European Journal of Immunology

Signaling events involved in cytokine and chemokine production induced by secretory phospholipase A_2 in human lung macrophages

Francescopaolo Granata[1], Annunziata Frattini[1], Stefania Loffredo[1], Annalisa Del Prete[2], Silvano Sozzani[2,3], Gianni Marone[1,4] and Massimo Triggiani[1,4]

[1] Division of Clinical Immunology and Allergy, University of Naples Federico II, Naples, Italy
[2] Institute of Pharmacological Research "Mario Negri", Milan, Italy
[3] Section of General Pathology and Immunology, University of Brescia, Brescia, Italy
[4] Center for Basic and Clinical Immunology Research (CISI), Naples, Italy

Secretory phospholipases A_2 ($sPLA_2$) are enzymes released during inflammatory reactions. These molecules activate immune cells by mechanisms either related or unrelated to their enzymatic activity. We examined the signaling events activated by group IA (GIA) and group IB (GIB) $sPLA_2$ in human lung macrophages leading to cytokine/chemokine production. $sPLA_2$ induced the production of cytokines (TNF-α, IL-6 and IL-10) and chemokines (CCL2, CCL3, CCL4 and CXCL8), whereas no effect was observed on IL-12, CCL1, CCL5 and CCL22. $sPLA_2$ induced the phosphorylation of the MAPK p38 and ERK1/2, and inhibition of these kinases by SB203580 and PD98059, respectively, reduced TNF-α and CXCL8 release. Suppression of $sPLA_2$ enzymatic activity by a site-directed inhibitor influenced neither cytokine/chemokine production nor activation of MAPK, whereas alteration of $sPLA_2$ secondary structure suppressed both responses. GIA activated the phosphatidylinositol 3-kinase (PI3 K)/Akt system and a specific inhibitor of PI3 K (LY294002) reduced $sPLA_2$-induced release of TNF-α and CXCL8. GIA promoted phosphorylation and degradation of IκB and inhibition of NF-κB by MG-132 and 6-amino-4-phenoxyphenylethylamino-quinazoline suppressed the production of TNF-α and CXCL8. These results indicate that $sPLA_2$ induce the production of cytokines and chemokines in human macrophages by a non-enzymatic mechanism involving the PI3 K/Akt system, the MAPK p38 and ERK1/2 and NF-κB.

Received 4/10/05
Revised 10/3/06
Accepted 4/5/06

[DOI 10.1002/eji.200535567]

Key words: Chemokines · Cytokines · Macrophages · Secretory phospholipases A_2 · Signal transduction

Supporting information for this article is available at http://www.wiley-vch.de/contents/jc_2040/2006/35567_s.pdf

Correspondence: Massimo Triggiani, Division of Clinical Immunology and Allergy, University of Naples Federico II, 80131 Naples, Via S. Pansini 5, Italy
Fax: +39-081-3722607
e-mail: triggian@unina.it

Abbreviations: **BPB:** bromophenacyl bromide · **GIA:** group IA $sPLA_2$ · **GIB:** group IB $sPLA_2$ · **HLM:** human lung macrophages · **IKK:** IκB kinase · **MDC:** Macrophage-derived chemokine · **Quinazoline-11q:** 6-amino-4-phenoxyphenylethylamino-quinazoline · **RPA:** RNase protection assay · **$sPLA_2$:** secretory phospholipases A_2

Introduction

Secretory phospholipases A_2 ($sPLA_2$) are emerging as a novel class of inflammatory mediators [1]. These molecules are released in large quantities in the blood and biological fluids of patients with inflammatory, allergic and autoimmune disorders. Furthermore, several $sPLA_2$ possess potent proinflammatory activities both *in vitro* and *in vivo* [2].

Secretory PLA_2 catalyze the hydrolysis of membrane phospholipids to produce free arachidonic acid and lysophospholipids. Indeed, several studies have shown that these enzymes contribute to the biosynthesis of lipid mediators in inflammatory cells [3]. However, $sPLA_2$ exert other biological effects relevant to the initiation and regulation of inflammatory and immune responses. These include the induction of exocytosis [4, 5], the production of cytokines [4, 6, 7], chemokines [7, 8] and NO [9, 10], the expression of surface molecules [7, 11, 12], the recruitment of inflammatory cells [13, 14] and the enhancement of cell survival [15, 16]. Most of these effects are unrelated to $sPLA_2$ enzymatic activity and involve the interaction of $sPLA_2$ with such membrane targets as heparin-containing proteoglycans or specific receptors such as the M-type [17].

We have previously shown that both human and venom $sPLA_2$ are potent stimuli for cytokine production in human lung macrophages (HLM) [4, 18], blood monocytes [6] and eosinophils [7]. Other groups have shown that $sPLA_2$ induce the release of CXCL8/IL-8 [19] from human neutrophils and of various CXC chemokines from endothelial cells [8]. The production of cytokines and chemokines in human inflammatory cells requires activation of gene transcription and protein synthesis [20]. These events are initiated and regulated by a number of intracellular kinases, which in turn drive membrane signals to nuclear factors, the most important of which is NF-κB [21]. Among signaling kinases, the phoshatidylinositol 3-kinase (PI3 K) and the MAPK p38 and extracellular-regulated kinases (ERK) 1/2 play a prominent role in the regulation of immune cell functions [22–24]. For example, they are involved in the pathways leading to cytokine production in human and murine macrophages stimulated with LPS or PMA [25, 26]. However, the involvement of these kinases in the intracellular signaling activated by $sPLA_2$ in human macrophages and their role in $sPLA_2$-induced production of cytokines and chemokines has not been defined yet. In this study we have examined several signaling events involved in the production of cytokines and chemokines in primary HLM activated by two distinct $sPLA_2$.

Results

Activation of cytokine and chemokine production in HLM by $sPLA_2$

In the first group of experiments, we examined the profile of cytokines and chemokines produced by HLM stimulated with $sPLA_2$. In this study we used group IA (GIA) $sPLA_2$ which is the most potent $sPLA_2$ activating HLM and human monocytes [4, 6]. Fig. 1A shows that GIA induced a concentration-dependent release of TNF-α, IL-6 and IL-10 from HLM. In these experiments, GIA did not induce IL-12 release, even when the cells were preincubated (37°C, 1 h) with IFN-γ (1000 U/mL). Under the same conditions, HLM stimulated with LPS (1 μg/mL) released significant amounts of IL-12 (LPS: 122 ± 16 pg/mg protein; unstimulated cells: 22 ± 3 pg/mg protein; $p<0.01$). Incubation of HLM with GIA also resulted in the release of the chemokines CCL2/monocyte chemoattractant protein-1 (MCP-1), CCL4/macrophage inflammatory protein-1β (MIP-1β) and CXCL8/IL-8 (Fig. 1B). No effect of GIA was observed on CCL22/macrophage-derived chemokine (MDC) release. Interestingly, HLM produced this chemokine when stimulated with 50 ng/mL IL-4 (IL-4: 7.73 ± 1.79 ng/mg protein; unstimulated cells 2.22 ± 0.54 ng/mg protein; $p<0.01$) [27]. These experiments indicated that GIA elicit the production of several cytokines (TNF-α > IL-6 > IL-10) and chemokines (CXCL8 >> CCL4 > CCL2) in HLM. Kinetics

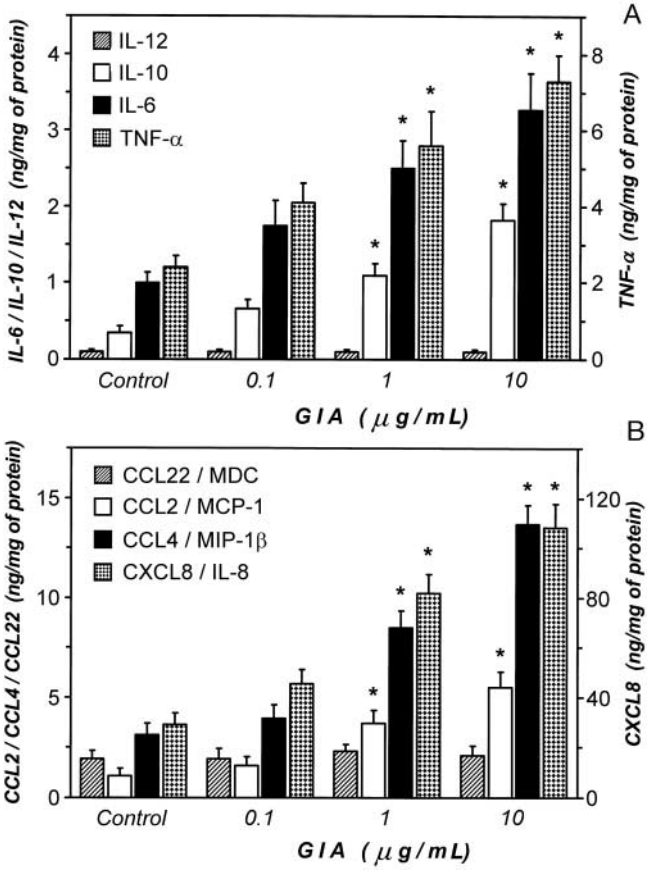

Figure 1. Effect of GIA $sPLA_2$ on cytokine and chemokine release from HLM. The cells were incubated (37°C, 6 or 24 h) with the indicated concentrations of GIA. (A) Cytokine (TNF-α, IL-6, IL-10 and IL-12) and (B) chemokine (CCL2/MCP-1, CCL4/MIP-1β, CCL22/MDC and CXCL8/IL-8) release was determined in the supernatants by ELISA. The data are the mean ± SE of six experiments. *$p<0.05$ *vs.* control

experiments revealed that the optimal secretion of cytokines and chemokines was reached at different time points after the addition of GIA. TNF-α, IL-6, CCL4 and CXCL8 release was maximum between 4 and 6 h of incubation, whereas the release of IL-10 and CCL2 peaked between 18 and 24 h (data not shown).

It has been previously shown that the release of cytokines induced by $sPLA_2$ is associated with the activation of their gene transcription [4, 6, 7]. To verify that this also occurred for $sPLA_2$-induced chemokine secretion, we examined chemokine mRNA expression by RNase protection assay (RPA). Fig. 2 shows a representative experiment out of three in which HLM were cultured for 6 h with medium alone (Ctr) or with the indicated concentrations of GIA or LPS. HLM constitutively express mRNA for CCL1/I-309, CCL3/MIP-1α, CCL4 and CXCL8. As expected on the basis of the results presented in Fig. 1B, GIA induced a concentration-dependent increase of the expression of CCL2, CCL4 and CXCL8 mRNA. In addition, CCL3 mRNA was also increased, whereas GIA had no effect on the expression of CCL1 and CCL5/RANTES. LPS, used as positive control, up-regulated the mRNA for all the chemokines examined. Data obtained from three different experiments indicated that the GIA, at the maximal concentration used (10 μg/mL), induced an enhancement of mRNA expression ranging from 1.9- to 3.7-fold for CCL2, from 4.3- to 6.2-fold for CCL3, from 3.1- to 5.5-fold for CCL4, and from 2.5- to 4.2-fold for CXCL8.

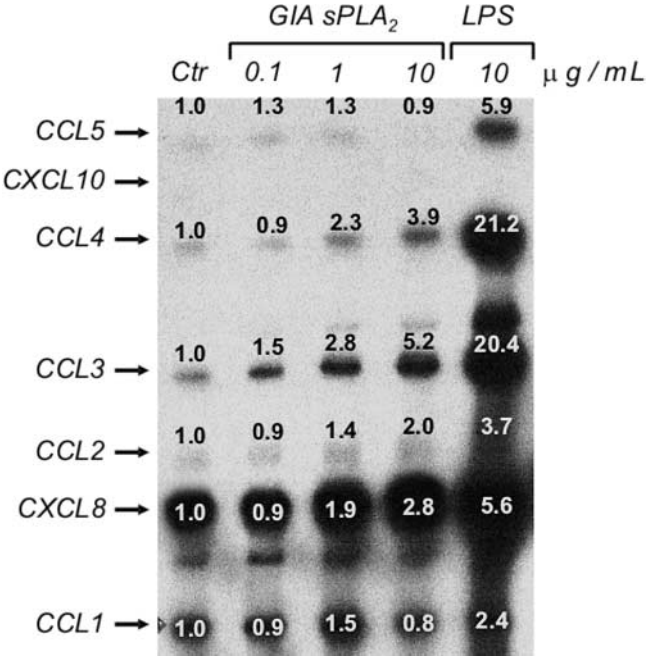

Figure 2. Effect of GIA $sPLA_2$ on chemokine mRNA expression in HLM. The cells were incubated (37°C, 6 h) with the indicated concentrations of GIA or LPS. Total RNA was extracted and expression of the various chemokine mRNA (CCL1/I-309, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL8/IL-8, CXCL10/IP-10) was evaluated by RPA as described in the Materials and methods. The expression enhancement of the various mRNA is indicated by the number reported on each sample representing the fold increase over untreated cells (Ctr). The experiment shown is representative of three.

Role of p38 and ERK1/2 in $sPLA_2$-induced production of cytokines and chemokines in HLM

Activation of p38 and ERK1/2 is crucial for IL-6 and TNF-α production induced by LPS in alveolar macrophages [25]. To clarify the intracellular mechanisms by which $sPLA_2$ promote cytokine and chemokine production in macrophages, we initially examined whether GIA activates p38 and ERK1/2. HLM were stimulated with GIA (5 μg/mL) for various times and the phosphorylation of p38 and ERK1/2 was evaluated using anti-phospho-specific Ab. Fig. 3A shows that GIA induced a time-dependent phosphorylation of both p38 (first gel) and ERK1/2 (third gel). Densitometric analysis of the signal intensity obtained in four different experiments revealed that the effect of GIA on both kinases was evident after 5 min of incubation, peaked at 90 min and persisted up to 120 min (Fig. 3B).

To verify that activation of MAPK was required for cytokine and chemokine production, we evaluated the effect of specific inhibitors of p38 (SB203580) [28, 29] and of ERK1/2 (PD98059) [29, 30] on the release of the major cytokine (TNF-α) and chemokine (CXCL8) produced by $sPLA_2$-stimulated HLM. Preliminary experiments showed that complete inhibition of p38 activation occurred with 30 μM SB203580, whereas ERK1/2 phosphorylation was completely blocked with 50 μM PD98059 (data not shown). The cells were preincubated (37°C, 1 h) with increasing concentrations of SB203580 (3–30 μM), PD98059 (10–50 μM) or with a combination of the two inhibitors (30 μM SB203580 + 50 μM PD98059) before stimulation with GIA. Both SB203580 and PD98059 inhibited GIA-induced release of TNF-α (Fig. 3C) and CXCL8 (Fig. 3D) in a concentration-dependent fashion. The inhibitory effect of the two compounds alone was slightly more evident on TNF-α release (30 μM SB203580, 13.4% of the maximal response; 50 μM PD98059, 21.5%) than it was on CXCL8 release (30 μM SB203580, 27.5%; 50 μM PD98059, 41.6%). Incubation of HLM with both inhibitors completely blocked the secretion of TNF-α and CXCL8 induced by GIA.

These results demonstrate that GIA activates p38 and ERK1/2 in HLM and that both kinases are involved in the production of TNF-α and CXCL8. Importantly, our data indicate that simultaneous inhibition of these kinases is required to fully suppress cytokine and chemokine production in human macrophages.

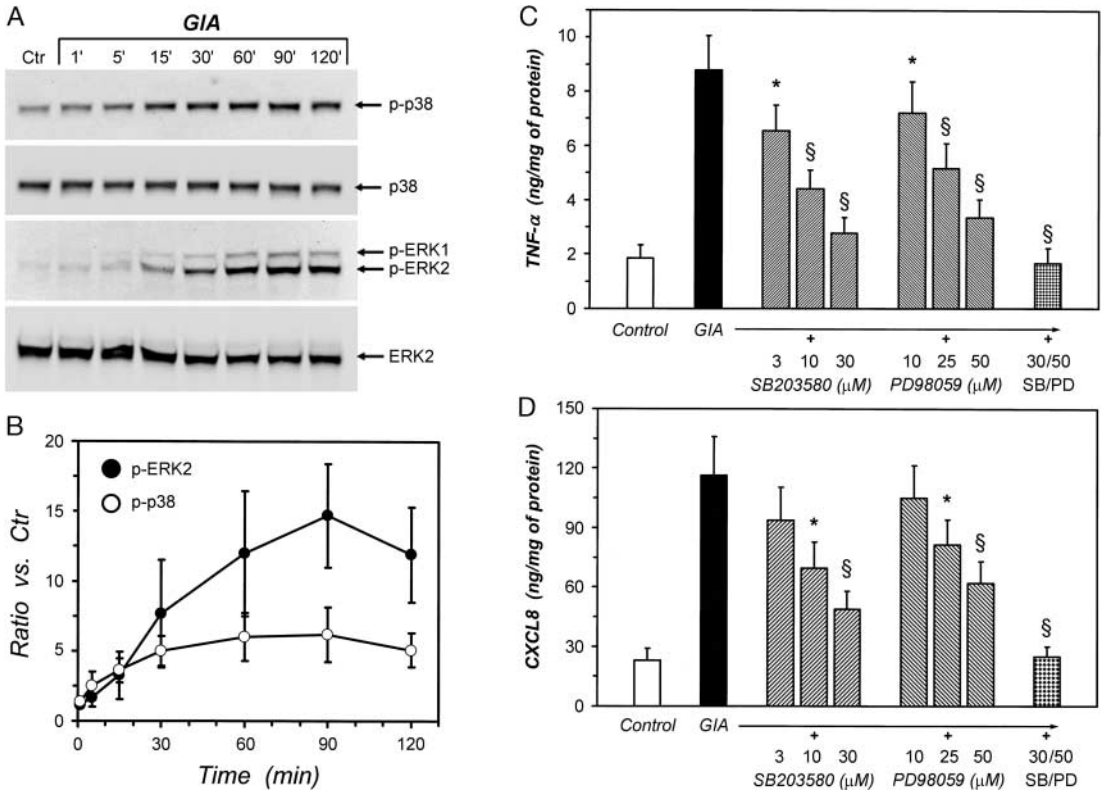

Figure 3. Role of p38 and ERK1/2 in GIA $sPLA_2$-induced activation of TNF-α and CXCL8 release in HLM. (A, B) Kinetics of p38 and ERK1/2 phosphorylation induced by GIA in HLM. The cells were stimulated with GIA (5 μg/mL) for the times indicated. Cellular protein extracts were subjected to Western blot analysis with anti-phospho-p38 (first gel) and -phospho-ERK1/2 (third gel) Ab. Stripped membranes were reblotted with anti-p38 (second gel) and -ERK2 (fourth gel) to confirm equal protein content of each sample. The Western blots shown are representative of four separate experiments. (B) Densitometric analysis of the phosphorylation kinetics of p38 and ERK1/2 was performed by measuring the signal intensity at the various time points and by calculating the ratio *versus* untreated samples (Ctr). Adequate normalization was accomplished by measuring the signal intensity of unphosphorylated forms of p38 and ERK2. The data shown in (B) are the mean ± SE of the four experiments. (C, D) Effect of p38 and ERK1/2 inhibitors on $sPLA_2$-induced TNF-α and CXCL8 release: The cells were preincubated (37°C, 1 h) with SB203580 (3–30 μM), PD98059 (10–50 μM) or both (30 μM SB203580 + 50 μM PD988059) and then stimulated (37°C, 6 h) with GIA (10 μg/mL). TNF-α and CXCL8 release was determined in the supernatants by ELISA. The data are the mean ± SE of four experiments. $^*p<0.05$ *vs.* GIA; § $p<0.01$ *vs.* GIA.

Role of enzymatic activity in $sPLA_2$-induced activation of p38 and ERK1/2 in HLM

Some of the biological responses induced by $sPLA_2$ are mediated by their enzymatic activity [3]. GIA mobilizes fatty acids from membrane phospholipids when added exogenously to intact mammalian cells [6, 31]. Therefore, we evaluated whether the effects of this $sPLA_2$ on HLM were related to its enzymatic activity using GIA inactivated by treatment with bromophenacyl bromide (BPB) or dithiothreitol (DTT). Both procedures irreversibly inhibit more than 98% of GIA enzymatic activity [6, 7]. BPB blocks $sPLA_2$ hydrolytic activity by binding to the catalytic site [32], whereas DTT alters the secondary structure of the molecule by breaking disulfide bridges [33]. Control experiments indicated that BPB- and DTT-inactivated GIA used in these experiments had 1.9% and 1.6% of the enzymatic activity remaining, respectively. HLM stimulated with BPB-inactivated GIA released TNF-α and CXCL8 in amounts comparable to those induced by the active GIA (Supplementary Table 1). In contrast, treatment with DTT completely abolished the capacity of GIA to induce the release of TNF-α and CXCL8. Similarly, when compared to the active $sPLA_2$, BPB-treated GIA was still able to induce the phosphorylation of p38 (Fig. 4A, first gel) and of ERK1/2 (Fig. 4A, third gel) whereas DTT-treated GIA lost its ability to activate both MAPK (Fig. 4B). In these experiments, incubation of HLM with BPB, DTT or DMSO (vehicle) in the absence of GIA did not influence the basal release of TNF-α and CXCL8 nor affected MAPK activation. In addition, under conditions in which DTT completely suppressed PLA_2-induced release, it did not affect the release of TNF-α and CXCL8 induced by LPS (Supplementary Table 1), revealing that DTT does not aspecifically influence the capacity of HLM to produce cytokines and chemokines.

These results indicate that selective blockade of $sPLA_2$ enzymatic activity by an active site-directed inhibitor does not prevent $sPLA_2$-induced effects, whereas alteration of $sPLA_2$ secondary structure by a reducing agent (DTT) abolishes its ability to activate HLM.

Activation of cytokine/chemokine production and p38 and ERK1/2 phosphorylation in HLM by human group IB $sPLA_2$

To verify a human $sPLA_2$ was able to activate cytokine/chemokine production and to induce phosphorylation of p38 and ERK1/2, HLM were stimulated with recombinant human group IB $sPLA_2$ (GIB). It has previously shown that GIB activates HLM independently from its enzymatic activity [18], and it is a ligand of the M-type receptor in a murine system [34]. Fig. 5A shows that GIB induced a concentration-dependent release of TNF-α and CXCL8. Maximal release induced by GIB was comparable to that induced by GIA. In addition, GIB induced phosphorylation of both p38 (Fig. 5B, first gel) and ERK1/2 (Fig. 5B, third gel). As for GIA, SB203580 and PD98059, when used alone, reduced the release of TNF-α and CXCL8 induced by GIB, whereas the combination of the two inhibitors completely blocked this effect. Me-indoxam is the only molecule so far reported to block the interaction of $sPLA_2$ with its specific M-type receptor [34]. Fig. 5B shows that Me-indoxam prevented phosphorylation of both p38 and ERK1/2 induced by GIB.

These results indicate that a $sPLA_2$ of human origin is able to activate TNF-α and CXCL8 release as well as p38 and ERK1/2 phosphorylation, and confirm that both MAPK are involved in $sPLA_2$-induced production of cytokines and chemokines in HLM. Activation of MAPK induced by GIB is inhibited by preventing the interaction of the $sPLA_2$ with its putative membrane target M-type receptor.

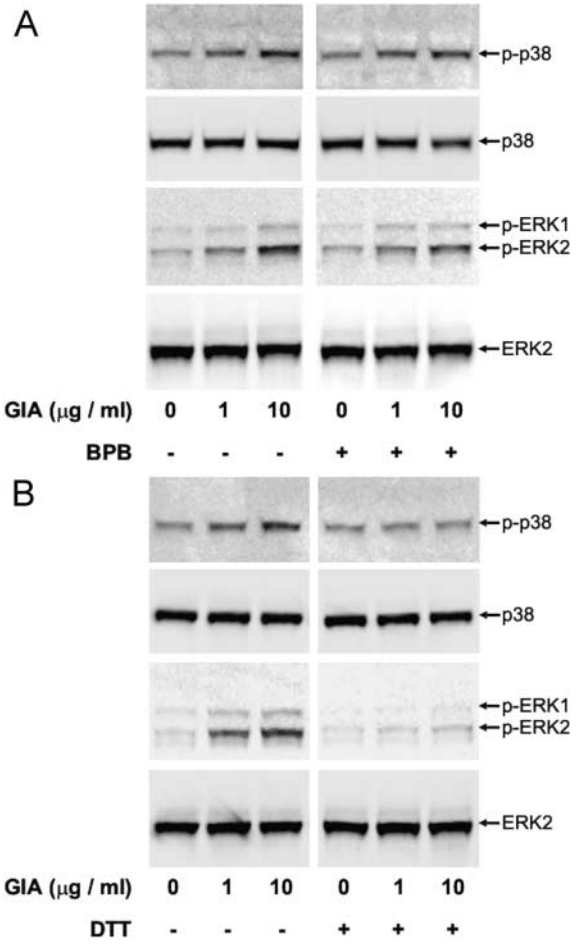

Figure 4. Effect of BPB- and DTT-inactivated GIA $sPLA_2$ on MAPK activation in HLM. The cells were incubated (37°C, 1 h) with the indicated concentrations of DMSO (vehicle)-, BPB- (A) and DTT-treated (B) GIA. Western blot analysis with anti-phospho-p38 (first gels) and -phospho-ERK1/2 (third gel) Ab was performed. Stripped membranes were reblotted with anti-p38 (second gels) and -ERK2 (fourth gels) to confirm equal protein content of each sample. The Western blots shown are representative of three separate experiments.

Role of PI3K in $sPLA_2$-induced production of cytokines and chemokines in HLM

It has been reported that activation of PI3 K is required for TNF-α and IL-6 production in RAW 264.7 macrophages stimulated with LPS or PMA [26]. To investigate whether PI3 K had a role in $sPLA_2$-induced activation of human macrophages, we initially studied the effect of GIA on the phosphorylation of the regulatory subunit (p85α) of PI3 K [35]. Protein extracts from HLM stimulated with GIA (5 μg/mL) were analyzed by immunoprecipitation with the anti-p85α Ab and subsequent immunoblot with anti-phosphotyrosine Ab (4G10). In these experiments a short incubation time (1–15 min) was used since phosphorylation of p85α has been characterized as an early event in the intracellular signaling cascade [24, 36, 37]. Stimulation of HLM with GIA resulted in the phosphorylation of p85α detectable at 1 and 5 min, which was no longer evident at 15 min (Fig. 6A, upper gel). However, even though a considerable amount of p85α was immunoprecipitated (Fig. 6A, lower gel), the phosphorylation signals in these experiments were relatively weak. Therefore, we confirmed that $sPLA_2$ effectively induced activation of PI3 K by exploring the phosphorylation kinetics of Akt. This Ser/Thr kinase is a major downstream target of PI3 K whose activation is necessary and sufficient for the phosphorylation of Akt at the residues Thr308 and Ser473 [38]. Fig. 6B shows the results of a typical

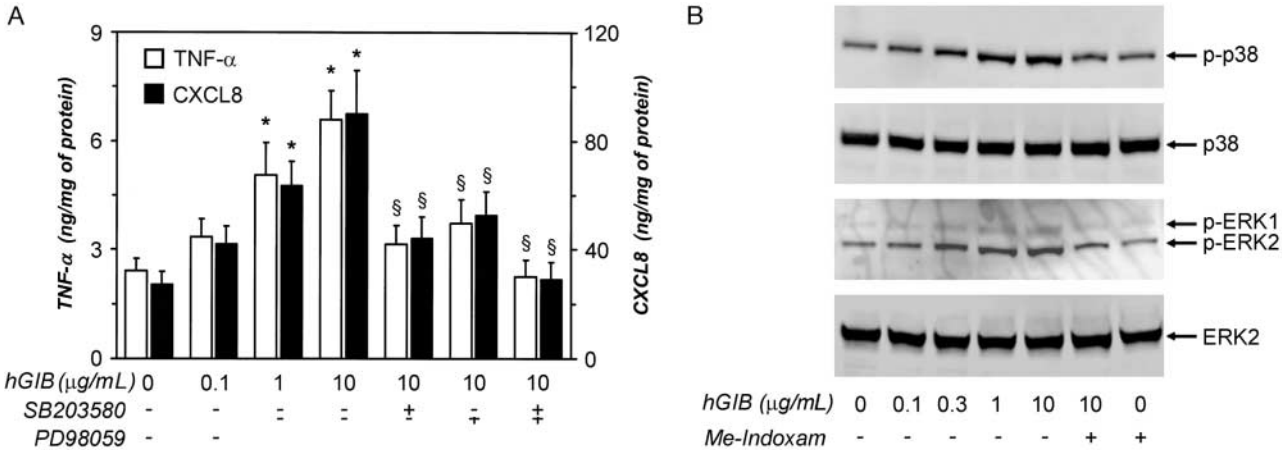

Figure 5. Activation of HLM by human GIB $sPLA_2$. (A) Effect on cytokine and chemokine release: The cells were preincubated (37°C, 1 h) with medium alone, SB203580 (30 μM), PD98059 (50 μM) or both (30 μM SB203580 + 50 μM PD988059) and then stimulated (37°C, 6 h) with the indicated concentrations of GIB. TNF-α and CXCL8 release was determined by ELISA. The data are the mean ± SE of four experiments. *$p<0.05$ *vs.* control; § $p<0.05$ *vs.* GIB. (B) Effect on p38 and ERK1/2 phosphorylation: hGIB (0.1–10 μg/mL) was incubated (37°C, 15 min) with or without Me-indoxam (10 μg/mL) before being added to HLM (37°C, 1 h). Western blot analysis with anti-phospho-p38 (first gel) and -phospho-ERK1/2 (third gel) Ab was performed. Stripped membranes were reblotted with anti-p38 and -ERK2 Ab to confirm equal protein content of each sample. The Western blots shown are representative of three separate experiments.

experiment in which GIA induced a rapid phosphorylation of Akt as detected by two different Ab directed against Akt when it is phosphorylated at Thr308 (first gel) and at Ser473 (second gel). Densitometric analysis performed on three different experiments confirmed that phosphorylation of Akt occurred early, peaking at 5 min and declining to the basal level within 60 min (Fig. 6C). Together these data indicate that GIA induces the early activation of the PI3 K/Akt pathway.

To explore the functional involvement of PI3 K in the production of TNF-α and CXCL8, HLM were preincubated (37°C, 1 h) with increasing concentrations (3–30 μM) of a specific PI3 K inhibitor (LY294002) [29, 39], and then stimulated with GIA (10 μg/mL). Fig. 7A shows that LY294002 concentration-dependently inhibited GIA-induced release of both TNF-α and CXCL8. However, LY294002 (30 μM) almost completely abolished the release of TNF-α (15.8% of the maximal response), whereas it only partially inhibited the release of CXCL8 (49.2% of the maximal response). These results are somehow reminiscent of those obtained with the inhibitors of p38 and ERK1/2 when used alone, suggesting that PI3 K could be involved in the activation of one of these MAPK. To verify this hypothesis, we analyzed the effect of LY294002 (30 μM) on GIA-induced activation of p38 and ERK1/2. The inhibitor blocked the phosphorylation of ERK1/2 (Fig. 7B, third gel), but it had no effect on GIA-induced phosphorylation of p38 (Fig. 7B, first gel). These results indicated that PI3 K is involved in $sPLA_2$-induced production of TNF-α and CXCL8 as well as in the activation of ERK1/2.

Role of NF-κB in $sPLA_2$-induced production of cytokines and chemokines in HLM

NF-κB is a major nuclear factor regulating the transcription of several genes encoding for inflammatory cytokines and chemokines [21]. This dimeric protein is complexed in the cytosol of resting cells with specific inhibitors belonging to the IκB family. Cell activation leads to the phosphorylation of IκB and its subsequent degradation via the ubiquitin-proteasome pathway [40]. This event allows the translocation of NF-κB to the nucleus, where it binds to specific DNA sequences. We tested the hypothesis that $sPLA_2$ activate NF-κB by exploring the kinetics of phosphorylation and degradation of IκB in HLM. Fig. 8A shows that GIA induced both the phosphorylation (upper gel) and the degradation (lower gel) of IκB. As expected, the phosphorylation of IκB preceded its degradation since phosphorylated IκB was evident after 5 min and peaked at 30 min, whereas degradation of IκB initiated after 30 min and was maximum at 120 min (31.2 ± 19.6% of the initial signal remaining) (Fig. 8B). These results are consistent with NF-κB activation and suggest its involvement in $sPLA_2$-induced cytokine/chemokine production. This hypothesis was explored by evaluating the effect of two different inhibitors of NF-κB on GIA-induced TNF-α and CXCL8 release. In these experiments, HLM were preincubated (37°C, 1 h) with the proteasome inhibitor MG-132 (0.3–30 μM) that prevents degradation of IκB [41], and with 6-amino-4-phenoxyphenylethylamino-quinazoline

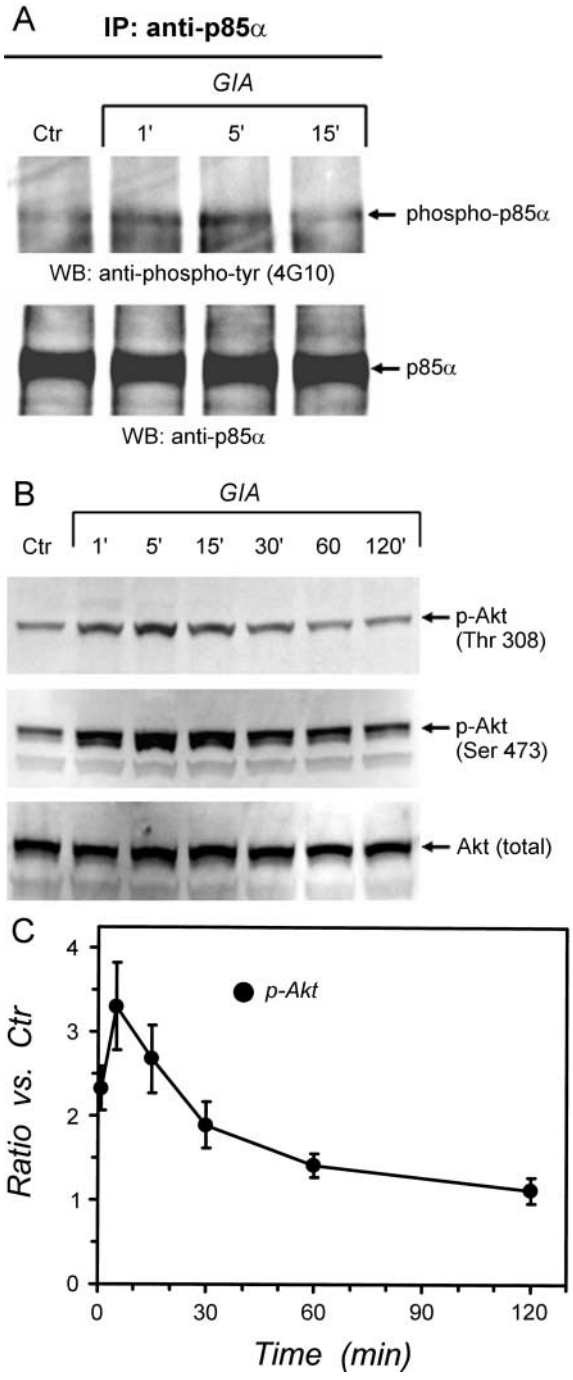

Figure 6. Effect of GIA $sPLA_2$ on the activation of PI3 K/Akt system in HLM. (A) Phosphorylation kinetics of p85α subunit of PI3 K. The cells were stimulated with GIA (5 μg/mL) for the times indicated. Reactions were stopped with the addition of ice-cold PIPES buffer. The cells were microfuged and immediately lysed. Precleared lysates were immunoprecipitated with anti-p85α Ab and subjected to Western blot analysis with anti-phospho-tyrosine Ab (first gel). Stripped membranes were reblotted with anti-p85α Ab (second gel) to confirm equal protein content of each sample. The Western blot shown is representative of three separate experiments. (B, C) Phosphorylation kinetics of Akt: (B) cells were stimulated with GIA (5 μg/mL) for the times indicated. Protein extracts were subjected to Western blot with two different Ab directed against Akt phosphorylated at Thr308 (first gel) or Ser473 (second gel) and, after stripping, with anti-unphosphorylated Akt Ab (third gel) to confirm equal protein content of each sample. The Western blot shown is representative of three separate experiments. (C) Densitometric analysis of the phosphorylation kinetics of Akt was performed by measuring the signal intensity at the various time points and by calculating the ratio *versus* untreated samples (Ctr). Adequate normalization was accomplished by measuring the signal intensity of unphosphorylated form of Akt. The data are the mean ± SE of the three experiments.

(Quinazoline-11q: 0.1–10 μM) that blocks NF-κB transcriptional activation [42], and were then stimulated with GIA (10 μg/mL). The release of both TNF-α (Fig. 8C) and CXCL8 (Fig. 8D) induced by GIA was completely inhibited by the two compounds with IC_{50} values (MG-132: 1.80 ± 0.48 μM for TNF-α and 1.71 ± 0.29 μM for CXCL8; Quinazoline-11q: 0.40 ± 0.10 μM for TNF-α and 0.63 ± 0.16 μM for CXCL8) consistent with those reported for inhibition of NF-κB activity [41–43]. In the same experiments, the two inhibitors completely blocked TNF-α and CXCL8 release induced by 1 μg/mL LPS, used as positive control (data not shown). These results indicate that activation of NF-κB is required for cytokine/chemokine production induced by $sPLA_2$ in HLM.

To gain further insight into the pathways leading to the activation of NF-κB, we analyzed the effect of ERK1/2, p38 and PI3 K inhibitors on $sPLA_2$-induced degradation of IκB. HLM were preincubated (37°C, 1 h) with optimal concentrations of PD98059 (50 μM), SB203580 (30 μM), a combination of PD98059 (50 μM) + SB203580 (30 μM), LY294002 (30 μM) or MG-132 (30 μM) and then stimulated with GIA for 120 min. At the end of the experiments, membranes were incubated with anti-IκB (Fig. 9, upper gel) and, after stripping, with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 9, lower gel) to verify equal protein loading. Degradation of IκB induced by GIA was partially prevented by the PI3 K inhibitor (LY294002), but it was not influenced by the inhibitor of ERK1/2 (PD98059), p38 (SB203580) or their combination. As expected, MG-132 completely blocked GIA-induced degradation of IκB. These results suggest that PI3 K, but not ERK1/2 and p38 kinases, is involved in $sPLA_2$-induced activation of NF-κB.

Discussion

In this study we demonstrate that two distinct $sPLA_2$ induce the production of a wide spectrum of cytokines and chemokines from primary macrophages isolated

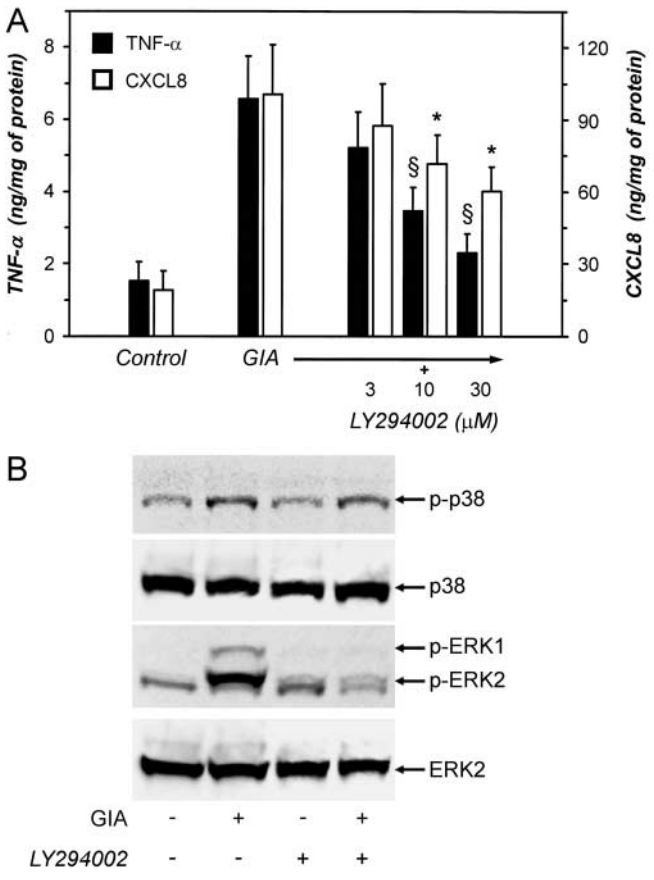

Figure 7. Role of PI3 K in GIA $sPLA_2$-induced TNF-α/CXCL8 release and MAPK activation in HLM. (A) The cells were preincubated (37°C, 1 h) with LY294002 (3–30 μM) and then stimulated (37°C, 6 h) with GIA (10 μg/mL). TNF-α and CXCL8 release was determined in the supernatants by ELISA. The data are the mean ± SE of four experiments. *$p<0.05$ *vs.* $sPLA_2$; § $p<0.01$ *vs.* $sPLA_2$. (B) The cells were preincubated (37°C, 1 h) with LY294002 (30 μM) and then stimulated (37°C, 1 h) with GIA (5 μg/mL).Western blot analysis with anti-phospho-p38 (first gel) and -phospho-ERK1/2 (third gel) Ab was performed. Stripped membranes were reblotted with anti-p38 (second gel) and -ERK2 (fourth gel) Ab to confirm equal protein content of each sample. The Western blots shown are representative of three separate experiments.

from human lung parenchyma. This spectrum comprises both proinflammatory (TNF-α and IL-6) and immunoregulatory (IL-10) cytokines as well as chemokines of the CC (CCL2, CCL3 and CCL4) and CXC (CXCL8) families. The activating properties of $sPLA_2$ are independent from the enzymatic activity and require activation of signaling events involving PI3 K, p38, ERK1/2 and NF-κB.

The profile of cytokines and chemokines induced in HLM indicates that $sPLA_2$ may have a major impact on the initiation/modulation of lung inflammation and immune responses. In particular, the release of large amounts of TNF-α and IL-6 from $sPLA_2$-activated HLM indicates that $sPLA_2$ may enhance the local inflammatory responses, whereas the induction of CXCL8, CCL2, CCL3 and CCL4 suggests that $sPLA_2$ induce the preferential recruitment of neutrophils and monocytes at sites of inflammation. These *in vitro* observations may help explain previous data obtained *in vivo* indicating that administration of $sPLA_2$ to the lung [44] and the skin [45] is characterized by intense inflammatory reactions and by influx of inflammatory cells, predominantly neutrophils and monocytes. The physiological relevance of our data is strengthened by the observation that human GIB $sPLA_2$ effectively activates HLM. This isoform is highly expressed in the human lung [46] and promotes severe inflammation when administered *in vivo* [45].

Interestingly, $sPLA_2$ display a certain degree of selectivity. In fact, not all the cytokines and chemokines produced by human macrophages are up-regulated by $sPLA_2$. For example, GIA has no influence on the production of IL-12, CCL1 and CCL5 that are up-regulated by LPS. Moreover, GIA did not induce the release of CCL22, which is induced by IL-4. These observations support the hypothesis that $sPLA_2$ generate a cascade of intracellular events that is highly regulated in human macrophages.

Previous studies have shown that $sPLA_2$ may activate just one of the MAPK p38 or ERK1/2 in inflammatory cells [5, 7, 9, 19]. For example, Baek *et al.* [9] have shown that group IIA $sPLA_2$ activates ERK1/2, but not p38, in RAW264.7 macrophages. Two other studies in neutrophils have shown that GIB may alternatively activate p38 [5] or ERK1/2 [19]. Here we report that p38 and ERK1/2 are activated simultaneously by $sPLA_2$ in human macrophages and that they are both required for optimal cytokine and chemokine production. In fact, inhibition of either ERK1/2 (with PD98059) or p38 (with SB203580) resulted in the reduction of TNF-α and CXCL8 release, whereas the simultaneous blockade of the kinases completely suppressed cytokine/chemokine secretion. These data indicate that p38 and ERK1/2 cooperate for optimal production of cytokines and chemokines in $sPLA_2$-activated human macrophages.

The enzymatic activity of $sPLA_2$ is not required for activation of p38 and ERK1/2 and for induction of cytokine/chemokine production. These results are in line with previous observations showing that catalytically inactive $sPLA_2$ are still able to induce cytokine production in human inflammatory cells [7, 16, 18]. However, we show that an intact secondary and tertiary structure of $sPLA_2$ is essential for macrophage activation given that disruption of the disulfide bridges completely abolishes the ability of GIA to induce MAPK activation and cytokine/chemokine release. Our data also indicate that Me-indoxam inhibits the activation of p38 and ERK1/2 induced by GIB at concentrations (10 μM) previously shown to block cytokine production in HLM [18]. Me-indoxam prevents the binding of GIB with the M-type receptor [34], which is expressed on HLM [18].

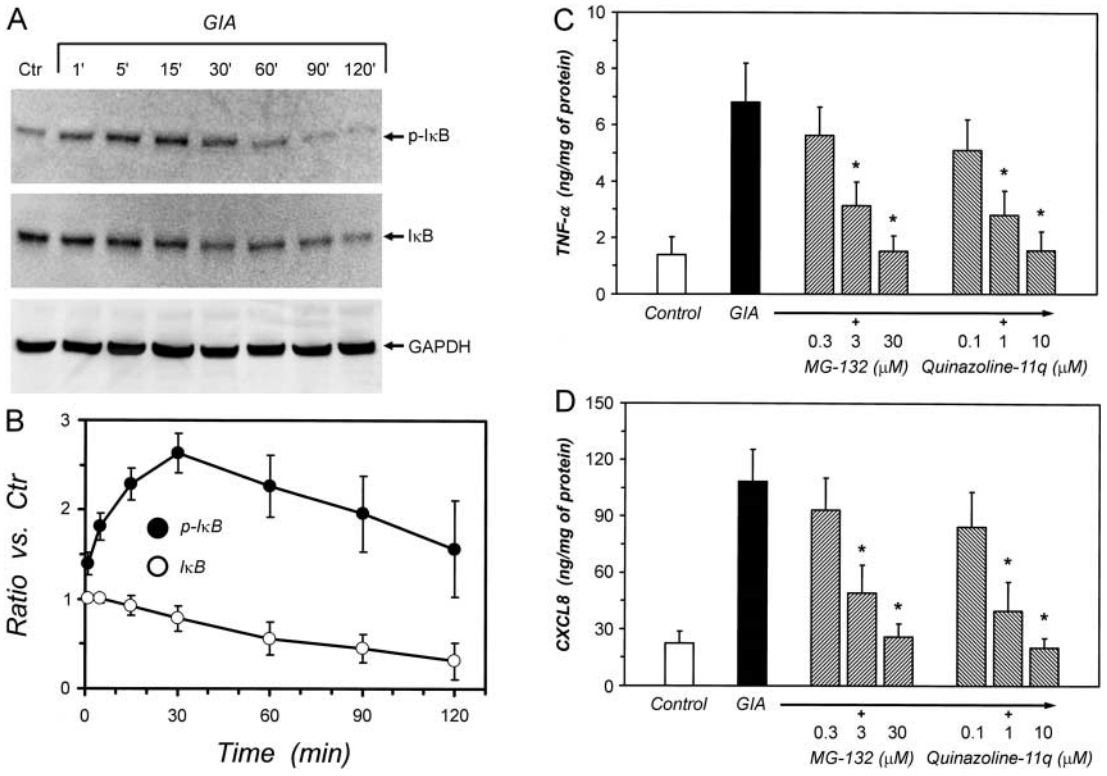

Figure 8. Role of NF-κB in GIA $sPLA_2$-induced release of TNF-α and CXCL8 in HLM. (A, B) Kinetics of phosphorylation and degradation of IκB. (A) The cells were stimulated with GIA (5 μg/mL) for the times indicated. Cellular protein extracts were subjected to Western blot analysis with anti-phospho-IκBα Ab (first gel) and, after stripping, with anti-IκBα Ab (second gel). The Western blot shown is representative of three separate experiments. (B) Densitometric analysis of the phosphorylation and degradation kinetics of IκB was performed by measuring the signal intensity at the various time points and by calculating the ratio *versus* untreated samples (Ctr). Adequate normalization was accomplished by measuring the signal intensity of GAPDH used to verify equal protein loading. The data are the mean ± SE of the three experiments. (C, D) Effect of NF-κB inhibitors on $sPLA_2$-induced TNF-α and CXCL8 release. The cells were preincubated (37°C, 1 h) with MG-132 (0.3–30 μM) or Quinazoline-11q (0.1–10 μM) and then stimulated (37°C, 6 h) with GIA (10 μg/mL). TNF-α and CXCL8 release was determined in the supernatants by ELISA. The data are the mean ± SE of four experiments. $^*p<0.01$ *vs.* GIA.

Thus, our results provide further evidence that $sPLA_2$ activate human macrophages by interacting with the M-type receptor or other membrane targets.

In mammalian cells, phosphorylation of MAPK is determined by several intracellular cascades triggered by membrane signals [47]. An early kinase mediating a number of cellular responses in leukocytes is PI3 K [22, 24]. Studies in murine systems reported the involvement of PI3 K and Akt in the up-regulation of iNOS induced by group IIA $sPLA_2$ in RAW 264.7 macrophages [10], and in the activation of matrix metalloproteinase-2 induced by GIB $sPLA_2$ in NIH3T3 fibroblasts [37]. Our data demonstrate that, in primary human macrophages, GIA activates the PI3 K/Akt pathway, which is required for $sPLA_2$-induced cytokine/chemokine production and activation of ERK1/2. Moreover, the observation that LY294002, an inhibitor of PI3 K, has no influence on p38 phosphorylation indicates that PI3 K is not involved in the activation of this MAPK.

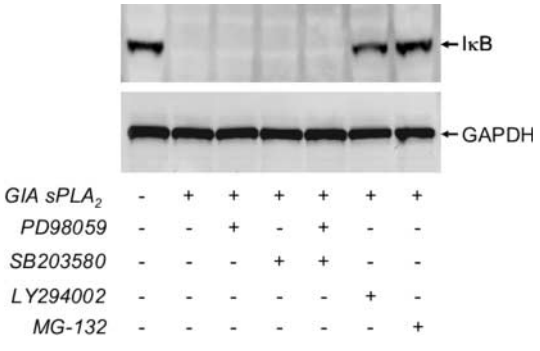

Figure 9. Role of p38, ERK1/2 and PI3 K in GIA $sPLA_2$-induced degradation of IκB. The cells were preincubated (37°C, 1 h) with PD98059 (50 μM), SB203580 (30 μM), PD98059 (50 μM) plus SB203580 (30 μM), LY294002 (30 μM) or MG-132 (30 μM) and then stimulated (37°C, 2 h) with GIA (5 μg/mL). Cellular protein extracts were subjected to Western blot analysis with anti-IκBα Ab (first gel) and, after stripping, with anti-GAPDH Ab (second gel). The Western blots shown are representative of three separate experiments.

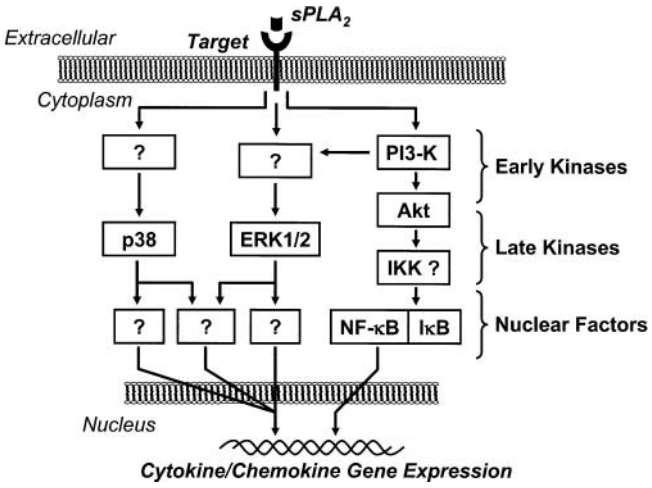

Figure 10. Schematic representation of the signaling pathways involved in $sPLA_2$-induced cytokine/chemokine production in HLM. The interaction of $sPLA_2$ with a membrane target expressed on the macrophage membrane activates at least three different signaling cascades involved in the production of cytokines and chemokines. The activation of PI3 K is an early event leading to the activation of two independent pathways involving the MAPK ERK1/2 and the nuclear factor NF-κB. The latter goes through Akt and, presumably, one of the member of the IKK family, which phosphorylates IκB. On the other hand, PI3 K-dependent activation of ERK1/2 likely involves the members of the PKC family able to drive the signal of PI3 K to Ras/Raf complexes, which in turn activate the MAPK cascade. The third signaling pathway includes the activation of p38 independently on PI3 K. Both the pathways involving p38 and ERK1/2 directly or indirectly activate nuclear factors other than NF-κB (*e.g.*, Elk-1, ATF-1/2, CREB, etc.).

The production of cytokines and chemokines induced by $sPLA_2$ in HLM is associated with the activation of gene transcription (Fig. 2), an event requiring the activation of specific nuclear factors [20]. NF-κB is the major nuclear factor regulating transcription of cytokine/chemokine genes in macrophages [21, 48]. Our results demonstrate that GIA induces the phosphorylation/degradation of IκB in HLM, and that two different inhibitors of NF-κB suppress $sPLA_2$-induced production of TNF-α and CXCL8. These data support a crucial role of NF-κB in $sPLA_2$-induced activation of HLM. Data obtained with inhibitors of the various kinases indicate that only LY294002, an inhibitor of PI3 K, prevents the signal to IκB, suggesting that PI3 K, but not p38 and ERK1/2, is involved in the activation of the NF-κB pathway by $sPLA_2$. This hypothesis is strengthened by the kinetics of phosphorylation of IκB, which is comparable to that of PI3 K and Akt and precedes the phosphorylation of p38 and ERK1/2. Current evidence supports the concept that the PI3 K/Akt system may participate in the pathways targeting NF-κB in immune cells [24, 26, 38, 49], whereas p38 and ERK1/2 are involved in cytokine/chemokine production through NF-κB-independent pathways [48, 50]. However, the full definition of the pathways leading to the activation of NF-κB in human macrophages stimulated with $sPLA_2$ requires further investigations.

Thus, multiple signaling cascades are activated by $sPLA_2$ in primary HLM and are involved in cytokine and chemokine production. The interaction of $sPLA_2$ with membrane targets, that still need to be completely characterized, activates at least three intracellular pathways (Fig. 10). The first leads to the activation of the early kinase PI3 K and the downstream Akt, which in turn induces the activation of NF-κB presumably by interacting with a member of the IκB kinase (IKK) family [38, 49]. PI3 K also participates to the activation of ERK1/2, which is not directly involved in the activation of NF-κB, but activates other nuclear factors (*e.g.*, Elk-1, ATF-1/2, CREB) regulating cytokine/chemokine gene transcription [20, 23]. This pathway likely involves the members of the PKC family able to drive the signal of PI3 K to Ras/Raf complexes and then to ERK1/2 [22–24, 47]. Finally, $sPLA_2$ activate p38 by a signaling cascade independent from the PI3 K/Akt system and leading to the activation of nuclear factors other than NF-κB. Further studies are necessary to characterize both the early transducers located upstream to p38 and the downstream nuclear factors targeted by this kinase.

In conclusion, this study identifies several intracellular events mediating $sPLA_2$-induced cytokine and chemokine production in primary human macrophages. These events may be potential targets for pharmacological modulation of $sPLA_2$-mediated responses in lung inflammatory diseases.

Materials and methods

Reagents and buffers

The following were purchased: GIA $sPLA_2$ (from *Naja mossambica mossambica* venom), LPS (from *E. Coli* serotype 026:B6), fatty acid-free human serum albumin (HSA), BSA, PIPES, L-glutamine, antibiotic-antimycotic solution (10 000 UI/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B) and Triton X-100 (Sigma, St. Louis, MO); BPB, DTT, RPMI 1640 and FCS (ICN, Costa Mesa, CA); arachidonic acid (Biomol, Plymouth Meeting, PA); rabbit Ab anti-phospho-ERK1/2 (Thr202/Tyr204), -phospho-p38 (Thr180/Tyr182), -phospho-Akt (Thr308), -phospho-Akt (Ser473), -phospho-IκBα (Ser32), -Akt and -IκB and the compounds PD98059 and LY294002 (Cell Signaling, Beverly, MA); rabbit Ab anti-ERK2 (C-14) and -p38 (C-20), goat Ab anti-GAPDH (V-18), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and rabbit anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-phosphotyrosine Ab (4G10) and rabbit anti-p85α subunit of PI3 K Ab (Upstate, Charlottesville, VI); HRP-conjugated donkey anti-rabbit Ig Ab

(Amersham Biosciences, Buckinghamshire, UK); SB203580, MG-132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal;Z-LLL-CHO) and 6-amino-4(4-phenoxyphenylethylamino) quinazoline (Quinazoline-11q) (Calbiochem, La Jolla, CA); IFN-γ and IL-4 (PeproTech, London, UK). Human recombinant GIB $sPLA_2$ and Me-indoxam were kindly donated by Michael H. Gelb (University of Washington, Seattle, WA). BPB- and DTT-inactivated $sPLA_2$ were prepared as previously described [6, 7]. All other reagents were from Carlo Erba (Milan, Italy).

PIPES buffer was made of 25 mM PIPES, 110 mM NaCl and 5 mM KCl. PCG buffer was made of PIPES buffer containing 1 mM $CaCl_2$ and 1 g/L glucose, pH 7.4. Lysis buffer for Western blot experiments was made of 20 mM Tris pH 7.5, 5 mM EDTA, 1 mM PMSF, 2 mM benzamidine, 30 μg/mL aprotinin, 30 μg/mL leupeptin, 10 mM NaF, 150 mM NaCl, 5 mM Na_3VO_4, 1% Nonidet P-40 and 5% glycerol.

Isolation and purification of HLM

Macrophages were obtained from the lung parenchyma of patients undergoing thoracic surgery as previously reported [4]. The study protocol involving the use of human lung tissue was approved from the Ethical Committee of the University of Naples Federico II, and informed consent was obtained from patients before surgery. The macrophage suspension was enriched (75–85%) by flotation over Percoll® density gradients. The cells were suspended (10^6 cells/mL) in RPMI 1640 containing 5% FCS, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution and incubated in 24-well plates. After 12 h, the medium was removed and the plates were gently washed with RPMI 1640. More than 98% of adherent cells were macrophages, as assessed by flow cytometry analysis and α-naphthylacetate esterase staining [18]. In the experiments with freshly isolated macrophages, the enriched macrophage suspension was layered onto additional Percoll gradients to reach a purity ranging between 95% and 99%, as assessed by CD71/HLA-DR double staining with flow cytometry [18].

Cell incubations

Adherent macrophages were incubated (37°C, 2–24 h) in RPMI 1640 containing increasing concentrations (0.1–10 μg/mL) of GIA or GIB $sPLA_2$. Commercial preparations of GIA were repurified by size exclusion chromatography [7] before use. All $sPLA_2$ preparations were routinely checked for LPS contamination *(Limulus Amebocyte* Test, ICN) and discarded if LPS concentration was above the detection limit of the assay (0.125 EU/mL). In selected experiments, the cells were preincubated (37°C, 1 h) with or without IFN-γ (1000 U/mL) and then incubated (37°C, 24 h) with optimal concentrations of GIA (10 μg/mL) or LPS (1 μg/mL). In other experiments, HLM were incubated (37°C, 6 h) with GIA (0.1–10 μg/mL) or LPS (1 μg/mL) either untreated or treated with DMSO (vehicle), BPB (37°C, 4 h) or DTT (37°C, 2 h). Control cells were incubated with DMSO, BPB or DTT in the absence of $sPLA_2$. Finally, in the experiments with the inhibitors of intracellular kinases or NF-κB, HLM were preincubated (37°C, 1 h) with various concentrations of PD98059, SB203580, LY294002, MG-132 or Quinazoline-11q and then stimulated (37°C, 6 h) with GIA or LPS. At the end of each experiment, cell viability was assessed by trypan blue exclusion and was always found to be greater than 95%.

At the end of incubation, supernatants were removed, centrifuged twice (1000×*g*, 4°C, 5 min) and stored at –80°C for the subsequent determination of cytokines (TNF-α, IL-6, IL-10, IL-12) and chemokines (CCL2, CCL4, CCL22 and CXCL8). The cells remaining in the plates were lysed with 0.1% Triton X-100 for the determination of total cellular content of proteins.

ELISA for cytokines and chemokines

The release of cytokines and chemokines in the supernatant of macrophage cultures was measured in duplicate determinations using commercially available ELISA kits for TNF-α, IL-6, IL-10, IL-12 and CXCL8 (Euro Clone, Devon, UK) and CCL2, CCL4 and CCL22 (R&D Systems, Minneapolis, MN). The linearity range of the assay was between 20 and 800 pg/mL (TNF-α), 6 and 200 pg/mL (IL-6 and IL-12), 12 and 400 pg/mL (IL-10), 15.6 and 1000 pg/mL (CCL2, CCL4), 7.8 and 500 pg/mL (CCL22), and 30 and 2000 pg/mL (CXCL8). The results were normalized for the total protein content in each well.

RPA for chemokine mRNA expression

Adherent macrophages (4×10^6/2 mL) were incubated (37°C, 6 h) with RPMI 1640 alone, with increasing concentrations (0.1–10 μg/mL) of GIA, or with LPS (10 μg/mL) that was used as a positive control. At the end of incubation, RNA was isolated by the TRIzol technique (Invitrogen, Milan, Italy). RPA was performed as previously described [51]. Briefly, chemokine mRNA were detected using the RiboQuant Multi-Probe RPA kit (template set hCK-5; Pharmingen, San Diego, CA) according the manufacturer's instructions. In brief, ^{32}P-labeled riboprobes were hybridized overnight with 5 μg RNA. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. Protected RNA were then resolved on a 5% denaturing polyacrylamide gel. The gel was adsorbed to filter paper, dried under vacuum, and exposed to film (X-AR; Kodak, Rochester, NY) with intensifying screens at –70°C.

Western blot analysis

Freshly isolated macrophages were suspended in PCG buffer. The cells (10^6/sample) were incubated (37°C, 1–120 min) with the indicated concentrations of GIA or GIB $sPLA_2$. In selected experiments macrophages were preincubated (37°C, 1 h) with PD98059, SB203580, LY294002 or MG-132 before stimulation with $sPLA_2$. At the end of incubation the reactions were stopped by adding ice-cold PIPES buffer and the samples were microfuged for 30 s. Cell pellets were immediately lysed in lysis buffer. Cell lysates were kept on ice for 20 min and then microfuged for 20 min at 4°C. Supernatant was collected as a protein extract containing lysed cell components without nuclei and diluted in an equal volume of 2× LDS Sample Buffer (LSB, Novex, Invitrogen, Milan, Italy) containing 2.5% 2-ME. Proteins were separated on 10% Bis-Tris gels (NuPAGE®, Novex) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After immersion overnight in TBST

(50 mM Tris pH 7.5, 150 mM NaCl and 0.05% Tween 20) containing 4% BSA, membranes were washed three times (10 min each) with TBST, and then blotted (4°C, overnight) with the indicated Ab. After washing, the membranes were incubated (22°C, 1 h) with HRP-conjugated secondary Ab. Membranes were washed four times and developed with the ECL Western blotting detection reagent (Amersham) under the image analysis system ChemidocXRS (Bio-Rad). Although comparisons were made on the basis of an equal number of cells, membranes blotted with Ab against phospho-kinases were stripped with stripping buffer (7 M guanidine hydrochloride in distilled water) and then reblotted with the Ab against the unphosphorylated proteins to verify equal protein content of each sample. Densitometric measurement of the signal intensity was performed with the Quantity One 4.5.0 software (Bio-Rad) and kinase phosphorylation rate at the various time points was obtained by calculating the ratio *versus* untreated samples. Normalization was accomplished by calculating the signal intensity of the unphosphorylated forms of the various kinases or of GAPDH.

Immunoprecipitation of p85α subunit of PI3K

Freshly isolated macrophages suspended in PCG buffer (5×10^6/sample) were incubated (37°C, 1–15 min) with GIA (5 μg/mL). At the end of incubation the reactions were stopped by adding ice-cold PIPES buffer and the samples were microfuged for 30 s. Cell pellets were immediately lysed in 1 mL lysis buffer. Cell lysates were kept on ice for 20 min and then microfuged for 20 min at 4°C. Lysates were precleared with Gammabind G Sepharose beads (Amersham) for 1 h at 4°C to remove any nonspecific binding. The clarified lysates were then incubated (1 h, 4°C) with the Ab against the p85α subunit of PI3 K prebound to Gammabind G Sepharose beads (1 h, 4°C). Immunoprecipitated proteins were eluted by boiling in LSB containing 2.5% 2-ME. Proteins were separated on 4–12% Bis-Tris gels and transferred to a nitrocellulose membrane. Immunoblotting with anti-phosphotyrosine Ab (4G10) or, after stripping, with the anti-p85α subunit Ab was performed as described above.

Statistical analysis

The data are expressed as the mean ± SE of the indicated number of experiments. *p* values were determined with Student's paired *t*-test [52].

Acknowledgements: This work was supported by grants from the *Ministero dell'Istruzione, dell'Università e della Ricerca* (G.M., M.T.), the *Ministero della Salute* "Alzheimer Project" (G.M.), the *Consiglio Nazionale delle Ricerche* (Rome, Italy) (G.M.). We thanks Michael H. Gelb (University of Washington, Seattle) for providing the recombinant human group IB $sPLA_2$ and Me-indoxam, and Tommaso Russo (University of Naples Federico II) for critically reading the manuscript.

References

1 **Granata, F., Balestrieri, B., Petraroli, A., Giannattasio, G., Marone, G. and Triggiani, M.,** Secretory phospholipases A_2 as multivalent mediators of inflammatory and allergic disorders. *Int. Arch. Allergy Immunol.* 2003. **131:** 153–163.

2 **Uhl, W., Nevalainen, T. J. and Buchler, M. W.** (Eds.) *Phospholipase A_2. Basic and clinical aspects in inflammatory diseases*. Karger, Basel 1997.

3 **Kudo, I. and Murakami, M.,** Phospholipase A_2 enzymes. *Prostaglandins Other Lipid Mediat.* 2002. **68–69:** 3–58.

4 **Triggiani, M., Granata, F., Oriente, A., De Marino, V., Gentile, M., Calabrese, C., Palumbo, C. *et al.*,** Secretory phospholipases A_2 induce β-glucuronidase release and IL-6 production from human lung macrophages. *J. Immunol.* 2000. **164:** 4908–4915.

5 **Silliman, C. C., Moore, E. E., Zallen, G., Gonzalez, R., Johnson, J. L., Elzi, D. J., Meng, X. *et al.*,** Presence of the M-type $sPLA_2$ receptor on neutrophils and its role in elastase release and adhesion. *Am. J. Physiol. Cell. Physiol.* 2002. **283:** C1102–1113.

6 **Triggiani, M., Granata, F., Oriente, A., Gentile, M., Petraroli, A., Balestrieri, B. and Marone, G.,** Secretory phospholipases A_2 induce cytokine release from blood and synovial fluid monocytes. *Eur. J. Immunol.* 2002. **32:** 67–76.

7 **Triggiani, M., Granata, F., Balestrieri, B., Petraroli, A., Scalia, G., Del Vecchio, L. and Marone, G.,** Secretory phospholipases A_2 activate selective functions in human eosinophils. *J. Immunol.* 2003. **170:** 3279–3288.

8 **Beck, G., Yard, B. A., Schulte, J., Haak, M., van Ackern, K., van der Woude, F. J. and Kaszkin, M.,** Secreted phospholipases A_2 induce the expression of chemokines in microvascular endothelium. *Biochem. Biophys. Res. Commun.* 2003. **300:** 731–737.

9 **Baek, S. H., Lim, J. H., Park, D. W., Kim, S. Y., Lee, Y. H., Kim, J. R. and Kim, J. H.,** Group IIA secretory phospholipase A_2 stimulates inducible nitric oxide synthase expression via ERK and NF-kB in macrophages. *Eur. J. Immunol.* 2001. **31:** 2709–2717.

10 **Park, D. W., Kim, J. R., Kim, S. Y., Sonn, J. K., Bang, O. S., Kang, S. S., Kim, J. H. *et al.*,** Akt as a mediator of secretory phospholipase A_2 receptor-involved inducible nitric oxide synthase expression. *J. Immunol.* 2003. **170:** 2093–2099.

11 **Takasaki, J., Kawauchi, Y., Yasunaga, T. and Masuho, Y.,** Human type II phospholipase A_2-induced Mac-1 expression on human neutrophils. *J. Leukoc. Biol.* 1996. **60:** 174–180.

12 **Perrin-Cocon, L., Agaugue, S., Coutant, F., Masurel, A., Bezzine, S., Lambeau, G., Andre, P. *et al.*,** Secretory phospholipase A_2 induces dendritic cell maturation. *Eur. J. Immunol.* 2004. **34:** 2293–2302.

13 **Rizzo, M. T., Nguyen, E., Aldo-Benson, M. and Lambeau, G.,** Secreted phospholipase A(2) induces vascular endothelial cell migration. *Blood* 2000. **96:** 3809–3815.

14 **Gambero, A., Landucci, E. C., Toyama, M. H., Marangoni, S., Giglio, J. R., Nader, H. B., Dietrich, C. P. *et al.*,** Human neutrophil migration *in vitro* induced by secretory phospholipases A_2: a role for cell surface glycosaminoglycans. *Biochem. Pharmacol.* 2002. **63:** 65–72.

15 **Zhang, Y., Lemasters, J. and Herman, B.,** Secretory group IIA phospholipase A(2) generates anti-apoptotic survival signals in kidney fibroblasts. *J. Biol. Chem.* 1999. **274:** 27726–27733.

16 **Fonteh, A. N., Marion, C. R., Barham, B. J., Edens, M. B., Atsumi, G., Samet, J. M., High, K. P. *et al.*,** Enhancement of mast cell survival: a novel function of some secretory phospholipase A_2 isotypes. *J. Immunol.* 2001. **167:** 4161–4171.

17 **Valentin, E. and Lambeau, G.,** Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. *Biochim. Biophys. Acta* 2000. **1488:** 59–70.

18 **Granata, F., Petraroli, A., Boilard, E., Bezzine, S., Bollinger, J., Del Vecchio, L., Gelb, M. H. *et al.*,** Activation of cytokine production by secreted phospholipases A_2 in human lung macrophages expressing the M-type receptor. *J. Immunol.* 2005. **174:** 464–474.

19 **Jo, E. J., Lee, H. Y., Lee, Y. N., Kim, J. I., Kang, H. K., Park, D. W., Baek, S. H. *et al.*,** Group IB secretory phospholipase A2 stimulates CXC chemokine ligand 8 production via ERK and NF-{kappa}B in human neutrophils. *J. Immunol.* 2004. **173:** 6433–6439.

20 **Holloway, A. F., Rao, S. and Shannon, M. F.,** Regulation of cytokine gene transcription in the immune system. *Mol. Immunol.* 2002. **38:** 567–580.

21 **Blackwell, T. S. and Christman, J. W.,** The role of nuclear factor-kappa B in cytokine gene regulation. *Am. J. Respir. Cell. Mol. Biol.* 1997. **17:** 3–9.

22 **Wymann, M. P., Sozzani, S., Altruda, F., Mantovani, A. and Hirsch, E.,** Lipids on the move: phosphoinositide 3-kinases in leukocyte function. *Immunol. Today* 2000. **21:** 260–264.

23 **Dong, C., Davis, R. J. and Flavell, R. A.,** MAP kinases in the immune response. *Annu. Rev. Immunol.* 2002. **20:** 55–72.

24 **Fruman, D. A. and Cantley, L. C.,** Phosphoinositide 3-kinase in immunological systems. *Semin. Immunol.* 2002. **14:** 7–18.

25 **Carter, A. B., Monick, M. M. and Hunninghake, G. W.,** Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell. Mol. Biol.* 1999. **20:** 751–758.

26 **Park, S. J., Lee, S. C., Hong, S. H. and Kim, H. M.,** Degradation of IkappaBalpha in activated RAW264.7 cells is blocked by the phosphatidylinositol 3-kinase inhibitor LY294002. *Cell. Biol. Toxicol.* 2002. **18:** 121–130.

27 **Bonecchi, R., Sozzani, S., Stine, J. T., Luini, W., D'Amico, G., Allavena, P., Chantry, D. *et al.*,** Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* 1998. **92:** 2668–2671.

28 **Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. *et al.*,** SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 1995. **364:** 229–233.

29 **Davies, S. P., Reddy, H., Caivano, M. and Cohen, P.,** Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 2000. **351:** 95–105.

30 **Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R.,** PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* 1995. **270:** 27489–27494.

31 **Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M. *et al.*,** Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2. *J. Biol. Chem.* 2002. **277:** 48535–48549.

32 **Verheij, H. M., Volwerk, J. J., Jansen, E. H., Puyk, W. C., Dijkstra, B. W., Drenth, J. and de Haas, G. H.,** Methylation of histidine-48 in pancreatic phospholipase A_2. Role of histidine and calcium ion in the catalytic mechanism. *Biochemistry* 1980. **19:** 743–750.

33 **Ruegg, U. T. and Rudinger, J.,** Reductive cleavage of cystine disulfides with tributylphosphine. *Methods Enzymol.* 1977. **47:** 111–116.

34 **Hanasaki, K. and Arita, H.,** Phospholipase A_2 receptor: a regulator of biological functions of secretory phospholipase A_2. *Prostaglandins Other Lipid Mediat.* 2002. **68–69:** 71–82.

35 **Fruman, D. A., Meyers, R. E. and Cantley, L. C.,** Phosphoinositide kinases. *Annu. Rev. Biochem.* 1998. **67:** 481–507.

36 **Hawes, B. E., Luttrell, L. M., van Biesen, T. and Lefkowitz, R. J.,** Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.* 1996. **271:** 12133–12136.

37 **Choi, Y. A., Lim, H. K., Kim, J. R., Lee, C. H., Kim, Y. J., Kang, S. S. and Baek, S. H.,** Group IB secretory phospholipase A_2 promotes matrix metalloproteinase-2-mediated cell migration via the phosphatidylinositol 3-kinase and Akt pathway. *J. Biol. Chem.* 2004. **279:** 36579–36585.

38 **Coffer, P. J., Jin, J. and Woodgett, J. R.,** Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* 1998. **335:** 1–13.

39 **Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F.,** A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 1994. **269:** 5241–5248.

40 **Karin, M. and Ben-Neriah, Y.,** Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol.* 2000. **18:** 621–663.

41 **Fiedler, M. A., Wernke-Dollries, K. and Stark, J. M.,** Inhibition of TNF-alpha-induced NF-kappaB activation and IL-8 release in A549 cells with the proteasome inhibitor MG-132. *Am. J. Respir. Cell. Mol. Biol.* 1998. **19:** 259–268.

42 **Tobe, M., Isobe, Y., Tomizawa, H., Nagasaki, T., Takahashi, H., Fukazawa, T. and Hayashi, H.,** Discovery of quinazolines as a novel structural class of potent inhibitors of NF-kappa B activation. *Bioorg. Med. Chem.* 2003. **11:** 383–391.

43 **Adams, J. and Stein, R.,** Novel inhibitors of the ptoteasome and their therapeutic use in inflammation. *Annu. Rep. Med. Chem.* 1996. **31:** 279–287.

44 **Tocker, J. E., Durham, S. K., Welton, A. F. and Selig, W. M.,** Phospholipase A_2-induced pulmonary and hemodynamic responses in the guinea pig. Effects of enzyme inhibitors and mediators antagonists. *Am. Rev. Respir. Dis.* 1990. **142:** 1193–1199.

45 **Pruzanski, W., Vadas, P. and Fornasier, V.,** Inflammatory effect of intradermal administration of soluble phospholipase A_2 in rabbits. *J. Invest. Dermatol.* 1986. **86:** 380–383.

46 **Higashino, K., Ishizaki, J., Kishino, J., Ohara, O. and Arita, H.,** Structural comparison of phospholipase-A_2-binding regions in phospholipase-A_2 receptors from various mammals. *Eur. J. Biochem.* 1994. **225:** 375–382.

47 **English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S. and Cobb, M. H.,** New insights into the control of MAP kinase pathways. *Exp. Cell. Res.* 1999. **253:** 255–270.

48 **Guha, M. and Mackman, N.,** LPS induction of gene expression in human monocytes. *Cell. Signal.* 2001. **13:** 85–94.

49 **Lu, Y. and Wahl, L. M.,** Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKK{alpha}/NF-{kappa}B pathway. *J. Leukoc. Biol.* 2005. **78:** 259–265.

50 **Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H. and Kracht, M.,** Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 2002. **72:** 847–855.

51 **Del Prete, A., Vermi, W., Dander, E., Otero, K., Barberis, L., Luini, W., Bernasconi, S. *et al.*,** Defective dendritic cell migration and activation of adaptive immunity in PI3Kgamma-deficient mice. *EMBO J.* 2004. **23:** 3505–3515.

52 **Snedecor, G. W.** (Eds.) *Statistical Methods*. Iowa State University Press, Ames 1980.

