

Original Paper

# Sphingosine-1-Phosphate Contributes to TLR9-Induced TNF- $\alpha$ Release in Lung Tumor Cells

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## Key Words

Sphingosine-1-phosphate (S1P) • Toll-like Receptor 9 (TLR9) • TNF- $\alpha$  • Lung inflammation • Lung cancer

## Abstract

**Background/Aims:** Sphingosine-1-phosphate (S1P) is a membrane-derived bioactive phospholipid involved in many lung physiological and pathological processes. Higher levels of S1P have been registered in a broad range of respiratory diseases, including inflammatory disorders and cancer. The aim of our study was to understand the role of S1P in healthy versus tumor cells after Toll-Like Receptors (TLRs) activation, well-known modulators of sphingolipid metabolism. **Methods:** Lung adenocarcinoma cells and non-pathological human fibroblasts were stimulated with unmethylated Cytosine phosphate Guanosine (CpG), the TLR9 ligand, and S1P-dependent TNF- $\alpha$  release was evaluated by means of ELISA. Immunofluorescence and LC-MS/MS analysis were performed to evaluate/quantify S1P generation following TLR9 activation. **Results:** We found that S1P was involved in TLR9-induced TNF- $\alpha$  release in that the inhibition of both ceramidase and sphingosine kinase I/II (SPHK I/II) significantly reduced the levels of TNF- $\alpha$  after TLR9 triggering in lung adenocarcinoma cells. These results were not observed in healthy fibroblasts, implying that this pathway was mainly involved in pathological conditions. Moreover, the activation of TLR4 by means of LPS did not have similar effects as in the case of CpG-stimulated TLR9. Importantly, the activation of TLR9 induced S1P generation and allowed it to interact on the outside membrane receptor S1P<sub>1</sub> and S1P<sub>3</sub> via the efflux through its membrane transporter SPNS2. Indeed, both the blockade of S1P<sub>3</sub> and the transporter SPNS2 significantly reduced the activity of S1P on TNF- $\alpha$  release from lung adenocarcinoma cells. **Conclusion:** Our study identifies a novel inflammatory pathway in that TLR9 increases the pro-inflammatory cytokine release, such as TNF- $\alpha$ , via the induction of a ceramide/S1P imbalance in favor of S1P, adding a novel puzzle piece in TLR9-orchestrated inflammatory pathway and shedding more light on the role of the higher levels of S1P during inflammatory conditions.

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## Introduction

Sphingolipids are bioorganic compounds that have been extensively considered as solely involved to modulate the physical properties of membranes, including fluidity and thickness, as well as to influence the activity and spatial organization of membrane proteins [1]. However, recent advances recognized sphingolipids as second messengers or as secreted ligands for cell-surface receptors. The primary signaling sphingolipids belong to the family of sphingosines, long chain bases, and ceramides, as well as their phosphorylated derivatives, which can regulate cellular homeostasis, being involved in apoptosis, cell survival, cell architecture, proliferation and differentiation, immune cell trafficking, endothelial barrier integrity, smooth muscle tone [2, 3]. One of the best characterized sphingolipids signaling is about sphingosine-1-phosphate (S1P). Sphingosine, a backbone structure of sphingolipids, is generated by ceramidase, an enzyme that converts ceramide into sphingosine, which can be generated by *de novo* synthesis through the condensation of serine with palmitoyl-CoA, or it can be generated by sphingomyelin hydrolysis due the activity of the acid and/or neutral sphingomyelinases [4]. The phosphorylation of sphingosine to sphingosine-1-phosphate (S1P) is catalyzed by sphingosine kinases, SPHK I and/or SPHK II. Once S1P is generated, it is transported out of the cell by Spinster homolog 2 (SPNS2), a non-ATP-dependent organic ion transporter, and it can act in a paracrine and/or autocrine manner by activating sphingosine-1-phosphate receptors (S1PRs) in an inside-out mode [3]. Five G protein-coupled S1PRs have been identified, S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, S1P<sub>4</sub> and S1P<sub>5</sub>, involved in many physiological and pathological processes, including cell growth, apoptosis, trafficking, differentiation and activation of immune cells, inflammation, cellular architecture and vascular integrity regulation. In particular, sphingolipids are involved in the maintenance of normal lung structure and function, attributing a pro-survival role to S1P opposed to the pro-apoptotic activity of the upstream ceramide [5]. An imbalance between S1P and ceramide levels in favor of S1P, leads to a broad range of respiratory diseases, including inflammatory disorders and cancer [6-9]. Indeed, a growing body of studies suggests a crucial role of S1P in mediating and amplifying lung inflammatory responses associated to cell proliferation [9-11].

Nevertheless, the upstream stimulus/i that regulate the generation of S1P and its signaling are still unclear. Therefore, the aim of our study was to understand the role of the sphingosine rheostat in Toll-Like Receptors (TLRs) activation, well-known modulators of sphingolipids metabolism [12] and regulators of many pulmonary physiological and pathological responses [13-15]. We focused our attention on TLR4 and TLR9 as mainly involved in pro-inflammatory respiratory pathways [16-19].

We found that the activation of TLR9, but not of TLR4, induced ceramidase activation leading to S1P formation/overexpression, resulting in NF- $\kappa$ B-dependent TNF- $\alpha$  release by lung tumor cells but not by non-pathological cells.

## Materials and Methods

### Cell cultures

Human lung adenocarcinoma A549 cells line (ATCC® CCL-185™) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cambrex Biosciences, Microtech, Naples, Italy) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine (Cambrex Biosciences, Microtech, Naples, Italy), in an atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were seeded (3x10<sup>4</sup>/well) and treated for 8 hours with lipopolysaccharides (LPS 0.1  $\mu$ g/ml; Alexis, Vincibiochem, Italy), unmethylated Cytosine phosphate Guanosine-oligodeoxynucleotides, CpG-ODN (CpG 1  $\mu$ g/ml; Vincibiochem, Milan, Italy), sphingosine-1-phosphate (S1P 10 nM; Sigma-Aldrich, Merck Life Science S.r.l., Milan, Italy), anti-Spinster homolog 2 antibody ( $\alpha$ SPNS2 10 ng/ml; Abcam, Milan, Italy), ceramidase inhibitor (D-NMAPPD 5  $\mu$ M; Sigma-Aldrich, Merck Life Science S.r.l., Milan, Italy), proteasome inhibitor (MG132 10  $\mu$ M; Sigma-Aldrich, Merck Life Science S.r.l., Milan, Italy), W146, a S1P<sub>1</sub> receptor antagonist (S1P<sub>1</sub>a 1  $\mu$ M; Tocris Bioscience,

Ellisville, MO), JTE-013, a S1P<sub>2</sub> receptor antagonist (S1P<sub>2</sub>a 100 nM; Tocris Bioscience, Ellisville, MO), TY52156, a S1P<sub>3</sub> receptor antagonist (S1P<sub>3</sub>a 10  $\mu$ M; Tocris Bioscience, Ellisville, MO), SKI II, a selective inhibitor of sphingosine kinases (SKI II 10  $\mu$ M; Tocris Bioscience, Ellisville, MO). The experimental time point for TNF- $\alpha$  evaluation was 8 hours; it was chosen upon preliminary time-dependent data in which we performed experiments at 5, 8 or 18 hours.

Human primary fibroblast (ATCC® PCS-201-012™), used as control since non-pathological cells, were cultured in DMEM supplemented with 20% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine, in an atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were seeded (1x10<sup>4</sup>/well) and treated for 8 hours with LPS 0.1  $\mu$ g/ml or CpG 1  $\mu$ g/ml.

### *Cytokine measurements*

TNF- $\alpha$  was measured in cell-free supernatants, obtained after 8 hours of cell treatment, by means of a commercially available enzyme-linked immunosorbent assay kit (ELISAs) (Diaclone SAS, France). The absorbance wavelength was 450 nm. Cytokine levels were expressed as pg/ml.

### *Western blotting analysis*

The expression of the receptor S1P<sub>1</sub> (Santa Cruz Biotechnologies, Heidelberg, Germany), S1P<sub>2</sub> (Santa Cruz Biotechnologies, Heidelberg, Germany), S1P<sub>3</sub> (Alomone labs, Jerusalem, Israel) and SPNS2 (Abcam, Milan, Italy), a crucial lysosphingolipid S1P transporter involved in S1P secretion, was evaluated on A549 cells, by means of SDS-PAGE. The expression of ceramidase (N-acylsphingosine amidohydrolase 1, ASAH1; Elabscience; US) was evaluated in S1P (10nM), LPS (0.1  $\mu$ g/ml) or CpG (1  $\mu$ g/ml) treated cells, 3 hours after treatment.

### *Immunofluorescence analysis*

A549 cells (5x10<sup>5</sup>/well) were analyzed for the presence of FITC-conjugated S1P (Echelon Biosciences Inc., Salt Lake City, UT), after 3 hours of treatment with CpG (1  $\mu$ g/ml). To stain cell nuclei, DAPI (4',6-diamidino-2-phenylindole) was used. Images were observed by means of Carl Zeiss confocal microscopy (magnification: 40X).

### *S1P extraction*

Lipid extraction followed the Folch method under acidic conditions [20]. Briefly, 45 $\mu$ l of 18% hydrochloric acid, 900 $\mu$ l of mixture CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (4:1:0.8) were added to 75 $\mu$ g of cell lysates. Samples were stirred for 1 hour, sonicated for 30 minutes and centrifuged at 21000 g for 30 minutes. The lower organic phase was well separated, transferred to a new centrifuge tube and evaporated to dryness at 60°C in the vacuum rotator. Samples were reconstituted in 35 $\mu$ l MeOH, vortexed for 5 minutes, sonicated for 30 minutes, centrifugated for 10 minutes at 10000 g and 10 $\mu$ l were loaded on the UPLC-MSMS system.

### *LC-MS/MS analysis*

S1P was quantified by liquid chromatography coupled to tandem mass spectrometry detection (LC-MS/MS) on Shimadzu LC-20A and Auto Sampler systems and QTRAP 6500 instrument from AB-Sciex. C18 chromatographic column (Kinetex C18, 50 x 2.1 mm, 5 $\mu$ m, Phenomenex) was used for chromatographic separation. The mobile phase was composed of water 0.1% formic acid (mobile phase A) and methanol 0.1% formic acid (mobile phase B). The flow rate was set at 400 $\mu$ l/minute. The mass spectrometer was set in the positive ion mode (ESI<sup>+</sup>) with an electrospray voltage of 5500 V at 400°C of the heated capillary temperature. The multiple reaction monitoring (MRM) mode and the Analyst 1.6.2 software has been used. S1P was analysed with the mass transition 380 m/z to 264m/z and it was observed at the rt of 8.20.

Nitrogen was used as the air curtain gas (20psi), atomizing gas (30psi), auxiliary gas (60psi) and collision gas (4psi). Dwell time was 100ms, DP was 74v, EP was 10v, CE was 22v and CXP was 15v. For quantitative analysis, a standard curve with S1P amounts of 100pg, 25pg, 10pg and 2.5pg was generated. In parallel, 1ng, 250pg, 100pg and 25pg of S1P were added to lysates, extracted as reported above and resuspended in 37.5 $\mu$ l MeOH and 10 $\mu$ l were applied to the column.

## Statistical analysis

Data are reported as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed by using Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test, or by Mann-Whitney test where appropriate; p values less than 0.05 were considered as significant. The statistical analysis was performed by using GraphPad prism 9.0.0 version (San Diego, USA). All values in text are shown as median [95% confidence limit].

## Results

### *Sphingosine-1-Phosphate is involved in CpG-mediated TNF- $\alpha$ release*

It is known that TLR4 and TLR9 are involved in physiological and pathological pulmonary conditions [13, 14]. Therefore, we treated lung epithelial adenocarcinoma cells (A549 cells) and non-pathological human fibroblasts with a TLR4 ligand, LPS (0.1  $\mu$ g/ml), or a TLR9 ligand, CpG (1  $\mu$ g/ml). The stimulation of cells with LPS statistically increased TNF- $\alpha$  release from A549 cells (Fig. 1A), but not from fibroblast (Fig. 1B). Similarly, the stimulation of A549 cells with CpG significantly increased the levels of TNF- $\alpha$  (Fig. 1C), effect that was not observed in fibroblasts (Fig. 1D). Because sphingolipid metabolism was suggested in TLR triggering [12], we went on by using a polyclonal antibody to block SPNS2 transporter ( $\alpha$ SPNS2, 10 ng/ml) or/and a potent ceramidase inhibitor [21], D-NMAPPD (5  $\mu$ M). The inhibition of the ceramidase (Fig. 1E) or of the SPNS2 transporter (Fig. 1F) did not alter LPS-induced TNF- $\alpha$  release, as well as the simultaneous inhibition of both SPNS2 and ceramidase (Fig. 1G). In sharp contrast, the blockade of ceramidase (Fig. 1H) or SPNS2 (Fig. 1I) significantly reduced the levels of TNF- $\alpha$  in CpG-stimulated A549 cells. In addition, the levels of TNF- $\alpha$  after CpG treatment were almost completely abolished when both SPNS2 transporter and ceramidase were blocked (Fig. 1J).

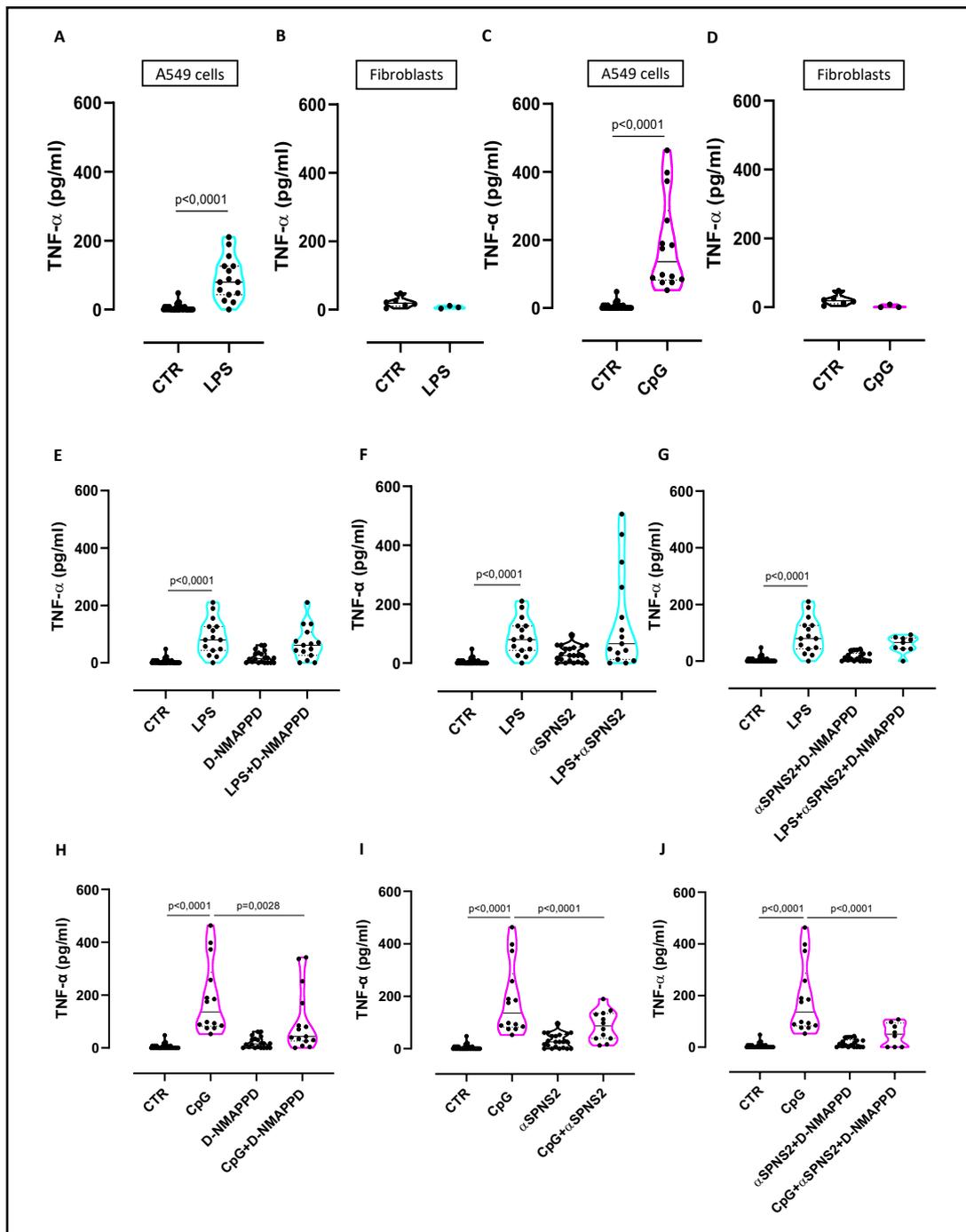
Taken together, these data imply that LPS-dependent TNF- $\alpha$  release from lung adenocarcinoma cells was regardless of S1P signaling. On the contrary, the release of TNF- $\alpha$  induced by TLR9 stimulation was S1P dependent, in that the blockade of both ceramidase and the inside-out S1P transporter, completely reduced the levels of the cytokine.

### *TLR9 activation modulates S1P metabolism*

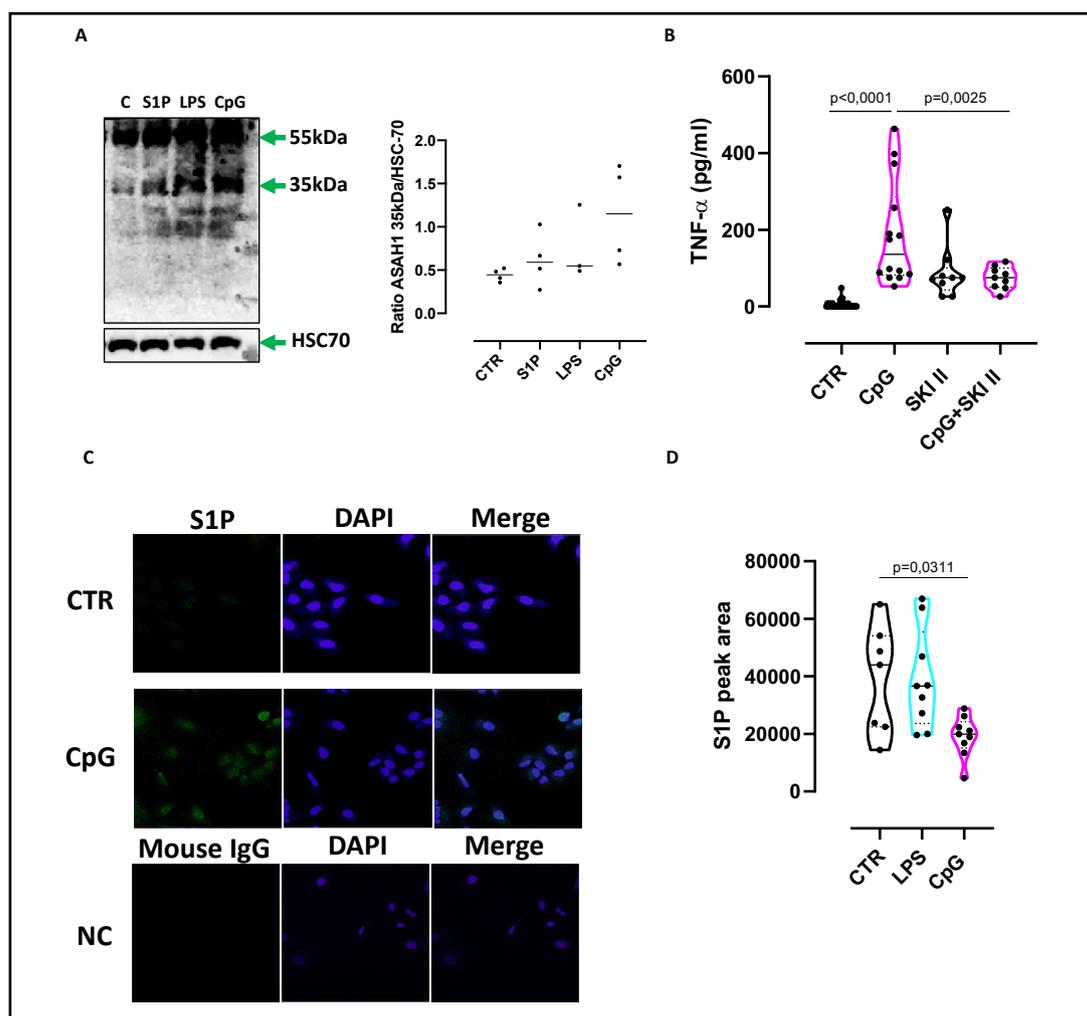
To prove the existence of a link between TLR9, but not TLR4, activation-dependent TNF- $\alpha$  release and S1P signaling, we moved on verifying whether CpG was able to induce S1P synthesis.

A549 cells were first treated with LPS, CpG or S1P for 3 hours and ceramidase expression was evaluated (Fig. 2A). Ceramidase was expressed in its inactive form (55kDa) at all performed treatments (Fig. 2A). Interestingly though, the active ceramidase, identified by the 35kDa band by means of western blot, was higher expressed when cells were stimulated with CpG compared to control and LPS- or S1P-stimulated cells (Fig. 2A). Since the activation of ceramidase does not necessarily imply S1P synthesis, but the sole formation of sphingosine, we treated cells with an inhibitor of sphingosine kinase (SPHK) I/II (SKI II 10  $\mu$ M) [22], responsible of the phosphorylation of sphingosine once generated by ceramidase. The inhibition of SPHK I/II significantly reduced CpG-induced TNF- $\alpha$  release (Fig. 2B).

To further prove of S1P generation followed to TLR9 triggering, we analyzed the levels of S1P by using two different methodologies, immunofluorescence and LC-MS/MS. Immunofluorescence experiments revealed that the stimulation of A549 cells with CpG increased the levels of membrane S1P after 3 hours of CpG treatment compared to control (Fig. 2C). Similarly, LC-MS/MS resulted in lower S1P after CpG treatment in cell lysate (free of membrane components) (Fig. 2D), further supporting the inside-out mode of action of TLR9-induced S1P that could interfere with outer membrane receptors, confirming our previous data on the efflux of S1P through SPNS2. The difference between these two latter experiments was based on the fact that immunofluorescence was performed on intact cells, whereas LC-MS/MS was performed on membrane-free cell lysates, implying an inside-out signaling of S1P on its outer receptors after its generation due to TLR9 triggering.



**Fig. 1.** S1P is involved in CpG-mediated TNF- $\alpha$  release. Lung adenocarcinoma A549 cells and normal human fibroblasts were stimulated with TLR4 ligand, LPS 0,1  $\mu$ g/ml or TLR9 ligand, CpG 1  $\mu$ g/ml for 8 hours and TNF- $\alpha$  release was evaluated. LPS (A and B) and CpG (C and D) stimulation induced TNF- $\alpha$  release from A549 cells (A and C), but not from fibroblast (B and D). TNF- $\alpha$  release from A549 was not altered when ceramidase (E) or/and SPNS2 transporter (F and G) were blocked, after LPS addition. Ceramidase (H) or/and SPNS2 transporter (I and J) inhibition induced a statistically significant reduction of TNF- $\alpha$  release, after CpG stimulation of adenocarcinoma cells. Data are presented as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed with Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test. Ceramidase inhibitor (D-NMAPPD, 5  $\mu$ M); anti-Spinster homolog 2 antibody ( $\alpha$ SPNS2, 10 ng/ml).



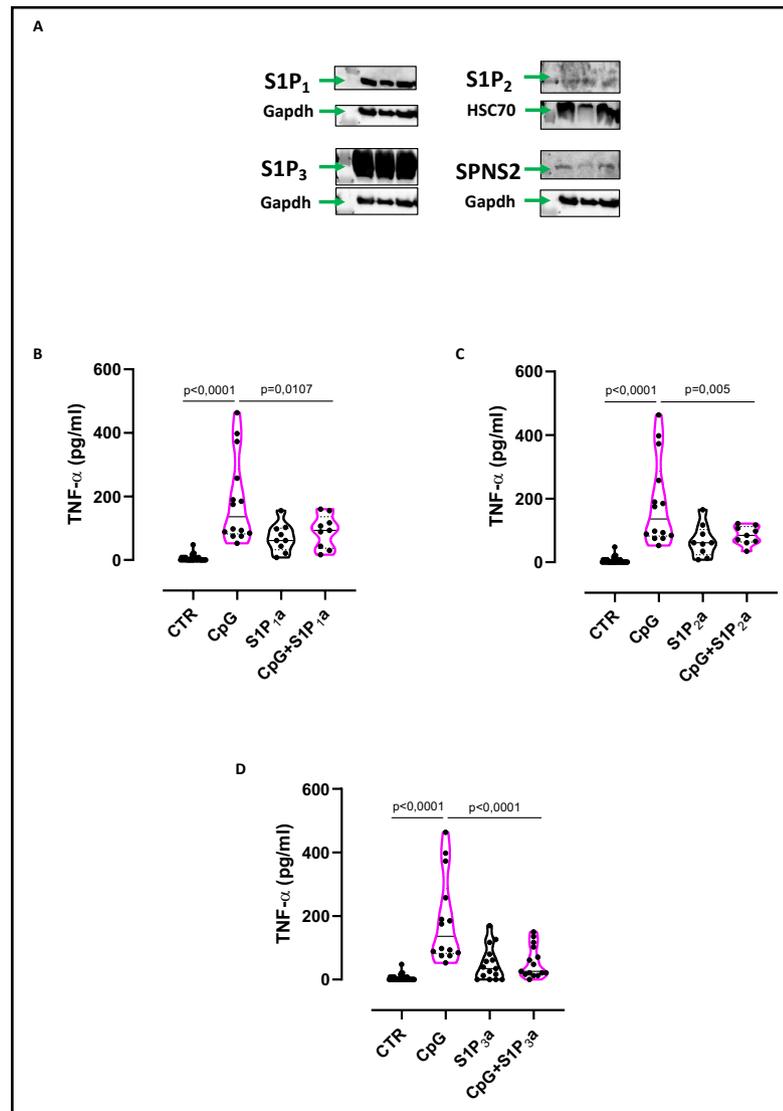
**Fig. 2.** CpG signaling alters S1P metabolism. Lung adenocarcinoma A549 cells were stimulated with TLR9 ligand, CpG 1  $\mu$ g/ml for 3 hours. A) Western blotting analysis show that CpG 1  $\mu$ g/ml 3 hours treated A549 cells express high levels of ceramidase active form (35kDa) compared to control and S1P 10 nM- or LPS 0,1  $\mu$ g/ml-stimulated cells. HSC70 was used as loading protein. Densitometric analysis is shown. B) TNF- $\alpha$  release from 8 hours-treated A549 was significantly reduced when SPHKs were inhibited by means of SKI II, in presence of CpG. C) Immunofluorescence analysis show S1P overexpression after CpG treatment compared to control; negative control (NC) was also shown. D) LC-MS/MS experiment show that the levels of S1P in cell lysates (expressed as S1P peak area) were reduced after 3 hours of CpG treatment compared to control and LPS-stimulated cells. Data are presented as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed with Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test. SKI I/II, selective inhibitor of sphingosine kinases (SKI I/II 10  $\mu$ M).

Taken together, these data demonstrate that CpG is involved in the alteration/modulation of sphingolipid metabolism, in that it induces ceramidase activation and SPHK activity, leading to S1P formation responsible of TLR9-dependent TNF- $\alpha$  release.

#### *TLR9/S1P axis-mediated TNF- $\alpha$ release is mainly S1P<sub>3</sub>-dependent*

To further prove the inside-out signaling of S1P after TLR9 activation, we first evaluated the expression of S1P receptors and SPNS2 transporter on A549 cells, and then evaluated whether the blockade of S1PRs could alter TLR9-induced TNF- $\alpha$  release. We found that A549 cells expressed S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub> receptors and SPNS2 transporter (Fig. 3A); however, it looked like that S1P<sub>1</sub> and S1P<sub>3</sub> were higher expressed than S1P<sub>2</sub> (Fig. 3A).

**Fig. 3.** TNF- $\alpha$  release mediated by TLR9/S1P-axis is S1P3 dependent. A) A549 expression of S1P<sub>1</sub> (43kDa), S1P<sub>2</sub> (42kDa), S1P<sub>3</sub> (55-70kDa) and SPNS2 (70kDa) evaluated by western blotting assay. GAPDH and HSP70 were used as loading protein. Lung adenocarcinoma A549 cells were stimulated with TLR9 ligand, CpG 1  $\mu$ g/ml for 8 hours and TNF- $\alpha$  release was evaluated after the inhibition of S1P receptors. The inhibition of both S1P<sub>1</sub> (B), S1P<sub>2</sub> (C) and S1P<sub>3</sub> (D) significantly reduced the release of TNF- $\alpha$  after CpG stimulation. Data are presented as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed with Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test. W146, S1P<sub>1</sub> receptor antagonist (S1P<sub>1</sub>a, 1  $\mu$ M), JTE-013, S1P<sub>2</sub> receptor antagonist (S1P<sub>2</sub>a, 100 nM), TY52156, S1P<sub>3</sub> receptor antagonist (S1P<sub>3</sub>a, 10  $\mu$ M).



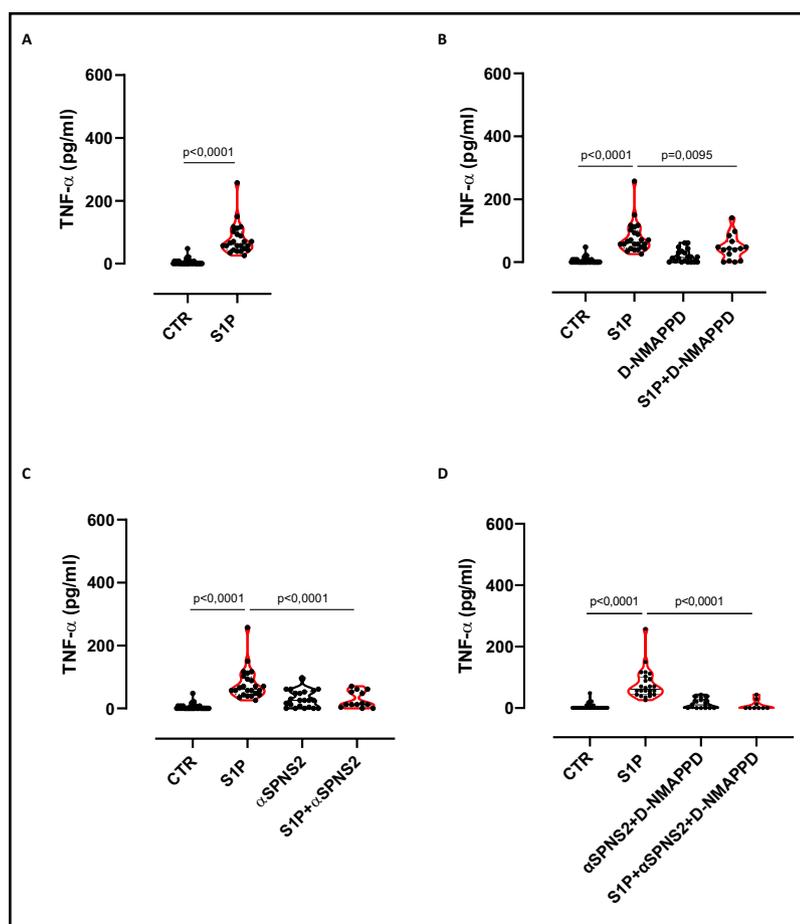
To determine which S1P receptor was mainly involved in TLR9/S1P/TNF- $\alpha$  axis, A549 cells were treated with S1P receptors antagonists in the presence of CpG. The inhibition of S1P<sub>1</sub> (Fig. 3B), S1P<sub>2</sub> (Fig. 3C) and S1P<sub>3</sub> (Fig. 3D) significantly reduced the release of TNF- $\alpha$  induced by CpG stimulation. However, it is noteworthy that among the three used antagonists, the S1P<sub>3</sub>a, in presence of CpG, markedly greater reduced TNF- $\alpha$  levels (25.62 [16.78-102.60] pg/ml; Fig. 3D) compared to the inhibition of S1P<sub>1</sub> (93.37 [30.06-155.00] pg/ml; Fig. 3B) and S1P<sub>2</sub> (84.16 [61.39-116.70] pg/ml; Fig. 3C), with respect to the CpG production (136.3 [75.01-372.9] pg/ml).

Altogether, these data imply that TNF- $\alpha$  release induced by TLR9/S1P axis is predominantly S1P<sub>3</sub>-dependent.

#### TLR9/S1P-axis mediated TNF- $\alpha$ release is NF- $\kappa$ B-dependent

In order to understand whether S1P was a step in TLR9-induced TNF- $\alpha$  release, we treated cells with exogenous S1P (10 nM). S1P significantly increased TNF- $\alpha$  levels (Fig. 4A), although this effect was much higher when CpG was added to cells (136.3 [75.01-372.9] pg/ml) than exogenous S1P stimulation (61.40 [47.89-93.37] pg/ml). Nevertheless, exogenous S1P-induced TNF- $\alpha$  release was reverted by the inhibition of the ceramidase (Fig. 4B) or of S1P transporter (SPNS2) (Fig. 4C) or both (Fig. 4D), implying that the exogenous S1P could

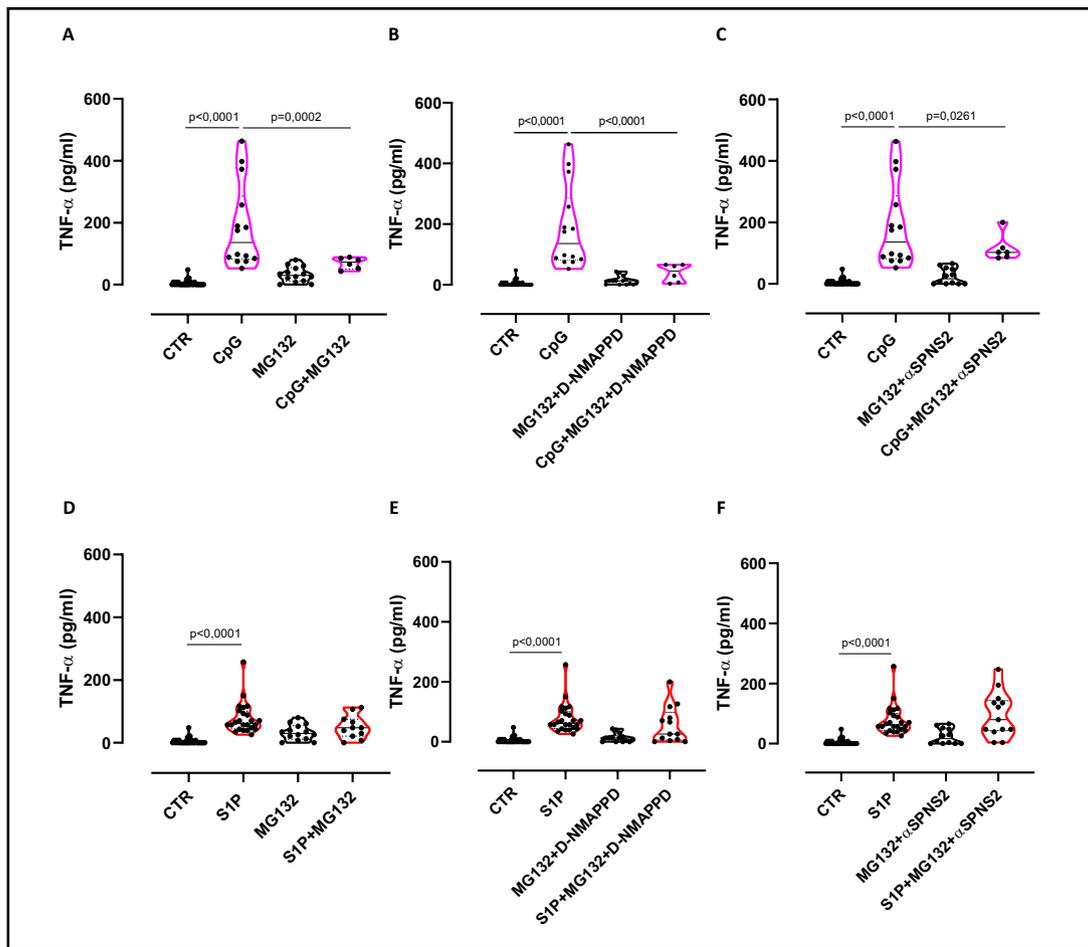
**Fig. 4.** S1P induces TNF- $\alpha$  release from A549 cells. A) S1P induced the release of TNF- $\alpha$  from A549 cells after 8 hours treatment. A significantly reduction of TNF- $\alpha$  levels was detected when ceramidase (B) or/and SPNS2 transporter (C and D) were inhibited after S1P stimulation. Data are presented as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed with Mann-Whitney test and Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test. Ceramidase inhibitor (D-NMAPPD, 5  $\mu$ M), anti-Spinster homolog 2 antibody ( $\alpha$ SPNS2, 10 ng/ml).



be able to induce the synthesis of endogenous S1P via ceramidase activation. This data is further supported by the induction of the active form of the ceramidase after S1P addition to the cells (Fig. 2A). Therefore, we were prompted to suppose that either in TLR9 signaling or after exogenous S1P addition (as well as after S1P ligation to its receptor), the endogenous S1P synthesis could affect TNF- $\alpha$  levels.

To understand the molecular mechanism/s by which TLR9 signaling could be associated to S1P-correlated TNF- $\alpha$  release, and because TLR9-dependent TNF- $\alpha$  release underlies NF- $\kappa$ B [23], lung adenocarcinoma cells were treated with a proteasome inhibitor MG132, blocking NF- $\kappa$ B activation. The inhibition of NF- $\kappa$ B significantly reduced TNF- $\alpha$  release induced by CpG treatment (Fig. 5A). The inhibition of both NF- $\kappa$ B and ceramidase, still reduced TNF- $\alpha$  release after CpG treatment (Fig. 5B). Similar data were observed when SPNS2 and NF- $\kappa$ B were inhibited (Fig. 5C).

The release of TNF- $\alpha$  was not statistically altered when cells were co-treated with S1P and MG132 (Fig. 5D); however, when ceramidase (Fig. 5E) or SPNS2 transporter inhibitor (Fig. 5F), were added, inactive NF- $\kappa$ B reverted what observed in Fig. 4B and 4C. These data may imply that NF- $\kappa$ B activation is relevant for exogenous S1P-induced TNF- $\alpha$  release.

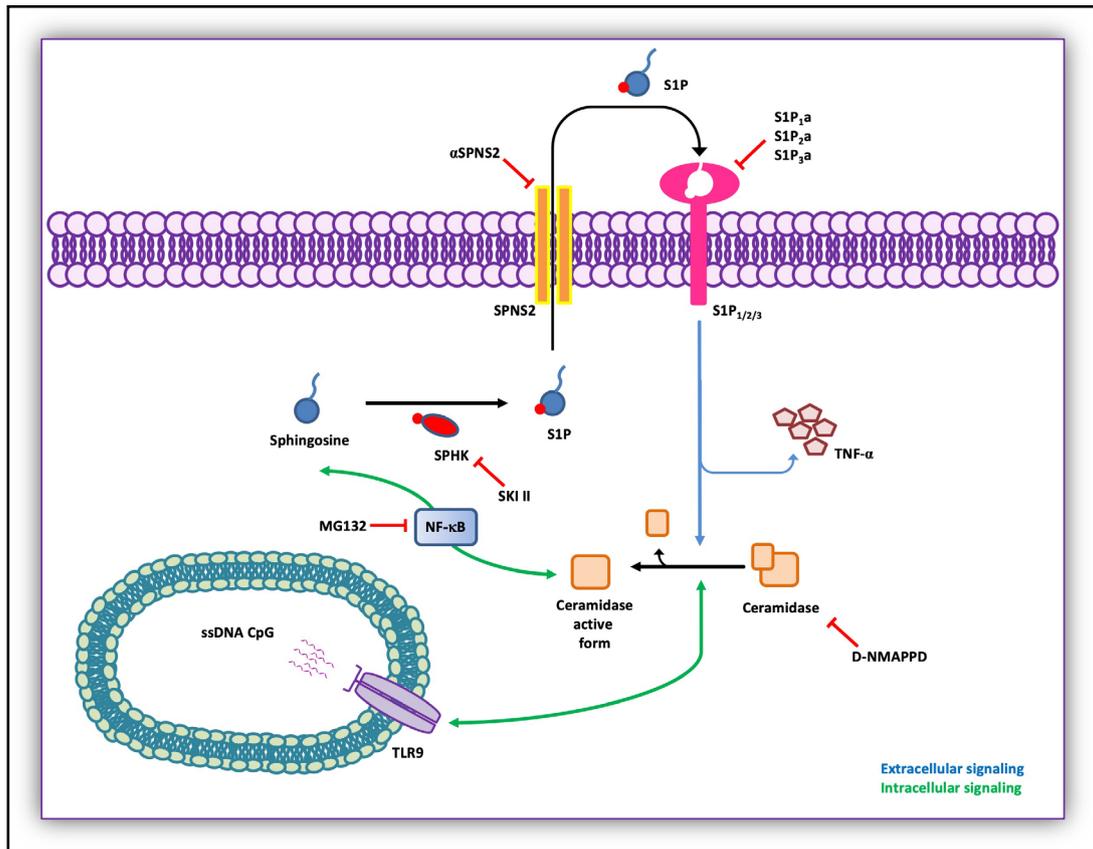


**Fig. 5.** TNF- $\alpha$  release mediated by TLR9/S1P-axis is NF- $\kappa$ B-dependent. Lung adenocarcinoma A549 cells were stimulated with TLR9 ligand, CpG 1  $\mu$ g/ml or S1P 10nM for 8 hours and TNF- $\alpha$  release was evaluated. TNF- $\alpha$  release was significantly reduced after CpG+MG132 (A), CpG+MG132+D-NMAPPD (B), CpG+MG132+ $\alpha$ SPNS2 (C) treatment, compared to the sole CpG addition. D) S1P-induced TNF- $\alpha$  release was not NF- $\kappa$ B-dependent. When both ceramidase and NF- $\kappa$ B (E) or SPNS2 transporter and NF- $\kappa$ B (F) were inhibited, TNF- $\alpha$  levels were not significantly decreased after S1P addition. Data are presented as violin plot, showing the median  $\pm$  interquartile range. Statistical differences were assessed with Ordinary ONE-Way ANOVA followed by Tukey's multiple comparison test. Proteasome inhibitor (MG132, 10  $\mu$ M), ceramidase inhibitor (D-NMAPPD, 5  $\mu$ M), anti-Spinster homolog 2 antibody ( $\alpha$ SPNS2, 10 ng/ml).

## Discussion

In this study we found that the activation of TLR9, but not of TLR4, is involved in the generation of S1P by means of ceramidase and SPHK I/II activation in tumor cells but not in normal, healthy cells. Through an inside-out signaling, newly formed S1P supports TLR9/NF- $\kappa$ B-mediated TNF- $\alpha$  release mainly via S1P<sub>3</sub> receptor interaction (Fig. 6).

Despite the bioactive phospholipid S1P is a well-known lung pro-inflammatory/proremodeling/pro-survival agent [7, 11, 24, 25], what is known about its upstream regulators is really fuzzy and unclear. We propose a new and never investigated mechanism, to our knowledge, that identifies S1P as one of the second messenger in TLR9 signaling for the induction of pro-inflammatory cytokines, such as TNF- $\alpha$ . Recently, TLRs have been identified on tumor cells, where their activation may orchestrate the downstream signaling pathways that play crucial functions in tumorigenesis and tumor progression. Specifically, TLR4 and



**Fig. 6.** S1P is involved in TLR9-mediated TNF- $\alpha$  release. TLR9 stimulation by means of CpG induces ceramidase activation and an increased formation of S1P through SPHKs. S1P extruded from the cell through its transporter SPNS2 induces TNF- $\alpha$  release interacting on its receptors.  $\alpha$ SPNS2, Anti-Spinster homolog 2 antibody blocking SPNS2 transporter; D-NMAPPD, a ceramidase inhibitor; SKI II, selective inhibitor of sphingosine kinases; S1P1a, S1P1 receptor antagonist (W146), S1P2a, S1P2 receptor antagonist (JTE-013), S1P3a, S1P3 receptor antagonist (TY52156), proteasome inhibitor (MG132).

TLR9 are among the most characterized TLRs that have been related to the growth of lung cancer cells [17-19], although their roles and fundamental mechanisms in lung cancer progression need to be further elucidated. We found that LPS-dependent TNF- $\alpha$  release from lung adenocarcinoma cells was not related to S1P signaling. Instead, TLR9 stimulation by means of CpG increased the activation of the ceramidase and led to endogenous S1P formation that through its membrane transporter, SPNS2, mediated TNF- $\alpha$  release, a well-known pro-inflammatory cytokine [26], predominantly via the interaction with S1P<sub>3</sub> (Fig. 6). Indeed, the exogenous S1P was able to induce TNF- $\alpha$ , although not at the same extent as CpG. It is noteworthy that, in our preliminary data, we found that higher concentration than 10 nM of exogenous S1P were not able to induce further release of TNF- $\alpha$ , describing a bell-shaped curve which could be expression of G-coupled receptors which undergo to saturation or even to desensitization mode resulting in the receptor inactivation. Nevertheless, the exogenous S1P was still able to induce ceramidase activation, implying a pro-inflammatory loop after the generation of endogenous S1P. The exogenous S1P could mimic TLR9-induced S1P release that via the efflux through SPNS2 can interact with outer membrane receptors amplifying the pro-inflammatory loop. However, we cannot exclude a CpG-induced TNF- $\alpha$  release also in a S1P-independent way. This hypothesis could justify the capability of CpG to induced more release of TNF- $\alpha$  compared to the exogenous S1P. Besides the role of S1P in TLR9 signaling, we have to point out that NF- $\kappa$ B was also responsible for the cytokine release. In support, the inhibition of NF- $\kappa$ B completely reduced CpG-induced TNF- $\alpha$  release, which instead was not observed after exogenous S1P addition, implying that the active form

of NF- $\kappa$ B is involved in S1P-mediated TNF- $\alpha$  release. However, while it is well-known that NF- $\kappa$ B is relevant for CpG activity, its involvement in S1P receptor/s signaling needs further elucidation. One of the limitations of this study is that we were not able to demonstrate whether the induction of NF- $\kappa$ B after CpG treatment could be able to induce S1P generation via the induction of ceramidase and SPHKs activity. However, we do not think that NF- $\kappa$ B was able to induce ceramidase and SPHKs activation, in that we observed ceramidase activation at 3 hours post CpG addition. Instead, because TLR9 signaling induces the release of calcium from intracellular stores [27], and because sphingomyelinase upstream ceramidase, is calcium-dependent [28], we suggest that the induction of S1P generation by the activity of ceramidase and SPHKs activation is mainly due to calcium-dependent way. Moreover, we believe that according to the presence of SPHK I into the cytosol and of SPHK II into the nucleus, it was likely that S1P was generated by SPHK I. However, the limitation was related to the use of a non-specific SPHK inhibitor in our study.

In addition, it is noteworthy highlight that TLR9-dependent endogenous S1P formation leads to an inside-out signaling of S1P. In particular, the release of S1P by TLR9 activation reflects that S1P may act as an intracellular second messenger to regulate calcium mobilization, cell growth and suppression of apoptosis in response to a variety of extracellular stimuli [29]. However, S1P can also be transported out of the cell by means of Spinster homolog 2 (SPNS2), and acts in a paracrine and/or autocrine manner by activating sphingosine-1-phosphate receptors (S1PRs) in an inside-out mode. Therefore, in the context of TLR9 stimulation we could support the hypothesis that TLR9 triggering by unmethylated DNA-derived CpG motifs, which could be physiologically higher after cell death, does not induce TNF- $\alpha$  release in an S1P-dependent manner as to promote a physiological cell defense (as in the case of fibroblasts); in pathological conditions, instead, an immunogenic process is favored in that S1P release via TLR9 could favor tumor cell proliferation, further pointing at TLR9/S1P axis as a rheostat for determining cell fate after the recognition of unmethylated DNA-derived CpG motif, highly present in the tumor environment [16, 19].

Another important issue in this study is that the increase of TNF- $\alpha$  levels were likely due to S1P<sub>3</sub> and S1P<sub>1</sub> activation. These two receptors are both Gi-coupled, which signaling pathway diminishes the levels of TNF- $\alpha$  via the inhibition of cAMP which negatively regulates TNF- $\alpha$  release. On the other side, S1P<sub>3</sub>, mainly expressed in lung adenocarcinoma cells, is also coupled to Gq, which supports calcium release by intracellular storage and protein kinase C (PKC) activation [28]. Similarly, TNF- $\alpha$  release could be strengthened by both calcium and PKC-dependent signaling. In support, a very recent study on murine peritoneal macrophages proposed a pro-inflammatory signaling for S1P in an NF- $\kappa$ B-dependent manner via TLR4 activation. Specifically, TLR4 stimulation induced SPHK I activation and S1P<sub>3</sub> expression via NF- $\kappa$ B. S1P, produced by SPHK I, activated a pro-inflammatory signaling through S1P<sub>3</sub>, resulting in increased production of prostaglandins, nitric oxides, IL-6, TNF- $\alpha$  and IL-1 $\beta$  [30]. To note though, in our study, TLR4 was not involved in S1P signaling, most likely because of the different nature of the cells. We used epithelial lung cancer cells, while Dr. Heo's group, focused on healthy macrophages [30], which express higher levels of TLR4. In support, we found that in non-pathological human fibroblasts the activation of TLR9/S1P axis, as well as of TLR4, was not associated to TNF- $\alpha$  release, in that these cells did not respond to CpG or LPS stimulation, confirming S1P pro-inflammatory role in pathological conditions.

## Conclusion

In conclusion, our findings open up new scenarios on the physiological and pathological role of S1P and shed light on a new and never investigated TLR9/S1P axis-dependent inflammatory pathway, that could play a fundamental role in the exacerbation of inflammatory pulmonary conditions through the induction of a ceramide/S1P imbalance in favor of S1P. Therefore, the identification of a novel pathway that focuses on TLR9-S1P axis could represent the metabolic rheostat that decides for TNF- $\alpha$ -dependent pathways, which could be responsible of inflammation-based pathologies.

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### Author Contributions

MT and CC designed and performed experiments; GF and MCM performed LC-MS/MS data; IC and FR performed antagonist experiments; MT, AP and RS statistically analyzed and interpreted data and wrote the manuscript.

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### Statement of Ethics

The authors have no ethical conflicts to disclose.

## Disclosure Statement

The authors have no conflicts of interest to declare.

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