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Research paper

## Mechanisms of activation induced by antiphospholipid antibodies in multiple sclerosis: Potential biomarkers of disease?

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## ARTICLE INFO

## Keywords:

Antiphospholipid antibodies

Multiple sclerosis

Signaling pathways

Astrocytes

## ABSTRACT

Multiple sclerosis (MS) is a chronic, multifactorial, inflammatory disease of the central nervous system where demyelination leads to neurodegeneration and disability. The pathogenesis of MS is incompletely understood, with prevalence of antiphospholipid antibodies (aPL) speculated to contribute to MS pathogenesis. In fact, MS shares common clinical features with the Antiphospholipid Syndrome (APS) such as venous thromboembolism. Consequently, the presence of aPL which are associated with blood clot formation in the APS need to be further investigated for a possible pro-coagulant role in the development of thrombosis in MS. The effects of IgG aPL from patients with MS upon astrocyte activation has never been characterized. We purified IgG from 30 subjects. A human astrocytic cell line was treated with 100 µg/ml IgG for 1 h, and cell extracts were examined by immunoblot using antibodies to p38 MAPK and NFκB to further examine intracellular signaling pathways induced by these IgGs. Only IgG from patients who are positive for aPL caused phosphorylation of p38 MAPK and NFκB in astrocytes. These effects were not seen with IgG from patients with MS but with no aPL or healthy controls. Understanding the intracellular mechanism of aPL-mediated astrocyte activation may help to establish new therapeutic approaches, such as selective inhibition of the mitogen-activated protein kinases, to control MS activity or possible thrombotic states.

## 1. Introduction

Multiple Sclerosis (MS) is a chronic demyelinating/degenerating disease affecting predominantly the white matter of the central nervous system (CNS). Antibody responses in MS are a prominent characteristic and the types of antibodies detected in MS patients so far comprise a large and heterogeneous collection (Schirmer et al., 2014). Although there is no specific biomarker strongly correlated in the diagnostic criteria, the intensive search for biomarkers in MS continues and disease-specific patterns of autoantibody profiles obtained by microarrays suggest their further use in the characterization of disease type and direction of research paths (Quintana et al., 2008). Several cell types are implicated in antigen-specific antibody responses in MS, including neurons, oligodendrocytes and astrocytes (Fraussen et al., 2014). Naturally, myelin-associated antigens were the first to be suspected to elicit antibody responses and numerous studies have confirmed antibody incidence against myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and more

(Belogurov Jr. et al., 2008; Reindl et al., 2017). Strikingly, a study by Ho et al. (2012), reported the antibody reactivity against phosphate groups of certain phospholipids (PL) of the myelin sheath and suggested the pharmacological use of these PL for their protective properties. Prevalence of aPL including IgG isotype anti-cardiolipin (anti-CL) antibodies in MS have been extensively described by us and others (Filippidou et al., 2016; Ricarte et al., 2018). The location of a particular epitope within the cell or the site where it is exposed on the surface, can distinguish between highly specific, pathogenic responses, and less pathogenic but highly informative; this dichotomy may determine the antibody's value as a possible biomarker (Stathopoulos et al., 2015). Furthermore, the identification of the effects of auto-antibodies in autoimmune pathogenesis is a subject of investigation since it implicates different pathways and presents characteristic disease phenotypes.

One of the most studied pathways in immune responses is p38MAPK, which plays a key role in activation of the physiological processes of inflammation and oxidative stress. There are a number of

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<https://doi.org/10.1016/j.jim.2019.112663>

Received 27 June 2019; Received in revised form 3 September 2019; Accepted 10 September 2019

Available online 13 September 2019

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potential mechanisms for p38 involvement in MS pathogenesis (Huang et al., 2012). Early evidence for its involvement in autoimmune neuroinflammation came from microarray studies showing that the expression of MAPK14 (encoding p38a) was elevated approximately 5-fold in MS lesions in the CNS (Huang et al., 2012; Lock et al., 2002). Most importantly, recent studies using pharmacologic inhibitors and genetic approaches have demonstrated a functional role of p38 MAPK in the experimental autoimmune encephalomyelitis (EAE) model and dissected the roles of this kinase in different immune cells (Noubade et al., 2011; Namiki et al., 2012). These results suggest that p38 MAPK activity is necessary for the progression of clinical signs and EAE pathology and that this pathway has potential for pharmacologic intervention in MS, although it is unclear which cell types may be involved. Similarly, NF $\kappa$ B has also been revealed as being able to signal downstream of MAPK kinases and associated with autoimmunity and inflammation (Lambrianides et al., 2007; Brandt et al., 2014; de Groot and Urbanus, 2014).

Any identified molecules with a role in the pathways which mediate pathogenesis could comprise possible therapeutic targets. Overall, a variety of multiple cellular and humoral mediators have been identified as implicated in aPL pathology. The highlights of research so far, point mostly towards endothelial cells, platelets and monocytes as cellular contributors. The association of MS with an increased risk of venous thromboembolism described in epidemiological studies (Peeters et al., 2014), in addition to the correlation between MS and pro-thrombotic factors including aPL positivity, could imply that the MS pathogenic mechanisms may at least partly implicate thrombotic processes. With this knowledge in mind, it seems promising to investigate the use p38 MAPK inhibitors to treat MS. Along these lines, further research aimed at elucidating the precise mechanisms of how aPL via p38 MAPK and NF $\kappa$ B may control disease and a more broad understanding of the molecular players and signaling pathways implicated should provide other novel targets for intervention. Here we examine the molecular mechanisms of how aPL in patients from MS patients may illuminate potential therapeutic targets. Our results presented in this study, identify p38MAPK and NF $\kappa$ B signaling as central pathways, arguably the master regulators of the inflammatory responses.

## 2. Methods

### 2.1. Patients

Blood samples were collected prospectively from 127 MS patients that fulfilled the revised McDonald criteria (Polman et al., 2011). From a detailed history available for each patient, none of the patients included in the study had any underlying autoimmune disease, no neurological manifestations not attributable to MS and no evidence of thrombotic events or pregnancy morbidity, or any clinical manifestations otherwise linked to the Antiphospholipid Syndrome (APS) or any other pathological entity. The population of MS patients from which blood was taken after signing informed consent forms approved by the national bioethics committee, comprised of 88 patients in the relapsing-remitting (RR) phase of the disease, 11 primary progressive (PPMS) and 28 secondary progressive (SPMS) patients (Table 1). From the latter, 5 patients also presented relapses. The entire age range of the MS patients was between 22 and 79 years of age. Up to 64 of the 127 patients received treatment, from which 40 were being administered Interferon- $\beta$  (IFN- $\beta$ ), 11 were taking Natalizumab and 13 were receiving other types of medication such as mitoxantrone, fingolimod, azathioprine, citalopram, mycophenolate, glatiramer acetate, methotrexate, alprazolam or citalopram. Ninety-two healthy controls (HC) were also included in the study, which matched the MS patient population in gender and age (Table 1). These individuals did not present with any pathological condition and had no history of a long-term illness or evidence of an autoimmune disorder.

**Table 1**

Clinical features and immunological characterization of the subjects used as a source of IgG.

	MS (n = 127)	HC (n = 92)
Gender (Female:Male) <sup>a</sup>	89:38	55:37
Mean age (SD)	51.69 (12.19)	52.1 (17.75)
RRMS	88	N/A
SPMS	23	N/A
SPMS with relapse	5	N/A
PPMS	11	N/A
Interferon	40	N/A
Natalizumab	11	N/A
Anti-Cardiolipin IgM	27/127 (21.3%)	1/92 (1.1%)
Anti-Cardiolipin IgG	23/127 (18.1%)	1/92 (1.1%)
Anti- $\beta$ 2-Glycoprotein I IgM	6/127 (4.7%)	0/92 (0%)
Anti- $\beta$ 2-Glycoprotein I IgG	3/127 (2.4%)	0/92 (0%)
Anti-Domain I IgM	8/127 (6.3%)	1/92 (1.1%)
Anti-Domain I IgG	9/127 (7.1%)	1/92 (1.1%)

N/A; Not Applicable, MS; Multiple sclerosis, HC; Healthy controls.

<sup>a</sup> Values indicated represent number of subjects.

### 2.2. Purification and immunological characterization of IgG

The aCL activity of IgG was measured as previously described using international calibrators in G phospholipid units (GPLU, from Louisville APL Diagnostics, USA) (Giles et al., 2003). The nine most highly positive serum samples from the cohort of MS patients that were tested by ELISA for IgG anti-CL and the sera of nine healthy control age and gender matched, were selected for purification. The mean age of the IgG anti-CL positive group is 46.33 and 48.89 for the HC group. IgG was purified from all serum samples by protein G sepharose affinity chromatography (GE Healthcare Lifesciences, Sweden), passed through Detoxi-Gel™ Endotoxin removing columns (Thermo Scientific, UK) and the presence/absence of endotoxin was determined (< 0.06 endotoxin units/ml considered to be endotoxin free) by the *Limulus* Amoebocyte Lysate assay (Sigma, UK). All IgG preparations tested negative in this assay. The concentration of purified IgG was determined using the Nanodrop ND-1000 Spectrophotometer (LabTech International, UK).

### 2.3. In vitro exposure of astrocytes to MS and HC IgG

For *in vitro* studies to investigate the effects of IgG from MS patients positive for aPL compared to IgG from HC, we used pooled IgG derived from an equal concentration of IgG from nine individuals in each group (MS and HC). The human astrocytic (U87) cell line (CLS, Germany) was cultured in MEM medium containing 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100  $\mu$ g/ streptomycin at 37 °C in a humidified atmosphere consisting of 5% CO<sub>2</sub>. U87 cells were incubated with 100  $\mu$ g/ml MS or HC IgG, 3  $\mu$ g/ml LPS, 100 ng/ml TNF- $\alpha$  or media alone for one hour. The one-hour incubation period has been identified to be appropriate for experiments addressing the effects of purified IgG in astrocytes following time-course experiments carried out for time periods between 10 min, 1, 3, 6 and 12 h.

### 2.4. Immunoblot analysis for NF $\kappa$ B and p38 signaling pathways

Subsequently, cell extracts were obtained according to standard protocols and were used for western blotting. Cell extracts (9  $\mu$ g per lysate per well) were resolved by sodium dodecyl sulfate – 10% polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Phosphorylated and total p38 and NF $\kappa$ B protein levels were determined by Western blotting using monoclonal or polyclonal anti-phospho-p38 MAPK, anti-p38 MAPK, and anti-GAPDH antibodies. Protein levels were quantified using the image analysis software ImageJ 1.x (LOCI, NIH).

## 2.5. Statistical analysis

All data are expressed as the mean SEM. Statistical analysis was performed using SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk NY). A normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric test (student's paired *t*-test). If the normality conditions were not met, a comparison was made by a non-parametric test (Mann-Whitney U). *P* values < .05 were considered significant.

## 3. Results

### 3.1. Titers of aCL antibodies from MS patients and healthy controls

Table 1 summarizes the demographics and clinical features of the study population and controls. MS patients have been collected to participate in this study. Eighty-nine of them were female (70.1%) and thirty-eight were male (29.9%). Their mean age was  $51.69 \pm 12.19$  years. Ninety-two healthy individuals were recruited, fifty-five female (59.8%) and thirty-seven were male (40.2%). The mean age of the healthy individuals was  $52.1 \pm 17.75$  years. Noteworthy, there are no statistical differences in the gender proportion between MS patients and HC.

Anti-cardiolipin antibodies of IgG isotype were positive in 23 of 127 patients with MS (18.1%) and in 1 of 92 HC (1.1%).

### 3.2. Confirmation of IgG activity post purification

After purification of total IgG from the serum of MS patients positive for IgG anti-CL and healthy controls, the activity of these fractions was confirmed by ELISA, as performed initially for testing of seropositivity, and subsequently pooled to obtain one group for MS and one group for healthy controls. The pooled IgG samples were tested at a concentration of 100  $\mu$ g/ml dilution in triplicate and anti-CL titers were expressed in GPLU. The mean IgG anti-CL titer of pooled IgG for MS patients was calculated to be 50.64 GPLU, whilst pooled IgG from healthy controls did not bind CL.

### 3.3. Activation of p38 and NF $\kappa$ B in astrocytes following stimulation with MS IgG

To establish the effects of exposing astrocytes to IgG, we used pooled IgG samples from MS patients and healthy controls. U87 cells were treated with pooled IgG for 10 min, 1, 3, 6 and 12 h (data not shown). Maximal differences in phosphorylation of p38MAPK (Fig. 1) and NF $\kappa$ B (Fig. 2) were detected between MS patients and healthy controls after one hour exposure to IgG. Cells cultured with positive control showed maximal level of phosphorylation of both p38MAPK and NF $\kappa$ B after one hour incubation, while medium alone had no effect. To analyze the mechanism triggered by anti-CL antibodies in MS patients, levels of phosphorylated forms of p38 MAPK, as measured by

densitometry, were significantly higher in MS patients compared with controls (shown in Fig. 1). From a total of four individual experiments, there was an average 2.5-fold increase in phosphorylation in p38 MAPK and p65 NF $\kappa$ B. Representative Western blots showing phosphorylated and total forms of NF $\kappa$ B from MS patients and healthy controls are depicted in Fig. 2. IgG from MS patients significantly increased p65 phosphorylation in astrocytes at the protein level compared with IgG from healthy controls (*p* < .05).

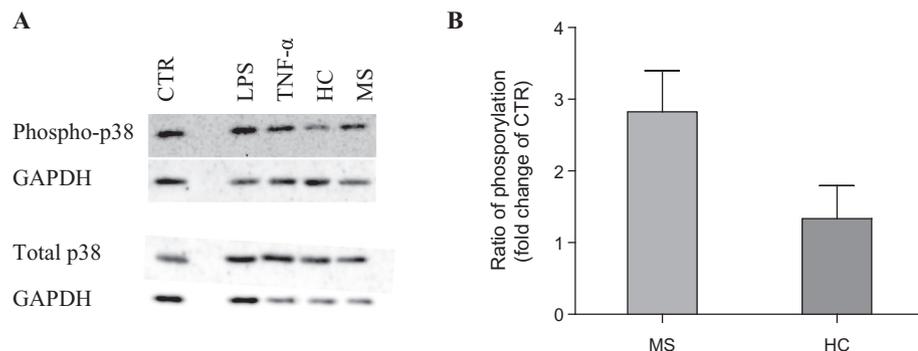
### 3.4. Intracellular pathways are only induced by MS positive for anti-CL antibodies

Given the evidence that p38 and NF $\kappa$ B are activated in astrocytes from MS patients, we sought to investigate whether these intracellular pathways are also activated in MS patients that are not positive for anti-CL antibodies and to what extent the anti-CL antibodies are involved. Pooled sera from ten patients with MS that were not positive for anti-CL antibodies were applied in all *in vitro* experiments (Fig. 3). Patient IgG preparations selected for this group had no IgG anti-CL activity. We confirmed that the presence of anti-CL antibodies in the serum of patients with MS is responsible for increased phosphorylation of p38 (Fig. 3A and C) and NF $\kappa$ B (Fig. 3B and D) compared with those of patients negative for anti-CL antibodies and healthy controls. More specifically, there was a 12-fold increase in phosphorylation of p38 MAPK and a 2-fold increase in phosphorylation of p65 NF $\kappa$ B following IgG stimulation with MS positive for anti-CL antibodies but no increase at all with IgG from a MS with no anti-CL antibodies or healthy controls. The lack of p38 MAPK and p65 NF $\kappa$ B activation in MS patients negative for anti-CL antibodies provides further proof that the observed NF $\kappa$ B activation is specifically due to anti-CL antibodies in those MS patients.

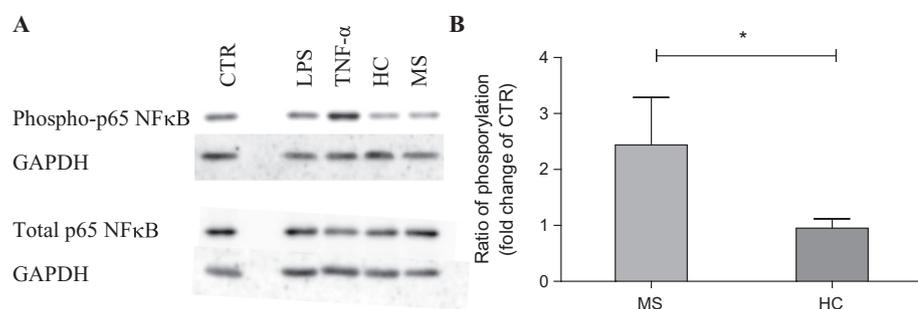
## 4. Discussion

The data obtained in this study strongly suggest that in MS patients, there is upregulation of certain signaling pathways as a consequence of anti-CL antibody activation. This is, to our knowledge, the first study in which aPL-induced intracellular signals that mediate the phosphorylation of signaling pathways in astrocytes have been further described.

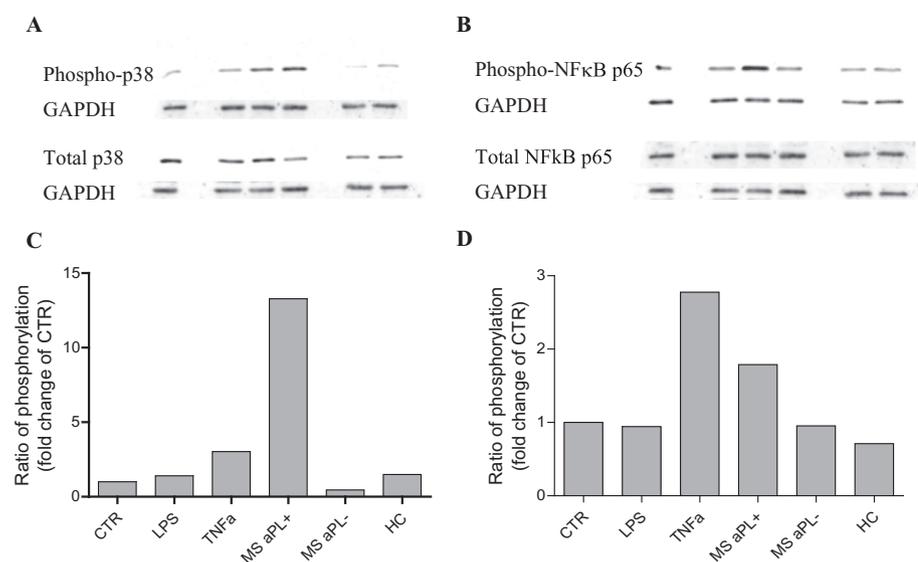
Use of pooled samples was necessary to carry out the multiple experiments to establish the initial responses with IgG in astrocytes, due to sample limitation. We tested different pools for each clinical group consisting of ten individual samples. In the case of the MS patients that were positive for anti-CL, we have selected IgG from the patients with the highest activity to CL. Our results primarily display that IgG fractions from MS patients who are positive for anti-CL antibodies induce p38 and NF $\kappa$ B phosphorylation in astrocytes. This was apparent for both phosphorylated proteins, with phosphorylation of p38 MAPK being more prominent. Stimulation of astrocytes with MS IgG showed an increase in p38 MAPK of about 2.5-fold and an increase in p65 NF $\kappa$ B of about 2.5-fold compared to the healthy control IgG. This study is the first to assess the effects of IgG from MS patients positive for aPL on



**Fig. 1.** IgG purified from patients with multiple sclerosis (MS) promotes phosphorylation of p38 MARK in U87 cells. U87 cells were treated with pooled IgG from ten individual patients with high anti-CL activity or purified IgG from healthy controls (HC) with no anti-CL activity. (A) Representative blots from a single experiment are illustrated and (B) quantitative analysis (from three independent experiments showing mean and SEM) with antibodies specific for human phosphorylated and total p38 MAPK.



**Fig. 2.** NFκB activation in U87 cells stimulated with IgG purified from patients with multiple sclerosis (MS). (A) Representative Western blotting results in ten MS patients and ten healthy controls (HC) from a single experiment and (B) quantitative densitometric analysis of the blots shown in A displaying ratio of phosphorylated to total protein against NFκB (p65). Statistically significant differences are shown (\* $p < .05$ ).



**Fig. 3.** Activation of intracellular pathways are only stimulated by multiple sclerosis (MS) patient IgG positive for anti-CL antibodies. Representative Western blotting results showing p38 MAPK (A) and NFκB (B) phosphorylation after treatment with IgG from MS patients who are antiphospholipid antibody (aPL) positive, IgG from MS patients who are aPL negative or IgG from healthy controls (HC) with no aPL activity.

cells of the human CNS. To date, one study tested the effects of aPL, namely anti-CL on astrocytes (Yu et al., 1991). The authors reported inhibition of astrocytic proliferation and contribution to the formation of blood clots by activation with anti-CL antibodies from patients with SLE (Yu et al., 1991). In that regard, the *in vitro* effects of rat astrocytes that were stimulated with IgG from patients with neuromyelitis optica (NMO), lead to induction of various inflammatory mediators and immune related genes. Interestingly, the upregulation of the chemokine C–C ligand 5 (CCL5) was disease-specific, in comparison to samples from patients with other diseases, including RRMS. However, samples from progressive MS patients were not included (Howe et al., 2014). It would be of great interest to compare the effects of NMO IgG against MS IgG and HC since it is known that NMO is antibody mediated (Howe et al., 2014; Parratt and Prineas, 2010), compared to the much more complex pathophysiology of MS which implicates a large scale of cellular and humoral immune mediators (Dendrou et al., 2015). Attributing specific molecular processes to certain diseases requires extensive research, assessing the induction of antibody-mediated effects across several clinically distinct conditions.

B cells in general, are shown to be highly involved in CNS inflammation and toxicity, as shown in a study by Lisak et al. (2012), where B cell supernatants from MS patients exert pathological effects on oligodendrocytes, regarding viability and morphology (Lisak et al., 2012). Consequently, one could argue that antibody-mediated effects in the CNS, are not a result of an isolated mechanism. Given our findings on U87 cells in response to IgG from MS patients and from healthy controls, we can conclude that MS IgG may initiate multiple inflammatory processes in astrocytes. Since the IgG fractions used in the present study originated from patients positive for aPL, our next aim was to confirm that these effects are aPL specific. Interestingly, we observed that IgG from patients who are not aPL positive do not

activate astrocytes concluding that activation of astrocytes is aPL specific. A possible limitation of this study would be that due to sample limitation, we were unable to deplete the anti-CL antibodies fraction from the MS patients. We feel given the clear difference between MS positive for anti-CL and MS negative for anti-CL allows us to speculate that these difference are attributable to the specific antibodies. Our findings support the hypothesis of astrocytes playing key roles in antibody-mediated pathogenicity. This is in accordance with a study evaluating MS CSF-derived monoclonal recombinant antibodies whereby describing loss of myelin, astrocyte activation and deposition of complement products (Blauth et al., 2015). As discussed by the authors, neither the abundance of such antibodies, nor the exact mechanisms by which they contribute to demyelination are clear. For instance, complement appears to be implicated following the binding of some of these antibodies to astrocytes and/or neurons (Blauth et al., 2015). There are recent studies available that associate the role of TLR2 with the proinflammatory profile of astrocyte cultures in CNS inflammation (Bellaver et al., 2018), therefore supporting the hypothesis that TLR2 and/or TLR4 can be involved in aPL signaling and astrocytic responses in MS. TLR-2 and TLR-4, are membrane targets of aPL, mediating the deleterious effects in the APS (Benhamou et al., 2014). In APS, there is extensive evidence where *in vitro* experiments have shown that activation of monocytes and endothelial cells by aPL involves downstream mediators of TLRs (Ruiz-Irastorza et al., 2010; Allen et al., 2012; Satta et al., 2011; Xie et al., 2013).

Activation of the NFκB transcription factor family plays a crucial role in inflammation through its ability to induce transcription of proinflammatory genes. Substantial evidence suggests that MAP kinases can contribute in the regulation of NFκB. Moreover, it has been demonstrated that the p38 pathway is implicated in the cytoplasm as well as in modulation of its transactivating potential in the nucleus.

Activation of p38 MAPK is of interest, since this kinase is essential in both inflammation and coagulation, making it an attractive candidate as a potential mediator of thrombotic effects in pathologic conditions.

Taken together, the above results suggest that IgG from MS patients positive for aPL promote phosphorylation of p38MAPK and NF $\kappa$ B signaling molecules. In this way we have extended our findings and observed that astrocytic stimulation with IgG from MS patients positive for IgG anti-CL further enhances the hypothesis that aPL may carry out inflammatory pro-thrombotic effects in the CNS of MS patients. Here, we describe how the aPL signaling mechanisms described may provide further evidence to the development of thrombosis in MS. The involvement of aPL in MS has been well documented by us and others which further supports this principle. Some aspects remain to be confirmed, for example whether following these signaling effects there is subsequent induction in tissue factor (TF), a cellular initiator of blood coagulation. Theoretically, the involvement of TF is an attractive avenue for development and may help to establish new therapeutic approaches, such as selective inhibition of MAP kinases, to reverse the possible prothrombotic state in patients with MS. Despite the fact that p38 MAPK inhibitors showed great promise in preclinical models of RA and Crohn's disease but so far the clinical results not as promising, the therapeutic efficacy of these does not necessarily mean the same for MS and this should be pursued further. At present, thirteen kinase inhibitors have been approved for oncologic indications. Regardless, the number of kinase inhibitors and the range of clinical indications are likely to expand dramatically in the next few years. At this point our results from *in vitro* stimulations provide further confirmation of the involvement of aPL in the pathogenesis of MS.

#### Declaration of Competing Interest

None.

#### Acknowledgements

We would like to especially thank all patients with MS and all healthy donors at the Cyprus Institute of Neurology and Genetics for donating blood for our research. We would like to thank Mrs. Eftyxia Gaglia and Mrs. Astero Constantinou, Cyprus Institute of Neurology and Genetics. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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