to understand in greater detail the regulation of *CIITA* expression.

In contrast to the expression of IRF-1, *CIITA* and *MHC II*, which depends on the JAK-STAT pathway, activation of PKC- α , PI3K and p38 MAPK occurred independently of JAK2 in IFN- γ -stimulated BMDM. These findings raise the issue of how IFN- γ activates signalling pathways independently of JAK. A recent study provided evidence that IFN- γ induces a rapid physical association between IFN- γ receptor 1 and the adaptor protein MyD88, as well as the formation of a signalosome containing p38 MAPK, mixed-lineage kinase (MLK) and c-Jun N-terminal kinase-interacting protein (JIP)-1.¹¹ A similar mechanism may account for the activation of PI3K, which was found to associate with MyD88 in LPS-stimulated macrophages.⁴⁰ Whether this is also the case for the activation of PKC- α by IFN- γ remains to be

demonstrated. Clearly, additional investigation will be required to address the potential role of MyD88 in mediating the activation of PKC- α and PI3K in macrophages stimulated by IFN- γ .

The finding that IFN- γ -stimulated phosphorylation of STAT1 on serine 727 is inhibited by Gö6976 suggests that PKC- α acts as a kinase for this phosphorylation event and is consistent with the observation that IFN- γ induces the association of PKC- α with STAT1. A similar role for PKC- δ has been reported in acute promyelocytic leukemia cells, where PKC- δ was shown to associate with and to phosphorylate STAT1.¹⁴ In contrast to the majority of nuclear proteins, PKC isoenzymes lack a canonical nuclear localization signal,⁴¹ indicating that other mechanisms are involved in the transport process. As phosphorylated STAT1 dimers translocate to the nucleus, it is possible that PKC- α uses STAT1 as a shuttle to access the nucleus.

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Disclosures

The authors declare no conflict of interest or financial interests.

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