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Identification of the Elusive Mammalian Enzyme Phosphatidylcholine-Specific Phospholipase C

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14. ABSTRACT

The long-term **purpose** of the present proposal aims at establishing the role of the elusive mammalian protein, phosphatidycholine-specific phospholipase C (PC-PLC) in the inflammatory processes involved in progression of rheumatoid arthritis (RA). Thus, the main **scopes** of this proposal are: 1. to identify the PC-PLC gene and protein; and 2. to test PC-PLC involvement in production of TNF alpha by monocytes. **Major findings.** . Identification of a novel experimental condition (High serum) that induces activation of PC-PLC. Besides being a novel observation in of itself, it allows us to overcome an unexpected obstacle (lack of activation following LPS stimulation) and allowed us to proceed further with our proposed transcriptome studies.

. Most of PC-PLC activity in basal and stimulated condition (high serum) resides in the cytosol. This is an important result since it greatly facilitates alternative approaches for the isolation of the PC-PLC protein (with affinity or traditional chromatographical purification) and it also refines the structural features of the putative PC-PLC protein (no or one transmembrane domain) that will help in the identification of the candidate PC-PLC clones from the transcriptome analysis.

. Provided for the first time clear-cut evidence of a potential role of PC-PLC activity in regulation of TNF-mediated inflammatory signaling.

15. SUBJECT TERMS

Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells

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1. INTRODUCTION:

The present proposal aimed at identifying novel players that are critically involved in the progression of rheumatoid arthritis (RA). The identification of these factors may ultimately provide alternative "druggable" targets for the treatment of this debilitating disease. The specific hypothesis that is addressed by this proposal is to test whether a mammalian protein called phosphatidycholine-specific phospholipase C (PC-PLC) might represent such a novel target. Since the mammalian PC-PLC gene has not been identified yet nor the PC-PLC protein isolated the main goals of this proposal were: 1. To identify the PC-PLC gene and protein; and 2. To test PC-PLC involvement in production of TNF alpha by monocytes.

2. KEYWORDS:

Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells.

3. OVERALL PROJECT SUMMARY:

Summary of Objectives.

Task 1. To identify mammalian PC-PLC.

In this task, we proposed to identify the gene responsible for PC-PLC activity in mammalian cells by complementing cell biology with genomic information. In order to identify mammalian PC-PLC, we proposed to take advantage of the following observations, recently reported in the literature [1, 2]. Certain stimuli, such as lipopolysaccharide (LPS) or oxidized LDLs (ox-LDLs), significantly increase PC-PLC protein level (tested using cross-reacting polyclonal antibodies raised against bacterial PC-PLC) and its enzymatic activity. Since the changes in both activity and protein level take place after 12-24 hours with either treatment, we hypothesized that the increase in PC-PLC is due to enhanced transcription of its gene.

In the course of the past two years, we have optimized new experimental approaches both to measure PC-PLC activity *in vitro* and to stimulate PC-PLC activity in HUVECs. In fact, after extensive experimentation, we found that the published *in vitro* assay for PC-PLC activity employed to demonstrate activation of PC-PLC in response to LPS [1] was not reliable/reproducible/specific and that treatment with LPS was not effective in stimulating PC-PLC activity once a reliable PC-PLC *in vitro* assay was performed (please refer to the "Summary of Results" section for details).

Also, during the initial experiments to determine the effect of oxidized LDLs on PC-PLC activity in HUVEC cells, we could not detect activation of the PC-PLC enzyme (as described in the second technical report). Importantly, after testing different batches of oxidized LDLs, we could observe a reproducible increase of cytosolic PC-PLC activity (please refer to the "Summary of Results" section for details).

Thus, we proceeded in the identification of PC-PLC by differential expression analysis using stimulation with high serum instead of LPS, and with oxidized LDLs treatment.

The major road blocks we encountered for the completion of this Task were the inability to reproduce published results and the unreliability of published PC-PLC assays. Importantly, after much troubleshooting, we were successful in overcome these road blocks and we were able to identify/optimize experimental conditions that allowed us to collect mRNA as proposed in the original application and to send it out for transcriptome sequencing. After we receive the results from the transcriptome analysis, it is our intention to proceed with the genetic analysis as proposed in the original Subtasks of the application.

<u>Subtask 1a: To perform whole transcriptome sequencing to identify unknown messages that are up-regulated in conditions that increase PC-PLC protein level (such as high serum and ox-LDL treatments)</u>.

For the identification of up-regulated transcripts, the high serum (20% FBS) treatment group will be first compared to the low serum (0.5% FBS) control group and the ox-LDL treatment group will be compared to the 0.5% FBS control group. Then, up-regulated transcripts from the 20% FBS and the ox-LDL groups will be compared to each other to find transcripts that are up-regulated in both groups. Informatic analysis of differentially expressed transcripts will be performed by the New York Genome that will also perform transcriptome analysis.

Up-regulated transcripts will be selected based on the following features, ordered by relevance: genes of unknown function, zero or one predicted transmembrane domain and potentially carrying one or more signature domains (C1: KxxxxxxR; C2: SGH; C3:SRxxxxxHxxxD) found in lipid phosphate phosphatases (LPPs, enzymes that cleave the phosphate group off lipids).

<u>Subtask 1b-c: To test the identified cDNA clones for PC-PLC activity in mammalian cells.</u>

CDNA clones corresponding to up-regulated transcripts will be then purchased from Applied Biosystems or other repositories and subcloned into a mammalian expression vector (i.e. pcDNA3.1 plasmid), transfected in Hela cells and tested for PC-PLC *in vitro* activity. Hela cells will be purchased from ATCC (with no access to identifiable information regarding the donor). The clones that confirm high PC-PLC activity as compared to the empty vector will be then down-regulated using siRNA in untransfected Hela cells (which have endogenous PC-PLC activity) to show that indeed the endogenous mammalian gene carries PC-PLC activity.

Task 2. To establish the role of PC-PLC in TNF-mediated signaling in monocytes.

In the timeframe that was necessary to identify new experimental conditions for the transcriptome analysis, we have provided clear, unprecedented evidence of the involvement of PC-PLC activity in the proinflammatory signaling of TNF alpha in human monocytic U937 cells (please refer to the "Summary of Results" section for details). These results are exciting as they confirm the potential involvement of PC-PLC in the inflammatory processes in part responsible for the progression of RA.

Summary of Results.

Task 1. To identify mammalian PC-PLC.

Based on results published by other groups, we proposed to identify candidate *PC-PLC* mRNAs by comparing transcriptome analysis between two PC-PLC activating conditions (treatment with LPS and oxidized LDLs).

Thus the first step in order to achieve the first task was to reproduce the published activation of PC-PLC by LPS. We followed the experimental protocol described by Zhang [1] for both treatment of HUVEC cells and for PC-PLC activity *in vitro* (**Figure 1A**). Briefly, HUVEC cells were either serum starved (with 0.5% of Fetal Bovine Serum, FBS) or serum starved and stimulated with LPS at different concentrations (0.1 and 0.5 ug/ml). After 24 hours, cells were collected and total lysates were prepared to measure PC-PLC activity as described by Zhang [1]. Unexpectedly, no activation of PC-PLC was observed (**Figure 1A**). To make sure that the source of LPS was not responsible for absence of activity, we tested different LPS preparations (from Sigma as indicated in the original publication and a more pure preparation of Lipid A) nerevtheless, none of the different LPS activated PC-PLC. Moreover, we figured out that the PC-PLC assay as described [1] was not reliable/reproducible and specific.

The limitation of the method described by Zhang [1] for measuring *in vitro* PC-PLC activity is that it indirectly measures PC-PLC activity determining the amount of inorganic phosphate (Pi) in the aqueous phase. This will account also for non-enzymatically derived Pi, like that associated with nucleotides or peptides. In this case, a dose response with different protein concentrations (**Figure 1B**) would result in false positives as it would be interpreted as an increase of PC-PLC activity with protein while the increase of Pi is merely due to non enzymatically derived Pi. On the other hand, if the detected Pi is a result of an enzymatic activity, it should increase with the time of incubation. Indeed, as shown in **Figure 1A**, the activity of the bacterial PC-PLC positive control (bPC-PLC) doubles at 30 minutes of incubation as compared to 15 minutes. Surprisingly, the Pi from cell extracts did not increase with time (30 vs 15 minutes) suggesting that it is rather a result a non-enzymatic source of aqueous Pi.

This indicated to us that no conclusions on activation of PC-PLC by LPS could be drawn using the published assay [1].

Thus we resorted to use a fluorimetric assay kit, commercially available from Molecular Probes (EnzChek® Direct Phospholipase C Assay Kit) which also distinguishes between PC-PLC and PC-PLD activities. Using this assay we could show that the PC-PLC activity of control HUVEC cells was increasing with time (**Figure 2**) and with protein (data not shown) indicating that the detected activity was indeed due to an enzyme. Importantly, we showed that, after separating total membranes from cytosol, most of the PC-PLC activity was in the cytosol. This is an important result since it greatly facilitates alternative approaches for the isolation of the PC-PLC protein (with affinity or traditional chromatographical purification) and it also refines the structural features of the putative PC-PLC protein (no or one transmembrane domain) that will help in the identification of the candidate PC-PLC clones from the transcriptome analysis. Also we excluded the possibility that a significant portion of PC-PLC activity was in the nuclear fraction (data not shown).

Thus with the optimized PC-PLC activity in hand, we evaluated the effect of increasing concentrations of LPS on the activity in the cytosolic fractions (**Figure 3**). Again unexpectedly given the published report [1], no significant changes in PC-PLC activity were observed either at different concentrations of LPS (**Figure 3**) or at different times of incubation (12, 24 and 48 hours) with 0.1ug/ml LPS (data not shown). We also tested different preparation of LPS with similar effects. These unexpected results excluded LPS as one of the stimuli that could be used for the proposed comparative transcriptome analysis.

While performing these experiments though, we noticed that the basal PC-PLC activity of serum starved HUVEC cells (0.5% FBS) was significantly lower than the basal activity of HUVECs grown in 20% FBS. Thus we set up to serum

starve HUVEC cells for 24 hours with 0.5% FBS and then one set of cells were stimulated with 20%FBS for 24 hours and one set was kept at 0.5% FBS. Membranes and cytosol were isolated and PC-PLC activity was measured (**Figure 4**). Indeed, a four-fold increase of PC-PLC activity was observed in serum stimulated samples. Moreover maximal stimulation was observed after 24 hours but it started at 12 hours. These results therefore support the fact that serum stimulation can be used instead of LPS for comparative transcriptome analysis. We have performed the experiment two more times and side-by-side we have collected samples after 16 and 24 hours of treatment for PC-PLC activity (as positive control) **(Figure 5A and B)** and for mRNA collection (**Table 1**).

We have also tested the effect of oxidized LDLs on PC-PLC activity in HUVECs cells.[2] Confluent HUVECs cells were serum starved for 24 hours with 0.5% FBS and then one set of cells were stimulated with different amounts of oxidized LDLs for different time while one set was kept at 0.5% FBS. Membranes and cytosols were isolated and PC-PLC activity was measured. After an initial lack of activation possibly due to a problem with a specific batch of oxidized LDLs, we tested other batches and found an approximately 6 fold increase of PC-PLC activity upon treatment with 80ug/ml for 16 hours which became 11 fold after 24 hours of incubation (**Figure 6**). Importantly, even with 20ug/ml oxidized LDLs we could observe a 5 fold stimulation of PC-PLC after 24 hours. These results instructed us that already at 16 hours of treatment, the PC-PLC activity is up suggesting that the message should go up even earlier and that the activity continues to increase at 24 hours suggesting that also the message will continue to increase. Moreover, a concentration between 20 and 80ug/ml will induce significant changes in PC-PLC. Thus we decided to treat the cells with 60ug/ml of oxidized LDLs for 16 hours and simultaneously collect samples for PC-PLC activity as positive control and for mRNA isolation (**Figure 7**). We performed two independent experiments and we collected therefore two independent sets of samples for transcriptome analysis (**Table 1**).

Table 1 reports the samples, the concentration and the quality of the mRNA that has been sent to the New York Genome Center for transcriptome analysis. Informatics analysis of differentially expressed transcripts will be also performed by the same center.
Up-regulated transcripts will be selected based on the following features, ordered by relevance: genes of unknown function, with one or no predicted transmembrane domains (as the results in the previous months have indicated that the PC-PLC activity that increases in response to serum stimulation resides in the cytosol) and potentially carrying one or more signature domains (C1: KxxxxxxR; C2: SGH; C3:SRxxxxxHxxxD) found in lipid phosphate phosphatases (LPPs), enzymes that cleave the phosphate group off lipids.

In order to evaluate whether the cross-reacting antibodies against bacterial PC-PLC [3-5] could be used to assist in identifying mammalian PC-PLC, we have obtained a preparation of these antibodies from Dr. Masataka Oda from the University of Tokushima Bunri in Japan. We tested these antibodies in cytosolic and membrane fractions of HUVEC cells. While the cross reacting antibodies were able to pick up a band of approximately 60kd in the cytosolic fraction (**Figure 8A**), the band was not more intense after serum stimulation suggesting that perhaps the band is non specific (**Figure 8B**). Moreover a similar band was detected also in other cell lines but its intensity did not match the level of the in vitro PC-PLC activity (data not shown). Thus it remains to be determined whether these antibodies do really cross react with mammalian PC-PLC.

Finally, as discussed earlier, we expect that the PC-PLC enzyme, given its type of biochemical reaction, will carry a signature motif found in lipid phosphate phosphatases (LPPs). Thus, as a complementary approach to the proposed transcriptome analysis, we also screened different already identified LPPs for PC-PLC activity. Briefly, we obtained constructs for the overexpression in mammalian cells of various LPPs-tagged with GFP or Myc (LPR3, LPR1, NLP1, NLP2, LPP3, ASL3, BC038108), and we transfected Hela cells with these constructs. After 24 hours from transfection, we verified their successful expression by western blotting (**Figure 9**) and collected cells and prepared total lysate for *in vitro* PC-PLC activity measured using tritiated phosphatidylcholine (PC). This method (total lysate and tritiated PC) should allow the use of less material, on the other hand, the low basal PC-PLC activity and high basal background did not allow to draw definitive conclusions. We repeated the overexpression experiment and then we separated cytosol from membranes for each sample (vector plasmid control and LPPs plasmids) and used the fluorescent assay for PC-PLC, as we performed for HUVEC cells. Preliminary experiments using this method uncovered basal PC-PLC activity in Hela cells (even using as low as 20ug of proteins), and that this activity is found in the cytosol (like HUVECs) (**Figure 10**). However, overexpression of LPPs did not affect basal PC-PLC neither in the cytosol nor in the membranes. Thus we can exclude that any of these enzymes posses PC-PLC activity.

Task 2. To establish the role of PC-PLC in TNF-mediated signaling in monocytes.

While we were making progress with Task 1, we established the role of PC-PLC in TNF-mediated signaling taking advantage of the PC-PLC pharmacological inhibitor D609.[6] Thus as stated in the alternatives, we determined the effect of D609 on the proinflammatory function of TNF in the monocytic cell line U937 by assessing the effects on TNF-mediated activation of NF-kB. To this aim, we pretreated U937 cells with 50ug/ml D609 for 24 hours, concentration and time that largely assures PC-PLC inhibition. After this time, cells were treated with 1nM TNF alpha for 20 minutes and nuclei were quickly separated from cytoplasm. The level of p65 NF-kB in the nucleus measured by western blotting was used as a read out for NF-kB activation (**Figure 11A**) together with the degradation of the inhibitory subunit, IKB alpha (**Figure 11B**). As show in Figure 11 and as expected, NF-kB translocates into the nucleus after TNF stimulation. Importantly, long term treatment with D609 significantly blocked NF-kB translocation. These results were confirmed by the fact that D609 partially prevented IKB alpha degradation, positioning PC-PLC upstream of IKB.

Since long-term preincubations may favor unspecific effects, we also tested the effect of short-term preincubation (2 hours) and, similarly to the previous results, D609 was able to inhibit NF-kB translocation to the nucleus suggesting that the effect of the drug may be specific (**Figure 12**).

One concern with D609 is that it is not completely specific for PC-PLC as it also targets another class of enzymes called sphingomyelin synthases (SMSs).[7-9] Thus to address this concern, we downregulated SMSs in U937 cells with siRNA to compare the effects on TNF alpha signaling to those of D609. We used the Neon transfection system and we electroporated 6ug of scrambled control siRNA (All Star from Ambion) or of already validated siRNA sequences specific for SMS1 or SMS2 as U937 cells express both SMSs isoforms.[10] After 72 hours of downregulation, 8 million cells were collected to test the efficacy of the downregulation on in vitro SMS activity while the rest were treated with TNF alpha for 20 minutes or left untreated. Nuclei and cytoplasm were then quickly isolated on ice and proteins were prepared for western blotting.

Downregulation with either SMS1 or SMS2 was efficient as each siRNA approximately decreased total SMS activity by 50% as compared to the scrambled control (**Figure 13**). Total SMS activity was determined by the ability of lysates to convert fluorescent ceramide (NBD-C6-Cer) into fluorescent sphingomyelin (NBD-C6-SM) (**Figure 13A**). Quantification of SMS activity is reported in **Figure 13B**. Importantly, as shown in Figure 14, downregulation of SMS1 or SMS2 did not affect TNF-induced activation of NF-kB compared to scrambled control as observed by its nuclear translocation (**Figure 14A**) or by IKB degradation (**Figure 14B**), thus implying that the inhibitory effects of D609 on NF-kB activation is not due to SMS and points to a specific role of PC-PLC.

Once mammalian PC-PLC is identified, it is our intension to overexpress and downregulate it in U937 cells to confirm its involvement in TNF-induced inflammatory signaling.

4. KEY RESEARCH ACCOMPLISHMENTS:

. Identification of a novel experimental condition (High serum) that induces activation of PC-PLC (**Figure 4**). Besides being a novel observation in of itself, it allows us to overcome an unexpected obstacle (lack of activation following LPS stimulation) and allows us to proceed further with our proposed studies.

. Most of PC-PLC activity in basal and stimulated condition (high serum) resides in the cytosol (**Figure 2** and **Figure 4**). This is an important result since it greatly facilitates alternative approaches for the isolation of the PC-PLC protein (with affinity or traditional chromatographical purification) and it also refines the structural features of the putative PC-PLC protein (no or one transmembrane domain) that will help in the identification of the candidate PC-PLC clones from the transcriptome analysis.

. Provided clear-cut evidence for the first time of a potential role of PC-PLC activity in regulation of TNF-mediated inflammatory signaling (**Figures 11-14**).

5. CONCLUSIONS:

Reumatoid arthritis is a significant medical challenge both in the military and general population. In fact, the limitation of physical activity or the acquisition of disability due to the disease together with high medical-related expenses [11, 12] determines a poor health-related quality of life [13, 14].

Since RA cannot be cured, current treatments aim at reducing the chronic inflammation to slow down the disease and reduce the damage to cartilage, bone and ligaments. Current treatments for RA include non-steroidal anti-inflammatory drugs, steroids, disease-modifying antirheumatic drugs (such as the widely used methotrexane), immunosuppressants, and TNFα inhibitors [15]. One limitation with these treatments (in addition to the fact that they are not curative) is that they manifest serious negative side effects, such as heart problems, liver and kidney damage, increased susceptibility to infections and even increased risk of certain cancers, such as non-melanoma skin cancer [16]. Hence the need and interest

in developing alternative treatments with a more targeted effect and less harmful side effects. One possible strategy would be to use agents with a narrower spectrum of action by blocking a specific target (i.e. PC-PLC) that acts in a specific cell type relevant to the progression of RA, such as monocytes.

The long-term objective of this proposal is indeed the investigation of whether PC-PLC might represent a safer alternative to current RA treatments. Thus the identification of PC-PLC through our proposed experiments will represent a potential new opportunity in the treatment against RA.

Importantly, the experimentation conducted so far has overcome obstacles ensuring that the proposed studies will proceed toward the identification of PC-PLC. In addition, they uncovered important new features of PC-PLC that can be instrumental in the pursue of alternative strategies and the refining of criteria for the planned experiments.

Moreover the results from Task 2 in cell culture on the role of PC-PLC in TNF signaling support a clear role for PC-PLC in the TNF-mediated inflammatory response via regulation of NF-kB. Once PC-PLC will be molecularly identified, we will be able to pin point the exact mechanism of action of PC-PLC in TNF-mediated signaling in monocytes.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS.** Nothing to report.

7. **INVENTIONS, PATENTS AND LICENCES.** Nothing to report.

8. **REPORTABLE OUTCOMES**. Nothing to report.

9. **OTHER ACHIEVEMENTS**. Nothing to report.

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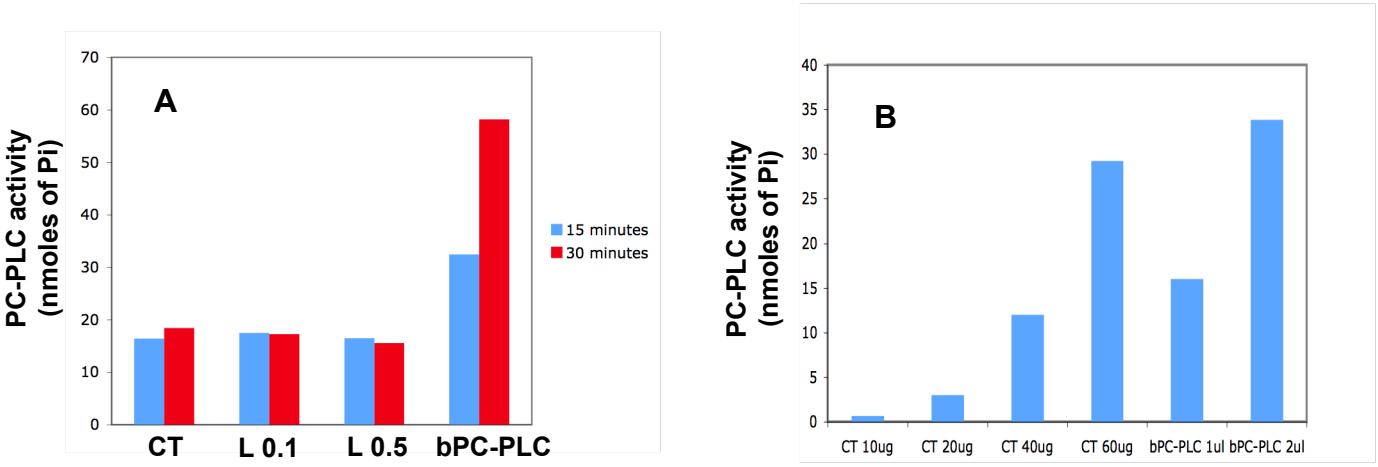

Figure 1. Absence of true enzymatic PC-PLC activity in total lysate of HUVECs as measured according to . A. HUVECs were treated with 0.1 and 0.5 ug/ml of LPS for 24 hours. Total lysates (40ug from each condition) were prepared to measure PC-PLC activity *in vitro* after 15 and 30 minutes of incubation at 37˚C. Purified bacterial PC-PLC (bPC-PLC) was included as positive control. **B.** Different amounts of lysate from control HUVEC cells (CT) were prepared to measure PC-PLC activity *in vitro* after 15 minutes of incubation at 37˚C. Results representative of 2 independent experiments.
CT= control cells; L0.1=cells treated with 0.1 ug/ml LPS; L0.5=cells treated with 0.1 ug/ml LPS.

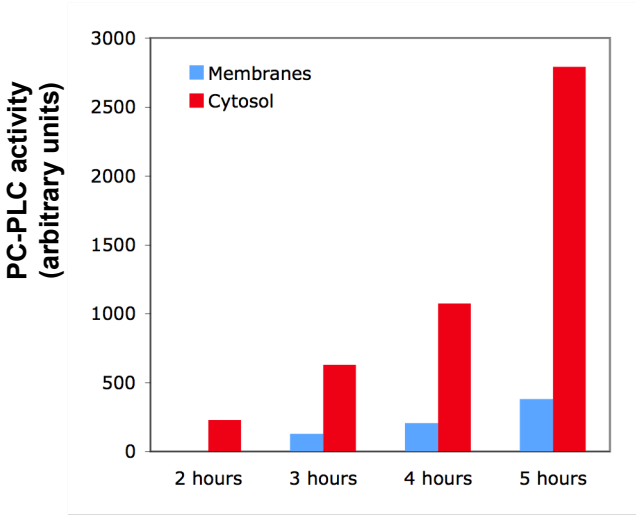

Figure 2. Optimization of an alternative method to measure PC-PLC activity in vitro showing maximum PC-PLC activity in cytosol. A. Total lysates from control HUVECs were fractionated to separate total membranes and cytosol. Equal fractions of membranes and cytosol (corresponding to 50ug of membranes) were compared for PC-PLC activity in vitro at different times of incubation.

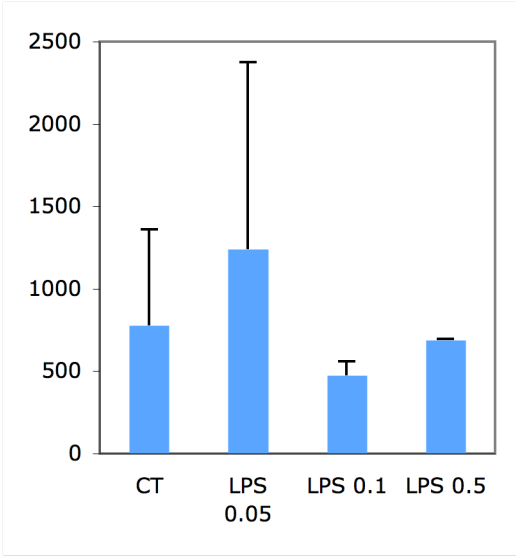

Figure 3. LPS did not activate PC-PLC in HUVECs. Serum starved HUVECs were treated with 0.05, 0.1 and 0.5 ug/ml of LPS for 24 hours. Cytosols (50ug from each condition) were prepared to measure PC-PLC activity *in vitro* and incubated for 4 hours at 37˚C.

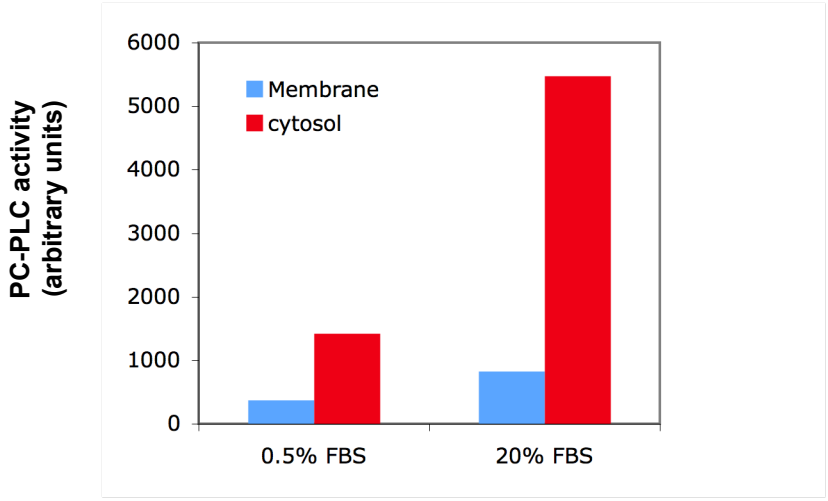

Figure 4. Serum activates PC-PLC in HUVECs. HUVEC cells were either serum starved (0.5% FBS) or serum stimulated (20% FBS) for 24 hours. Membranes and cytosol were prepared and PC-PLC activity was measured in equivalent fractions (corresponding to 50ug of membranes) for 4 hours at 37˚C. Results representative of two independent experiments.

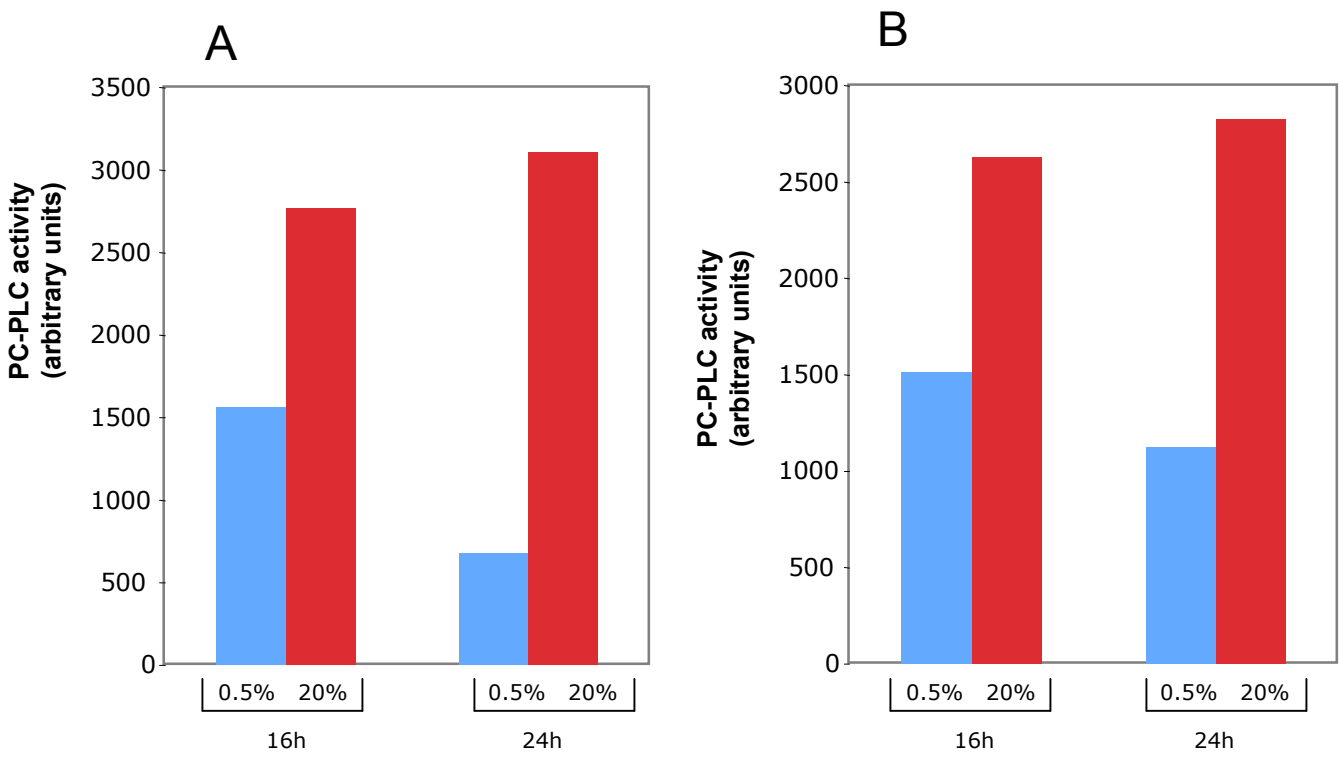

Figure 5. PC-PLC activation following serum stimulation in HUVECs. HUVEC cells were either serum starved (0.5% FBS) or serum stimulated (20% FBS). After 16 and 24hours of treatment, membranes and cytosol were prepared and PC-PLC activity was measured in cytosols. Results are representative of two independent experiments (A and B).

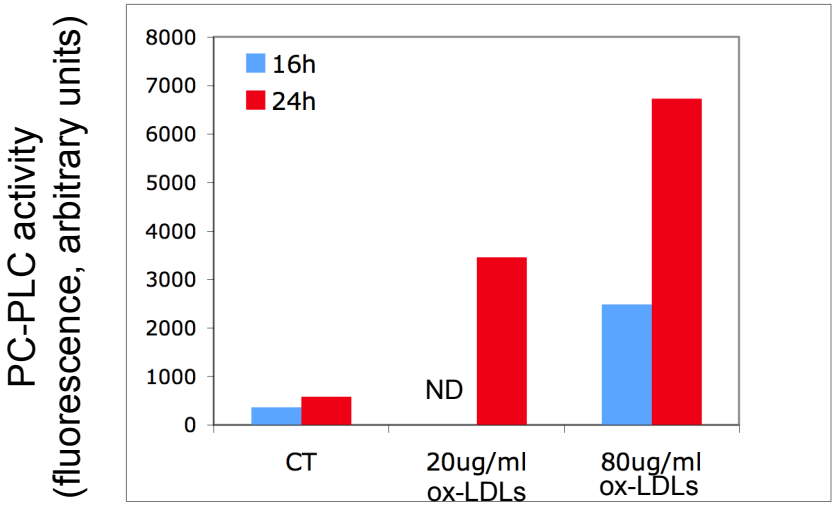

Figure 6. **PC-PLC activation following treatment with oxidized LDLs in HUVECs.** Cytosolic PC-PLC activity was measured in vitro using the Aplex Red Assay kit in HUVECs cells treated with or without 20 or 80ug/ml of oxidized LDLs (ox-LDLs) after 16 or 24 hours of incubation. ND: not determined.

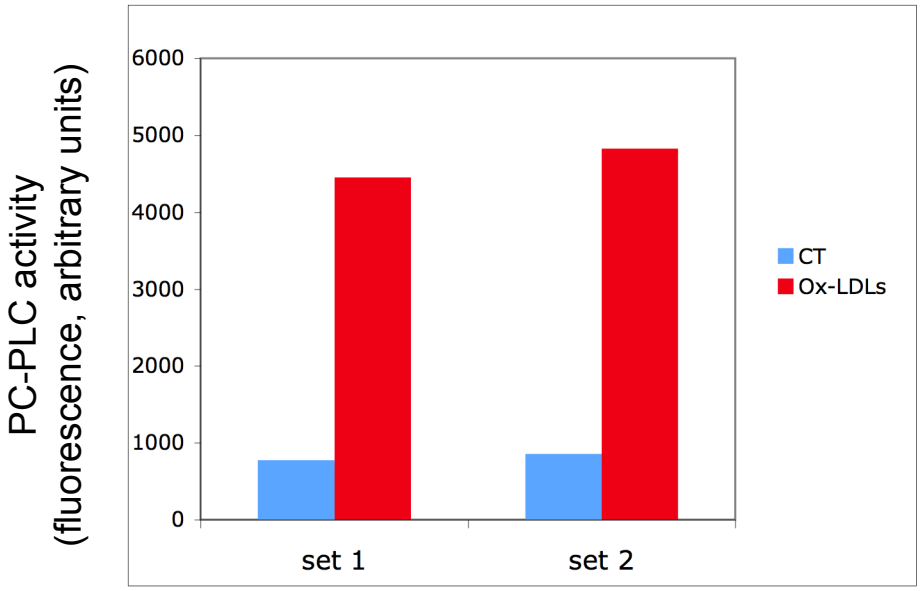

Figure 7. PC-PLC activation following treatment with oxidized LDLs in HUVECs. Cytosolic PC-PLC activity was measured in vitro using the Aplex Red Assay kit in HUVECs cells treated with or without 60ug/ml of oxidized LDLs (ox-LDLs) for 16 hours of incubation. Two sets of samples were collected from two independent experiments and processed for PC-PLC simultaneously.

Table 1. Extraction of mRNA from two sets of HUVECs incubated with 0.5% or 20% serum and corresponding to samples for which the PC-PLC activity was shown in Figure 2 and from two sets of HUVECs incubated with 60ug/ml oxidized LDLs for which the corresponding PC-PLC activity was shown in Figure.

Samples	[mRNA]ug/ml	260/280 nm	260/230 nm
Set 1 - Serum stimulation			
16 h 0.5% FBS	0.672	2.07	2.21
16 h 20% FBS	1.158	2.06	2.19
24 h 0.5% FBS	0.809	2.07	2.22
24 h 20% FBS	1.160	2.07	2.18
Set 2 - Serum stimulation			
16 h 0.5% FBS	0.921	2.08	2.13
16 h 20% FBS	0.982	2.08	1.88
24 h 0.5% FBS	0.731	2.08	2.18
24 h 20% FBS	1.170	2.07	2.18
Set 1 - Oxidized LDLs			
16 h 0.5% FBS	0.814	2.11	2.10
16 h Ox-LDLs	0.963	2.14	2.03
Set 2 - Oxidized LDLs			
16 h 0.5% FBS	0.708	2.11	2.16
16 h Ox-LDLs	0.781	2.11	2.26

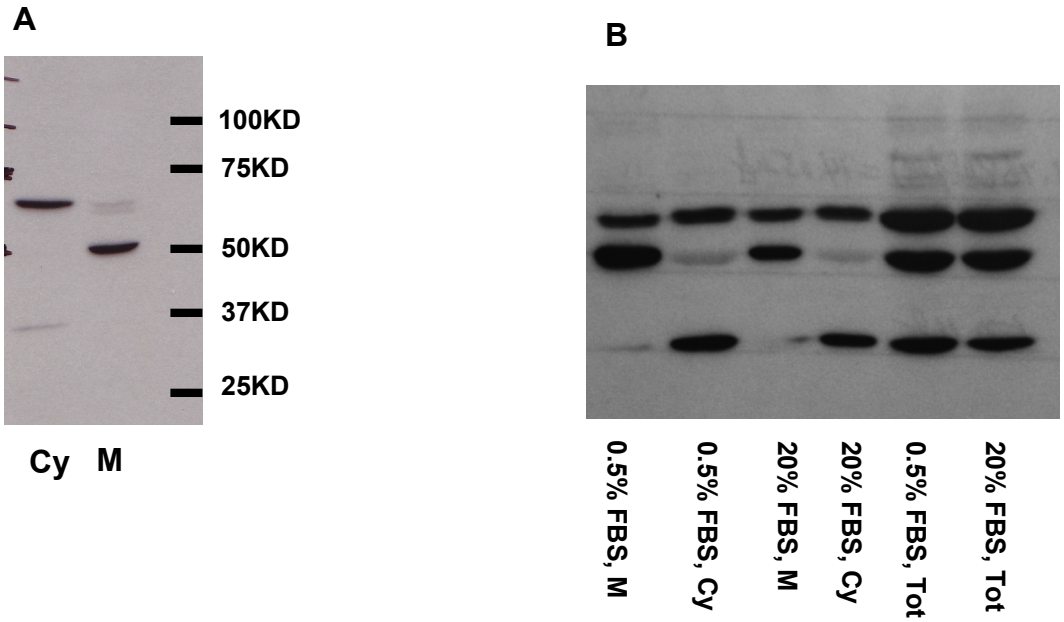

Figure 8. Western blotting using the antibodies against bacterial PC-PLC. A. Lysates from control HUVECs were fractionated to separate total membranes and cytosol. Equivalent fractions of membranes (M) and cytosol (Cy) from control cells (20% FBS) were probed using anti PC-PLC antibodies raised against bacterial PC-PLC. **B.** Membrane and Cytosols were isolated after serum starvation (0.5% FBS) or serum stimulation (20% FBS) and loaded in equal fractions. Equal amount of proteins were also loaded from total lysates (Tot).

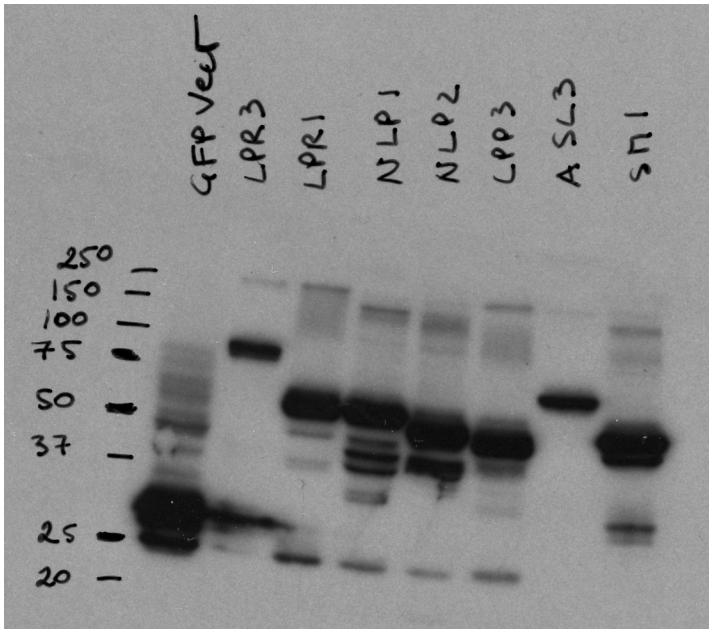

Figure 9. Transfection of different LPPs in Hela cells. Hela cells were transfected with expression plasmids for the different LPPs tagged with GFP and after 24 hours, total proteins were collected and LPP expression was monitored by western blotting using anti-GFP antibodies. SM1 corresponds to BC038108.

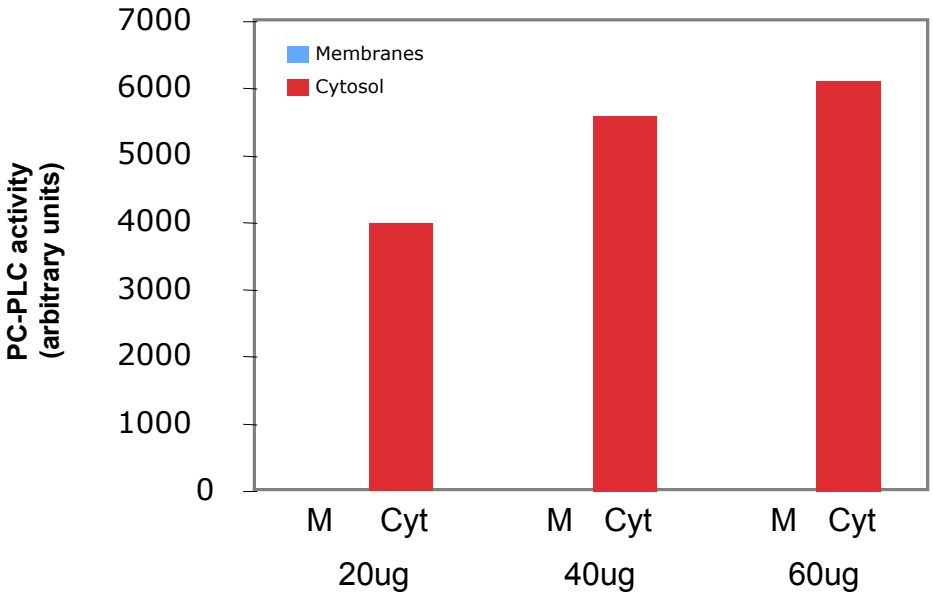

Figure 10. PC-PLC activity in vitro in Hela cells. Total lysates from control Hela cells were fractionated to separate total membranes and cytosol. Equal fractions of membranes (M) and cytosol (Cyt) (corresponding to different amounts of membranes) were compared for PC-PLC activity in vitro. Of note, PC-PLC activity in membrane fractions was undetectable.

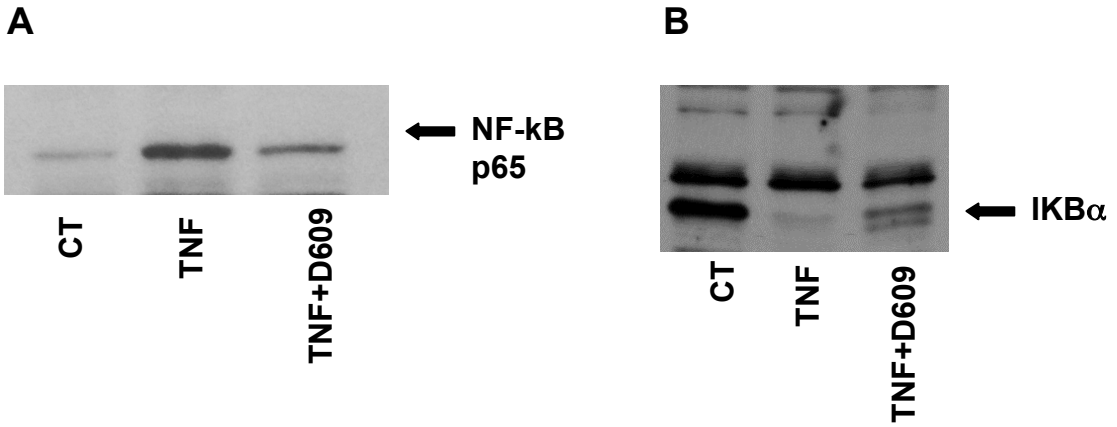

Figure 11. Western blotting of nuclear levels of NF-KB (**A**) and cytoplasmic levels of IKB alpha (**B**) in U937 cells after long term (24 hours) preincubation with 50ug/ml of D609 and 20 minutes treatment with 1nM TNF alpha.

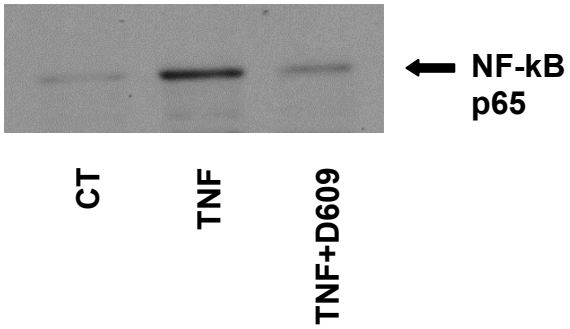

Figure 12. Western blotting of nuclear levels of NF-KB in U937 cells after short term (2 hours) preincubation with 50ug/ml of D609 and 20 minutes treatment with 1nM TNF alpha (T).

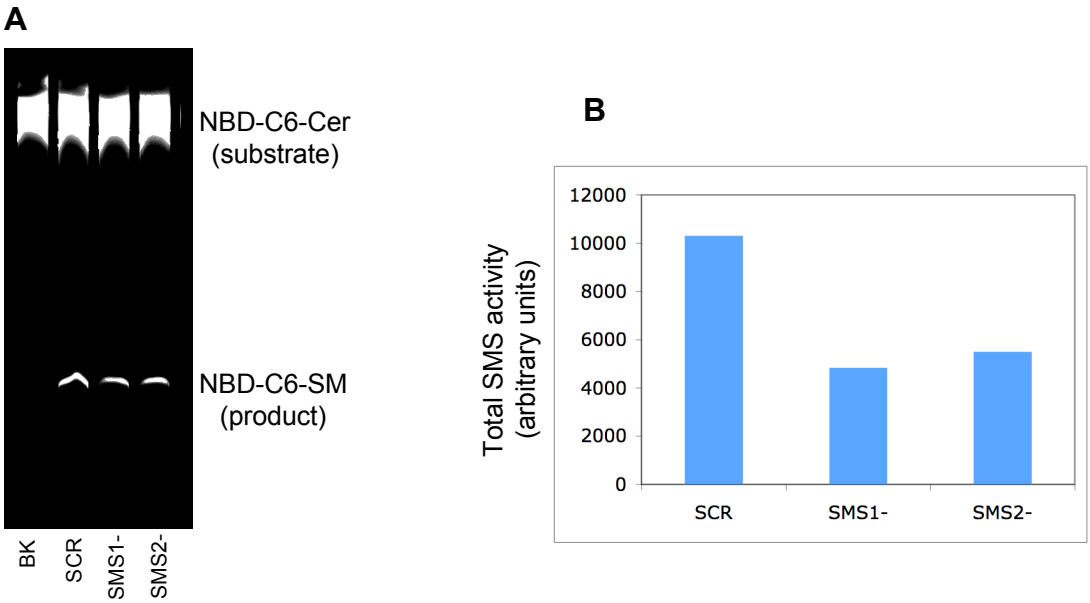

Figure 13. Total SMS *in vitro* activity in lysates of U937 cells after 72 hours of SMS1 (SMS1-) or SMS2 (SMS2-) knockdown with siRNA. **A**: TLC image; **B**: quantification of the fluorescence associated with the produced NBD-C6-SM, product of the SMS reaction. SCR: scrambled control siRNA.

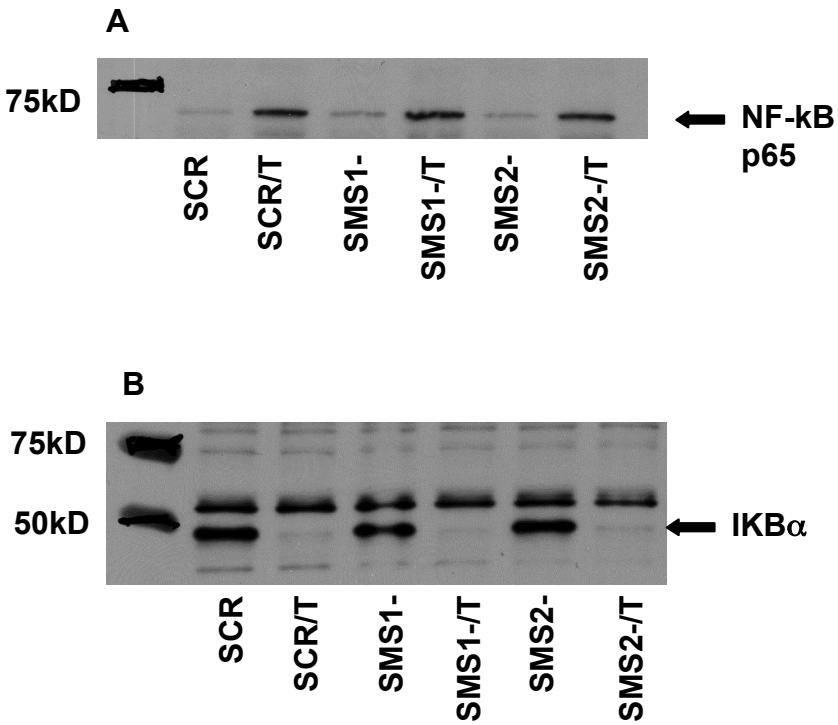

Figure 14. Western blotting of nuclear levels of NF-KB (**A**) and cytoplasmic levels of IKB alpha (**B**) in U937 cells after 72 hours of SMS1 (SMS1-) or SMS2 (SMS2-) knockdown with siRNA and 20 minutes treatment with 1nM TNF alpha (T). SCR: scrambled control siRNA.