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**NCS 613, a PDE4 inhibitor, by increasing cAMP level suppresses systemic inflammation and immune complexes deposition in kidney of MRL/lpr lupus- prone mice.**

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**Running head:** NCS 613 reduces kidney inflammation in lupus prone mice.

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**Key Words:** cAMP, PDE4, NCS 613, glomeruli nephritis, autoantibodies.

**Abstract:**

Nephritis remains the most common severe manifestation of systemic lupus erythematosus in which auto-antibodies mediate chronic inflammation and kidney damage. cAMP-phosphodiesterases regulate sodium excretion and inflammation in various tissues. How cAMP elevation can reduce systemic inflammation and suppress kidney inflammation and damage remains elusive. PDE4 signaling and cAMP metabolism were investigated along immune complex depositions in target tissues and kidney damage (histology). SLE disease progression is associated with changes in kidney PDE4 activity and expression. Moreover, lupus prone mice exhibit low kidney cAMP level which is associated to induction and relocation of nuclear and cytoskeleton PDE4 isoforms. Auto-antibodies-induced kidney damage was attested by mesangial proliferation and cellular infiltration. Interestingly, we reported that NCS 613 treatment decreases systemic auto-antibody secretion and their corresponding immune complex deposition in target tissues. Furthermore, NCS 613 is able to increase cAMP levels in the kidney; hence this compound rescues kidney PDE4 alterations in treated mice. NCS 613 overcomes disease progression in lupus prone mice by improving wellbeing and decreasing inflammation in treated mice. The PDE4 inhibitor, NCS 613, is a new anti-inflammatory compound that is believed to be a leading drug candidate for the treatment of inflammatory diseases such as lupus nephritis.

## Introduction

In systemic lupus erythematosus (SLE) autoimmune antibodies mainly targets nuclear compartments (spliceosome, phospholipids and DNA)[1]. SLE disease involves progressive and chronic inflammation of multiple joints and organs. In most severe manifestation autoantibody complex depositions in the kidney lead to lupus nephritis (LN) with poor outcome [2]. Indeed, the clinical future of SLE consists mainly in glomerulo-nephropathy inducing renal failure [3]. A murine model of SLE, such as MRL/lpr mice (characterized by lpr mutation, concerning Fas gene), develops lupus with similar symptoms like in human i.e. erythema, nephritis with proteinuria [4]. The high production of cytokine (TNF $\alpha$ ) and auto-antibodies is a morbid cause leading to multiple organ chronic inflammation and damage in both human and mice [5]. Currently, SLE is managed by symptomatic treatment, such as corticoids, as well as antibodies and peptides targeting T and B cells, anti-TNF $\alpha$  and anti-interleukin-10 antibodies [1]. Currently, excepting the development of the peptide P140, named Lupuzor [6], no specific treatment for SLE including oral administration of chemical drugs of low molecular weight exists. However, many studies reported cellular signaling alterations in SLE during disease progression, which lead to immune system deregulations [7-9].

Downstream receptor, cyclic nucleotide phosphodiesterases (PDEs) which hydrolyze cAMP and cGMP play a pivotal role in cell function including migration, proliferation, and inflammation [10-14]. Cyclic nucleotides are also recognized as critical mediators in many renal functions, including solute transport, regulation of vascular tone, proliferation of parenchymal cells, and inflammation [15-19]. We have previously reported that cAMP-PDE inhibitors improve sodium excretion in rats with cirrhosis and decreased proteinuria in these animals [20] and that NCS 613 treatment overcomes disease progression in MRL/lpr lupus prone mice [21]. Also, we have recently reported that NCS 613 reduces inflammation in

human lung tissues, and lupus disease progression in MRL/lpr lupus prone mice [12, 21, 22]. The compound NCS 613 is a new potent and specific PDE4 inhibitor which exhibits anti-inflammatory properties by decreasing TNF $\alpha$  secretion in guinea pigs [23, 24]. Interestingly, NCS 613 did not stimulate the gastric acid secretion suggesting that this compound may produce fewer gastrointestinal side effects [25]. These observations prompt us to investigate whether or not the cellular signaling alterations in SLE may be dependent on cAMP, and whether cyclic nucleotide phosphodiesterases might be involved. This study aims to investigate the role and consequences of PDE4 inhibition by *in vivo* administration of NSC 613 on lupus progression in MRL/lpr mice. This work shows that NCS 613 significantly increases kidney cAMP, whereby reduces auto-antibodies mediated inflammation and kidney damage in treated mice.

## 2. Material and Methods

cGMP, cAMP, Tris, and EGTA and EHNA were from Sigma (St. Louis, MO, USA). Rolipram, and NCS 613 (European patent No. 1 996 200), were generous gifts from Jean-Jacques Bourguignon and Claire Lugnier (Strasbourg Faculty of pharmacy). [8-<sup>3</sup>H] cAMP was purchased from New England Nuclear (Boston, MA, USA). Rolipram and NCS 613 were dissolved in dimethyl sulfoxide (100 % DMSO) and the final concentration of DMSO did not exceed 1 % for PDE activity assessments. Anti-PDE antibodies were from FabGennix Inc. (Frisco, TX, USA): anti- PDE4A (DP4-112AP), anti-PDE4B (PD4-201P), anti- PDE4C (PD4-301P) and anti- PDE4D (PD4-401P). Anti-GAPDH (#MAB374) was from Chemicon/Millipore (Molsheim, France). Anti-histone H3 (#1326-1), anti-EFGR (#1114-1) and anti-vimentin (#2707-1) antibodies were from Epitomics (Burlingame, California, USA). Horse radish peroxidase-conjugates were from Promega (Lyon, France, 1/60,000). ECL kit was from GE Healthcare (Saclay, France). Total RNA was isolated using RNeasy mini Kit

(Qiagen) and their expression analyzed by Q-PCR using BIO-RAD commercial kit (iScript™ cDNA Synthesis and iQ™ SYBR Green Supermix) in MyIQ™ thermocycler. Intracellular cAMP levels in mice kidney were assessed using enzyme immuno-assay (Abor, USA).

## **Animals**

Four weeks of age female CBA/J (H-2<sup>k</sup>) and MR/lpr (H-2<sup>k</sup>) mice were purchased from Harlan (Gannat, France). The animal experimentation was conducted according to the ‘‘Principles of Laboratory Animal Care’’ and with the approval of the Regional Ethics Committee of Strasbourg (CREMEAS, project nuAL/05/08/03/07).

## **Administration of the PDE4 inhibitor NCS 613**

The experiments were done with four different experimental groups: control CBA/J mice treated with drug vehicle (PBS, 10% ethanol) = C-, treated control CBA/J= C+, MRL/lpr control= L-, MRL/lpr treated= L+. Five-week-old female MRL/lpr lupus prone mice or healthy CBA/J mice received NCS 613 (30 µg /100µl / mouse) in 10% ethanol PBS via the i.v. route. Control groups were given saline only (100 µl PBS in 10% ethanol/ mouse). Four administrations were given at 5, 7, 9, and 13 weeks of age. Measurements of the outcomes included anti-ds-DNA antibodies levels. Animal were sacrificed by cervical dislocation.

## **Preparation of kidney extracts for PDE assays and Western blot**

After 2 weeks of the end of NCS 613 treatment, CBA/J control and MRL/lpr lupus-prone mice were sacrificed (15 weeks of age). Kidneys were isolated, cut in two parts and immediately frozen in liquid nitrogen and stored at –80°C. Frozen tissues were ground to powder in liquid nitrogen using a mortar/pestle. The resulting tissue-powders were

homogenized at 4°C with a glass-glass potter for 3 x 30 sec at 1,000 rpm (100-300 mg tissue/0.5 ml buffer) in solution of following composition (mM): 20 Tris, pH 7.5, 5 EGTA, 150 NaCl, 20 Na-β-glycerophosphate, 10 NaF, 1 NaVO<sub>3</sub> + 1 μM H-89 + 1% Triton X-100, 0.1% Tween 20. The protein inhibitor concentrations used were (μM): 166 Pefabloc, 133 aprotinin, 8.3 bestatin, 2.5 E64, 3.3 leupeptin, 1.6 pepstatin-A. The homogenates were centrifuged at 14000 g for 10 min at 4°C and the supernatants were stored as aliquots at -80°C until used. These extracts were used for PDE assay and Western blot analysis.

### **Preparation of kidney subcellular fractions**

Kidneys from 15-week-old CBA/J control and MRL/lpr mice were ground to powder in liquid nitrogen using a mortar/pestle. The crude homogenates as well as the cytoplasmic, nuclear, membrane and cytoskeletal proteins were obtained using the Compartmental Protein Extraction Kit from Millipore (Chemicon # 2145, Molsheim, France), according to the manufacturer protocol. The quality of subcellular fractions was assessed by Western blot using specific antibodies against compartmental proteins: GAPDH for cytoplasm, EGFR for membrane, histone H3 for nucleus and vimentin for cytoskeleton.

### **PDEs activities assessment in mice tissues**

PDE activity was measured by a radioenzymatic assay as previously detailed (Keravis et al., 2005) at a substrate concentration of 1 μM cAMP in presence of 10000 cpm [<sup>3</sup>H]-cAMP as tracer and hydrolysis between 10- 20%. Total cAMP-PDE activities were assessed in the presence of 1 mM EGTA and 1 μM cAMP. Contribution of different isozymes to total cAMP-PDE activity were tested in the presence of 1 μM cilostamide for PDE3 and 10 μM rolipram for PDE4 in each sample. Protein concentration allowed us to calculate specific activity in

pmol min<sup>-1</sup>mg<sup>-1</sup> of protein. The remained activity in presence of a specific inhibitor was subtracted from total activity for each PDE family contribution.

### **Determination of cAMP levels in mice kidney**

Tissue sample frozen in liquid nitrogen were ground to a fine powder under liquid nitrogen in mortar. After liquid evaporation, the tissue was weighted and homogenized in 10 volumes of 0.1 M HCl. Homogenates were centrifuge at 1000 x g at room temperature. Tissue cAMP level was measured by Assay Design<sup>TM</sup> Direct Cyclic AMP EIA Kit following manufacturer's recommendation. cAMP level was normalized by protein concentration in the sample.

### **Western blot analysis**

Protein from kidney extracts (30 µg) as well as protein from kidney subcellular fractions (cytoplasm, nuclei, membrane and cytoskeleton) was subjected to Western blot analysis as previously described [26]. Briefly, protein samples were denatured and solubilized for 5 min at 95°C in Laemmli buffer, electrophoresed on SDS 8%-polyacrylamide gel and electro-transferred onto polyvinylidene fluoride (PVDF) membranes. Immunodetections were carried out using: anti-PDE4A (1/2,500), anti-PDE4B (1/2,500), anti- PDE4C (1/2,500) and anti- PDE4D (1/2,500), anti-vimentin (1/1,000), anti-EGFR (1/1,000) and anti-GAPDH (1/60,000) antibodies. Immobilized antigens were detected by chemiluminescence using horse-radish peroxidase-conjugates as secondary antibodies (1/60,000), an ECL kit and autoradiography films. Autoradiography signals were captured on a GeneGenius Bio Imaging System (Syngene) using the GeneSnap software and analyzed using the GeneTools software.

## **Quantitative real time RT-PCR**

Frozen tissues were ground in liquid nitrogen using a mortar/pestle. The resulting tissue-powder was homogenized with a glass-glass potter 3 x 30 sec at 1500 rpm in Qiazol lysis buffer. Total RNA was isolated from kidney using the RNeasy<sup>®</sup> kit (Qiagen) following manufacturer's instructions including a RNase-free DNase treatment to prevent co-amplification of genomic DNA. The concentration of the purified RNA was determined spectrophotometrically at 260 nm. The integrity of purified RNA was assessed on 4% agar gel electrophoresis. The reverse transcription was performed with 1 µg of total RNA using iScript<sup>®</sup> cDNA synthesis kit from Bio-Rad.  $\beta$ -actin as house-keeping gene was used for normalization. The sequences of PDE oligonucleotides used as primers for real-time PCR are presented in **Table 1** and were bought from Invitrogen. Specific primers for conserved sequence of interest were designed with Primer3 Input (version 4.0). The transcript levels of PDE4 isoforms and  $\beta$ -actin were quantified by real-time PCR performed in the MyiQ<sup>®</sup> single-color detector system (Bio-Rad Laboratories) using the iQ SYBR<sup>®</sup> Green Supermix (Product no. 170-8884) containing the hot-start iTaq DNA polymerase. The amplification conditions were: 95°C for 5 min, 40 cycles at 94°C for 10 sec, 60°C for 10 sec and 72°C for 15 sec. Amplicon specificity was assessed by melting curve analysis and their size was verified on 4% agar gel electrophoresis. All samples were processed in according to the manufacturer's guidelines.

## **Auto-antibodies analysis**

Autoantibodies directed to double stranded-DNA were tested by ELISA as previously described [7]. Briefly Microtiter plates (cat. no. 3912; Falcon, Oxnard, CA) were coated overnight at 37°C with DNA diluted in 0.25 M citrate buffer, pH 5.4. In each assay, mouse

sera diluted at 1/500 were also tested in an uncoated well incubated with coating buffer as a control. Saturation of plates was obtained by adding PBS containing 0.05% Tween-20 (PBS-T) and 0.5% BSA. The subsequent steps of the test were performed using mouse sera and goat anti-mouse IgG conjugated to HRP diluted at 1/20,000 in PBS-T. All samples were systematically tested in at least two independent assays. The cut-off points of the assays as determined from a series of sera from 5 controls CBA/J mice were 0.036 OD units. When these threshold values were used, none of the sera from normal CBA/J mice was found positive.

Lupus prone mice develop many auto-antibodies and the heavy chains of their IgGs were detected by goat anti-mouse IgG. Tissue samples (20 µg of protein) were denatured and solubilized by heating for 5 min at 95°C in lysis buffer, electrophoresed on SDS 8% polyacrylamide gel and electro-transferred onto PVDF membranes. Membranes were processed for immunoblotting directly with secondary antibody (goat anti-mouse IgG) using horseradish peroxidase (HRP)-conjugates the ECL assay kit and autoradiography films.

## **Histology**

Fresh kidneys were fixed in buffered formalin (10 %, pH 7.0) for 48 h, then embedded in paraffin and dehydrated in 70 to 100 % ethanol/xylene. Thick sections were made (2–4 µm), deparaffinized, and stained with hematoxylin and eosin. Pathological changes in kidney were determined by kidney histology and measuring the size of glomeruli and microtubules with the Image J (<http://rsbweb.nih.gov/ij/>) software and picture calibration.

## **Statistical analysis**

Statistical analysis of digitized blots films was performed with the one-way analysis of variance (ANOVA) and the Bonferroni's multiple comparisons post-test. Relative mRNA

expression was calculated using the comparative threshold (Ct) method. Results were calculated as  $2^{-\Delta\Delta Ct}$ ,  $\Delta Ct$  being normalized with  $\beta$ -actin. Data are expressed as mean  $\pm$  S.E.M. from at least three independent experiments.

## **Results**

### **NCS 613 reduces systemic anti-ds-DNA antibodies and autoimmune complex deposition in lupus target tissues.**

During disease progression, MRL/lpr mice produce various antibodies against nuclear compartments. To test the hypothesis that chronic inhibition of PDE4 activity may impact on systemic inflammation, circulating anti-ds-DNA antibodies and their corresponding immune complex deposition in lupus target tissues were investigated. As early as 11 weeks, Untreated mice exhibit high levels of ds-DNA antibody which steadily increase to disease end stage (Fig. 1A). Auto-antibody secretion is delayed by NCS 613 treatment that significantly reduces circulating anti-ds-DNA antibody in MRL/lpr mice. Since circulating anti-ds-DNA auto-antibodies target multiple organs in SLE and play a pivotal role in disease progression, impact of NCS 613 treatment on immune complex deposition was evaluated. Heart, kidney, liver, and spleen are the main affected among organs tested. Of critical importance NCS 613 ameliorates disease outcomes by reducing autoimmune complex deposition in target tissues and thereby preventing organ damage (Fig. 1B & 1C). Therefore, these changes in auto-antibody production after treatment, attest NCS 613 potency on SLE and prompt us to investigate its effect on cAMP metabolism.

### **PDE4, the major kidney cAMP hydrolyzing enzyme is dysregulated in lupus**

Clinically significant kidney involvement occurs in about 20–40% of patients which considerably increased risks for morbidity and mortality. In this study, we focused on lupus nephritis to demonstrate the beneficial effects of chronic inhibition of PDE4 activities. In healthy CBA/J mice kidney, cAMP is mainly hydrolysed by PDE2, PDE3 and PDE4 (Fig. 2A). During this treatment no change in PDE2 or PDE3 were found. Kidney cAMP-PDE activity is the most elevated in control mice and PDE4 activity remains the most important cAMP hydrolysing enzymes (61 %) whereas PDE3 activity was very low (8%), the remaining PDE2 activity being only 31%. After chronic treatment by NCS 613, the total cAMP-PDE activity was significantly increased in control CBA/J ( $p= 0.027$ ) corroborating that PDE4 is the major cAMP hydrolyzing enzyme in kidney. This is similar in lupus prone mice which exhibit increase kidney cAMP-PDE activity upon NCS 613 treatment (Fig. 2B). Upon NCS 613 treatment, kidney PDE4 activity significantly increased by 16% in CBA/J mice, whereas an increase 24 % was found in MRL/lpr kidney.

### **NCS 613 is a cAMP alleviating agent that restores level in mice kidney**

To ascertain the putative change in cAMP level in response to NCS 613 treatment, cAMP levels were assessed in kidney, the main target organ in lupus (Fig. 3A). Without treatment, cAMP level in MRL/lpr mice kidney was lower than in CBA/J mice kidney ( $p=0.05$ ) which corroborates the increased inflammation in these mice kidney. As expected NCS 613 treatment increased cAMP levels by a 1.5-fold in CBA/J kidney ( $P=0.03$ ). In MRL/lpr mice, NCS 613 treatment significantly increased cAMP levels by 4-fold in ( $p=0.001$ ). Surprisingly, this data revealed that decreased PDE4 activity is associated with a low cAMP level in untreated MRL/lpr mice kidney suggesting a possible cAMP metabolism alteration. NCS 613 is an effective cAMP elevating agent since it increases *in vivo* cAMP level in MRL/lpr kidney.

## **NCS 613 restores altered PDE4 expression and dysregulated cAMP signaling in MRL/lpr mice kidney**

The expression of kidney PDE4 protein was analyzed by Western blot (Fig. 4). Any significant change in kidney PDE4s expressions was observed in CBA/J mice upon NCS 613 treatment. Conversely, significant decreases of PDE4 isoform proteins were reported in untreated MRL/lpr mice, when compared with untreated CBA/J mice, excepted for PDE4D. Interestingly, NCS 613 treatment increased PDE4 isozyme proteins in MRL/lpr to extent level of control mice (Fig. 4A- 4F). PDE4A-94 kDa, PDE4B-101 kDa, PDE4C-75 kDa and phospho-PDE4D-75kDa were significantly decreased in untreated MRL/lpr mice and NCS 613 reversed these alterations as it is shown in Western blot traces (Fig. 4G).

To further investigate PDE4 protein distribution during disease progression and effect of NCS 613 treatment, we analyzed their compartmentalization by Western blot (Fig. 5). Four subcellular fractions were analyzed: cytoplasm, nuclei, membrane and cytoskeleton. NCS 613 treatment did not modify the expression of PDE4s proteins in CBA/J mice kidney. Cytoplasmic PDE4A-94 kDa and nuclear PDEA-168 kDa are down-regulated in untreated lupus mice. Interestingly, these alterations are abolished by NCS 613 treatment re-establishing their expression to the extent level of control mice level. Surprisingly, nuclear PDE4D-128 kDa is upregulated in untreated MRL/lpr mice. This PDE4D long isoform could be implicated in nuclear cAMP pool breakdown allowing effector proteins activation. Interestingly, NCS 613 treatment decreased this PDE4D over-expression. NCS 613 also re-established membrane PDE4B-76 kDa level which was undetectable in the untreated group. In cytoskeleton, no change in PDE4 protein distribution was found in CBA/J mice. However, many PDE4 protein

alterations occurred in MRL/lpr mice and are sometimes reversed by the NCS 613 treatment. This was the case of up-regulated cytoskeleton PDE4B-70 kDa and -60 kDa, PDE4C-75 kDa and the long isoform of PDE4D-128 kDa that is induced in MRL/lpr mice.

NCS 613 treatment increased PDE4A and PDE4C mRNA levels in treated CBA/J and MRL/lpr mice (Fig. 6). The PDE4B and PDE4D mRNA remain unchanged during this treatment. Although the PDE4C isoforms are undetectable by Western blot; their mRNA transcripts are increased by NCS 613 treatment. Taking together, these results reveal that PDE4 regulation subsequent cAMP elevation lead to *de novo* synthesis of PDE4 isoforms.

### **Effects of NCS 613 treatment on glomeruli from CBA/J and MRL/lpr mice**

The kidney section stained with eosin-hematoxylin revealed no glomeruli histopathological change in NCS 613 treated CBA/J mice kidney (Fig. 7). As expected, in comparison with control mice kidney (Fig. 7A), MRL/lpr lupus-prone mice kidney exhibit increased proliferation of mesangial cells which is due to auto-antibody deposition. In MRL/lpr mice, glomeruli and microtubule size are significantly increased assessing glomerular injury and glomerulosclerosis characterizing lupus nephritis (Fig 7C). Interestingly, NCS 613 treatment reduces kidney damage in MRL/lpr lupus-prone mice (Fig. 7D) without altering control kidney mice (Fig 7B). Therefore, glomeruli and microtubules from NCS 613 treated MRL/lpr lupus-prone mice are significantly reduced, attesting the effectiveness of NCS 613 to decrease mesangium proliferation and lymphocyte infiltration. Altogether these data indicate a beneficial effect of NCS 613 treatment at the kidney level.

### **Discussion**

Lupus nephritis has been described as the most serious complication of SLE and is strongest predictor of poor outcome [2]. Increasing evidence recognized cyclic nucleotides as

critical mediators of many renal functions, including solute transport, regulation of vascular tone, proliferation of parenchymal cells and inflammation [14, 15, 27-29]. We recently reported that disease progression in MRL/lpr lupus-prone mice is reduced by a specific PDE4 inhibitor [21]. These findings prompted us to investigate whether kidney inflammation is modulated by cAMP. In this study, NCS 613 increases total cAMP-PDE and PDE4 hydrolytic activities in treated control as well as MRL/lpr mice that are correlated with cAMP up-regulation. These observations were supported by PDE4 increases in protein and mRNA levels. NCS 613 also decreases auto-antibodies secretions and their corresponding immune complexes depositions in lupus target tissues.

**NCS 613 reduces auto-antibody secretions and their corresponding immune complex deposition in target tissues.**

We are interested in auto-antibodies secretion, since their deposition in target tissues lead to complement activation and inflammation [30, 31]. Early, lupus prone mice without treatment spontaneously developed anti-double-stranded DNA antibodies; which level rapidly increased with disease progression compared to treated mice. Anti-ds-DNA antibodies are responsible of health degradation, renal failure in lupus mice and remain the leading cause of poor outcome [3]. Auto-antibody detection in the main target tissues of lupus mice allowed us to demonstrate the beneficial effect of NCS 613 on lupus disease management. Many therapies in lupus, target B and T cells to down-regulate the immune function and Abs secretion [1]. Our results support the fact that NCS 613 can modulate immune responses and consequently reduced tissue inflammation. The immunosuppressive effect of PDE4 inhibitor was previously reported by Kevin et al., 1985. These authors have shown that PDE4 inhibition by Ro 20-1724 reduces hyper-IgE synthesis by atopic dermatitis cells *in vivo*. We also recently reported that NCS 613 decreases TNF $\alpha$  secretion in peripheral blood

mononuclear cells from both lupus prone mice and patients revealing a potential immunosuppressive effect of this compound [22]. In all investigated tissue, NCS 613 significantly decreased immune complex deposition. Auto-antibody physiopathology has been well investigated and its clinical outcomes are ethnicity related [32]. Alba et al. (2003), have reported that higher proportion of anti-dsDNA antibodies is a strong predictor of nephritis in SLE patients of black ethnic origin.

### **NCS 613 reduces kidney damage in MRL/lpr mice**

Every component of the kidney can be affected in lupus nephritis, but glomeruli are the most altered structure in patients and mammals [31, 33, 34]. The site of deposition of immune complexes in different regions of the glomeruli concerns: mesangium, subendothelial or subepithelial space. Our study focusses on glomeruli structure changes and cellular infiltration. NCS 613 treatment has no effect on CBA/J mice kidney. Expectedly, many changes and hyper-remodeling were found in untreated lupus prone mice glomeruli. These changes concerned: mesangium proliferation, lymphocyte infiltration and hyper-cellularity around the glomeruli. Proliferation, or rather glomerular hyper-cellularity, is one of the most typical consequences of immune complex deposition in kidney [30]. The size of glomeruli increased in MRL/lpr mice subsequently to mesangium proliferation, which affects renal function mainly glomerular filtration [35]. These changes lead to the loss of kidney filtration function causing proteinuria in lupus-prone mice. In rats with cirrhosis and ascites, we previously demonstrated that cAMP up-regulation follows cyclic AMP-phosphodiesterase inhibitor treatments improved sodium excretion and decreased proteinuria [20]. Consistently, our data show that NCS 613 treatment decreased kidney damage in MRL/lpr mice since their glomeruli showed decreased size and mesangium proliferation compared to untreated mice.

The improved survival in treated animal could be justified by down-regulation of auto-antibody secretion due to anti-inflammatory and immuno-suppressive effect of NCS 613.

### **cAMP and PDE4 regulation upon NCS 613 chronic treatment**

PDE4 which contributes for 61 % of total cAMP-phosphodiesterase activity and is specifically inhibited by NCS 613 could be implicated in kidney inflammation. Low cAMP level is strongly correlated with inflammation [25]. NCS 613 increases cAMP level in both treated CBA/J and MRL/lpr mice. In the same way, PDE4 activity increased in treated mice kidney allowing breakdown of cAMP levels. When cAMP is increased in cells, the protein kinase A (PKA) catalytic subunit can phosphorylate PDE4 long isoform to increase the  $V_{max}$  and their capacity to rapidly breakdown intracellular cAMP [36-38]. Sustained PDE4 inhibition, by increasing cAMP level, can also activate PKA which phosphorylates and activates cAMP responsive element binding CREB allowing *de novo* PDE4 synthesis [26]. This is supported by the fact that long term PDE4 inhibition increases local cAMP level and triggers feedback mechanism to hydrolyze and break-down the substrate to normal level for cells homeostasis. In human umbilical vein endothelial cells (HUVECs), we reported previously that PDE4 sustained inhibition by rolipram (after 8h treatment) increased in PDE4 activity, possibly as a compensatory mechanism to accelerate cAMP degradation [26]. The lower PDE4 activity in untreated MRL/lpr mice kidney is associated with a lower level of cAMP. These data highlight the regulation of PDE4 activity in response to cAMP elevation or breakdown.

Western blot analysis revealed no change in PDE4 protein after 6 weeks of NCS 613 treatment in CBA/J healthy mice. This supports the fact NCS 613 did not modify PDE4 expression suggesting low side effects of this compound. PDE4 proteins are down-regulated

in untreated MRL/lpr mice, which exhibit low PDE4 activity and low cAMP level in kidney. Indeed, PDE4A 94 kDa and PDE4B 101 kDa are significantly decreased in untreated MRL/lpr mice; NCS 613 treatment rescues these modifications. Importantly, PDE4C-75 kDa which is turned off in lupus prone mice was induced with NCS 613 treatment. Decreased PDE4D phosphorylation associate to low cAMP level confirms that MRL/lpr mice kidneys exhibit cAMP signaling alteration. Consistently, this observation is also corroborated by the increased kidney PDE4C mRNA subsequent to NCS 613 treatment in MRL/lpr mice; supporting the fact that PDE4 enzymatic regulation in response to chronic inhibition might also involve in *de novo* synthesis of PDE4 proteins.

### **PDE4s isoforms relocation following NCS 613 treatment**

Intracellular cAMP is compartmentalized by spatio-temporal distribution of PDE4, its effectors PKA, ERK, and, small anchoring proteins such as AKAP [36, 39]. In CBA/J mice no change in cytoplasmic, nuclear and cytoskeletal PDE4 proteins were found upon NCS 613 treatment. However, there is induction of long isoform such as nuclear and cytoskeletal PDE4D-128 kDa in untreated MRL/lpr mice. These results suggest that during disease progression, correlated with inflammation in kidney, cytoplasmic and membrane-bound PDE4 proteins are down-regulated and subsequently re-localized in cytoskeleton and nucleus. This relocation contributes to hydrolyze cAMP, and CREB phosphorylation as well as activation of inflammatory gene transcription factor. The decreased cAMP levels in lupus prone mice might be associated with PDE4 relocation in cytoskeleton and nucleus. Conversely, NCS 613 treatment rescues PDE4 isoform alterations in MR/lpr mice kidney: i) by increasing the isoforms which are down- regulated in lupus mice kidney (cytoplasmic PDE4A-94 kDa, nuclear PDE4A-168 kDa, membrane-bound PDE4B-75 kDa); ii) by decreasing the aberrant induction and up-regulated isoforms such as nuclear, membrane and cytoskeletal PDE4D-128

kDa, cytoskeletal PDE4B and PDE4C. PDE4 alterations and relocations have been reported in others diseases [40, 41] like mAKAP induction occurring in cardiac myocytes upon hypertrophy, causing PDE4D3 to relocate around the nucleus [39]. Taking together, these data show that cAMP and PDE4 are deregulated in inflammatory disease. Therefore, they designate the PDE4 family as an interesting target for SLE management.

## **Conclusion**

Our study revealed the pivotal role of PDE4 isoenzymes in chronic inflammation using SLE as a model. Hydrolytic activities change and relocation of kidney PDE4 isoforms subsequent to cAMP breakdown is a hallmark of the inflammation process. The potent PDE4 inhibitor NCS 613, overcomes lupus disease progression by reducing systemic auto-antibody secretion and kidney damage in lupus prone mice. Moreover, NCS 613 increases kidney cAMP level which is correlated to a lower inflammation status and improved the renal function. This potent PDE4 inhibitor also rescues kidney PDE4s isoforms alterations in treated mice which return to control levels. NCS 613 is a potent anti-inflammatory compound which could be developed for the treatment of chronic inflammatory diseases.

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**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Table 1:** specific primers for mouse PDE4s isoforms amplification in Q-PCR.

Primers name	Sequence
PDE 4A Forward	5'GAT CAC AGG GCT GAA AAA GC 3'
PDE 4A Reverse	5' GCG TAC TCC GAC ACA CAA AA 3'
PDE 4B Forward	5' GAA CAA ATG GGG CCT TAA CA 3'
PDE 4B Reverse	5' GCA TGC AGG CTG TTG TGA TA 3'
PDE 4C Forward	5' ACA TGG TGA AAC CCC ATC TC 3'
PDE 4C Reverse	5'TGG AGT GCA GTG GCA TGA T 3'
PDE 4D Forward	5' GGA AAT CCC CTC TCC AAC TC 3'
PDE 4D Reverse	5' GCC CCA CTT GTT CAC ATC TT 3'

### Figure legends

**Figure 1: NCS 613 reduces circulating anti-ds-DNA antibody secretion and immune complex deposition in lupus target tissues.** Blood was collected at 5, 8, 11, 14 and 15 weeks of age and collected sera were subjected to ELISA. **A)** NCS 613 reduces circulating anti-ds-DNA antibody in MRL/lpr mice. **B & C)** The 50 kDa heavy chain of autoantibodies were detected by Western blot. NCS 613 reduces immune complex deposition in kidney, spleen, heart, and liver. Data are expressed as variation of percentage  $\pm$  s.e.m (n=3 mice).

**Figure 2: NCS613 restores normal PDE4 activity in mouse kidney.** **A)** NCS 613 treatment increases kidney cAMP-PDE activities in both control CBA/J and MRL/lpr mice. **B)** reduced hydrolytic activity of PDE4 returns to normal in NCS 613-treated mice. Data represent the mean  $\pm$  s.e.m. of 4 independent experiments.

**Figure 3: NCS 613 alleviates kidney cAMP levels in MRL/lpr mice.** Variation of kidney cAMP level upon NCS 613 treatment: total cAMP was extracted from frozen tissue and intracellular cAMP was assessed by competitive enzyme immuno-assay. Concentration of cAMP was normalized by protein concentration in the sample. **A)** NCS 613 treatment increases kidney cAMP levels in both control CBA/J and MRL/lpr mice. Data represent the mean  $\pm$  s.e.m. of 4 independent experiments.

**Figure 4: NCS 613 restores altered PDE4 signaling in lupus-prone mouse kidney.** Statistical analysis of interested subtype PDE4 isoforms show alteration of 94 kDa PDE4A (**A**), 101 kDa PDE4B (**B**), 75 kDa PDE4C (**C**), and 75 kDa p-PDE4D (**E**) in lupus mice while no change for 75 kDa PDE4D (**D**) and 37 kDa GAPDH (**F**). NCS 613 treatment restores the expression of altered PDE4 isoforms. **G):** representative Western blot traces. Data represent the mean  $\pm$  s.e.m. of 3 independent experiments.

**Figure 5: Abnormal subcellular PDE4 isoforms distribution in mouse kidney.** Cytoplasmic, nuclear, cytoskeletal and membrane-bound proteins were subjected to Western blot analysis with anti-PDE4A, anti-PDE4B, anti-PDE4C and anti-PDE4D antibodies (**A**). Lupus nephritis increases nuclear PDE4D and cytoskeletal PDE4B, 4C, and 4D which are reversed by NCS 613 treatment. Data are expressed as mean (%)  $\pm$  s.e.m (at least n=4).

**Figure 6: NCS 613 increases PDE4A and PDE4C mRNA in mouse kidney.** Quantitative real-time PCR carried out using specific primers shows that NCS 613 increases transcription of PDE4A (**A**), PDE4C (**C**) while no changes for PDE4B (**B**), and PDE4D (**D**). The figure displays relative mRNA levels normalized to beta-actin and the expression of beta-actin is

stable among the used mice and conditions. Statistical analysis was done by  $\Delta\Delta$ CT Method. Data are expressed as mean (%)  $\pm$  s.e.m (at least n=4).

**Figure 7: NCS 613 overcomes lupus nephritis progression in MRL/lpr mice.** Kidneys from control mice CBA/J untreated (**A**) and NCS 6136 treated (**B**) exhibit normal glomeruli histology. Untreated lupus prone mice exhibit glomerulonephritis with mesangium proliferation and infiltration of leucocytes (**C**). **D**) NCS 613 treatment prevents kidney inflammation and restores normal. Pictures were captured with a 40 x-magnification. Pathological changes in kidney concern: mesangium proliferation, lymphocyte infiltration and hyper-cellularity around the glomeruli (from 5 mice of each group).

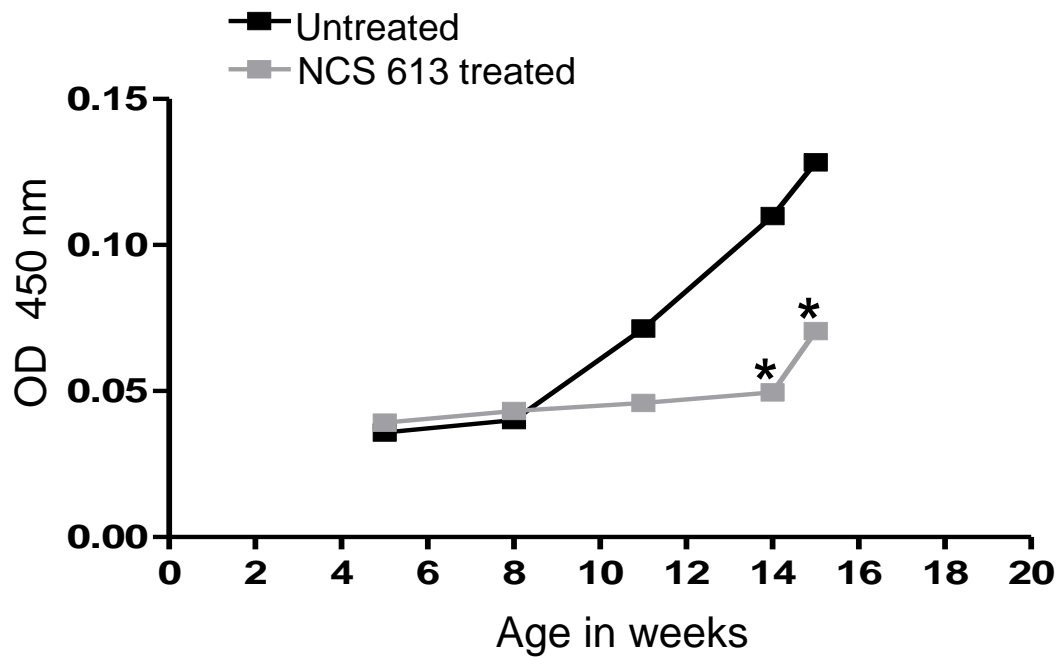
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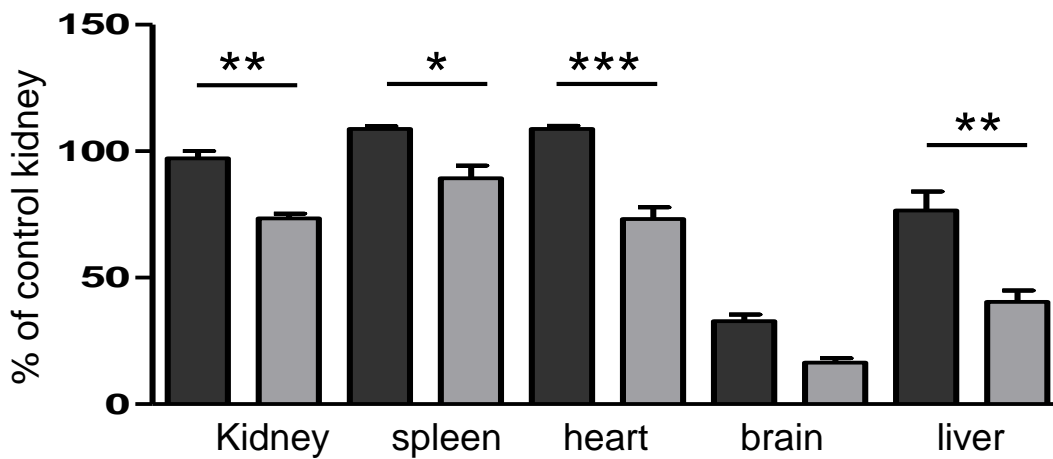
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### A) Anti-ds-DNA antibody production



### B) Immune complex deposition in targets tissues



### C)

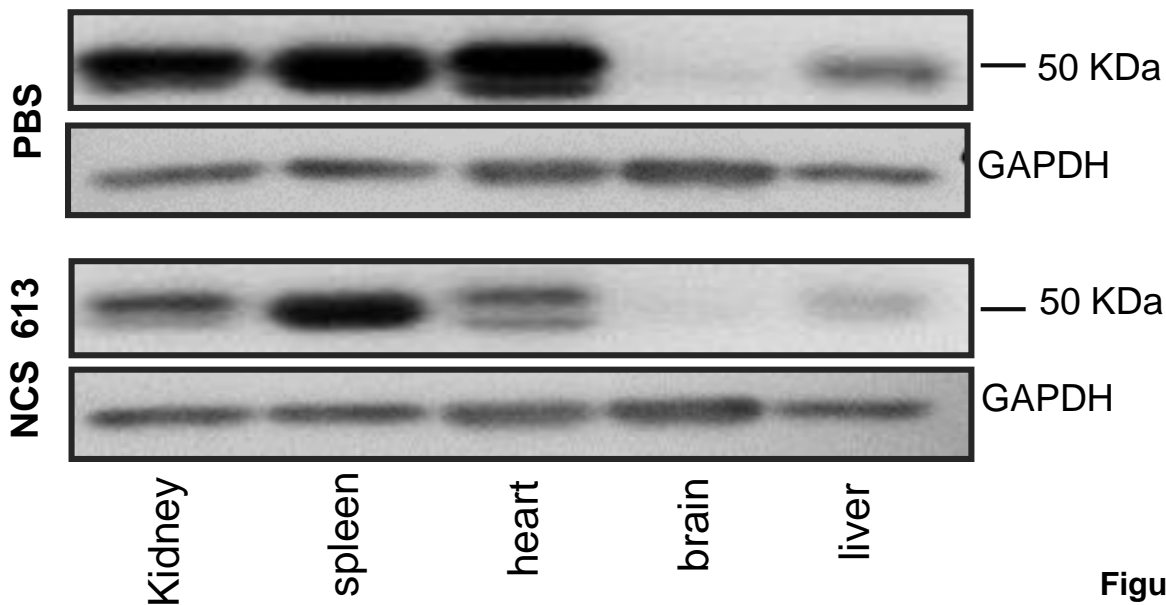
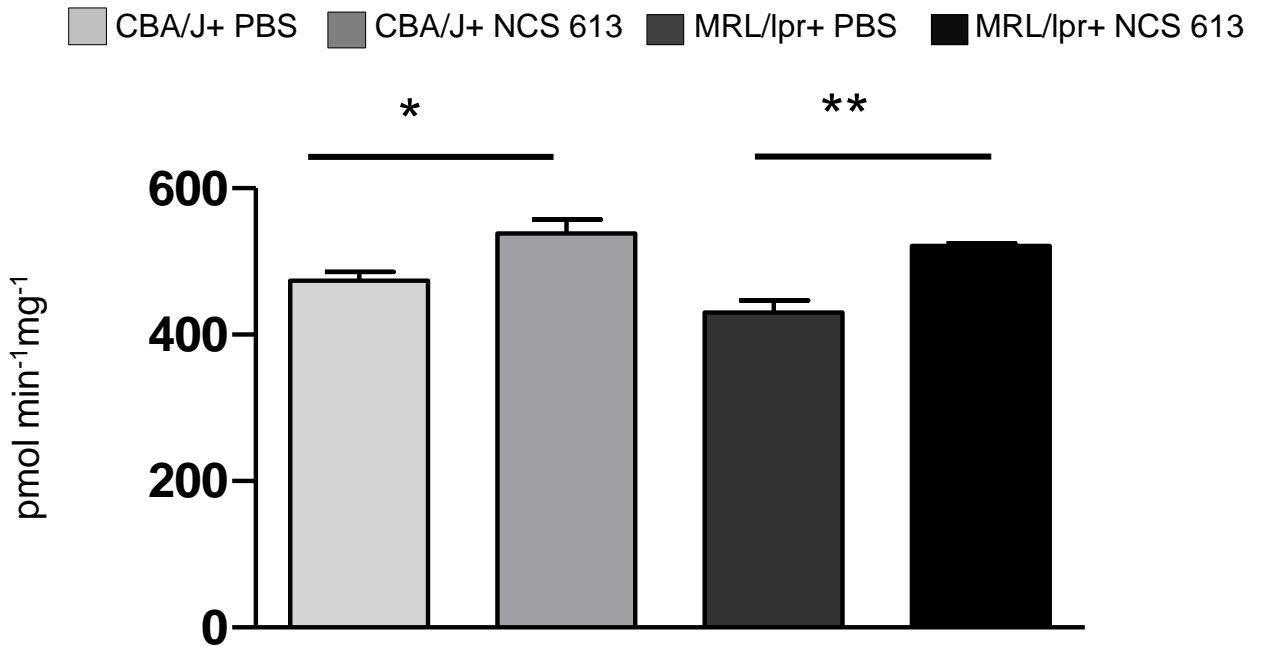


Figure 1

### A Kidney cAMP-PDE activity



### B Kidney PDE4 activity

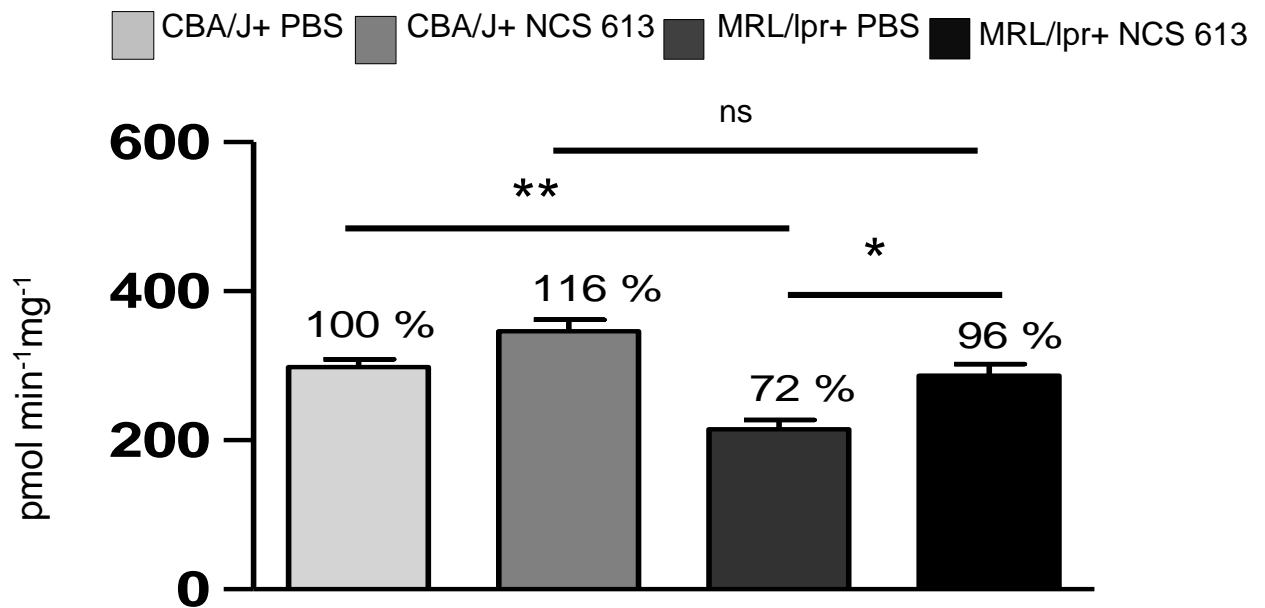


Figure 2

# A kidney cAMP levels

■ CBA/J+ PBS   ■ CBA/J+ NCS 613   ■ MRL/lpr+ PBS   ■ MRL/lpr+ NCS 613

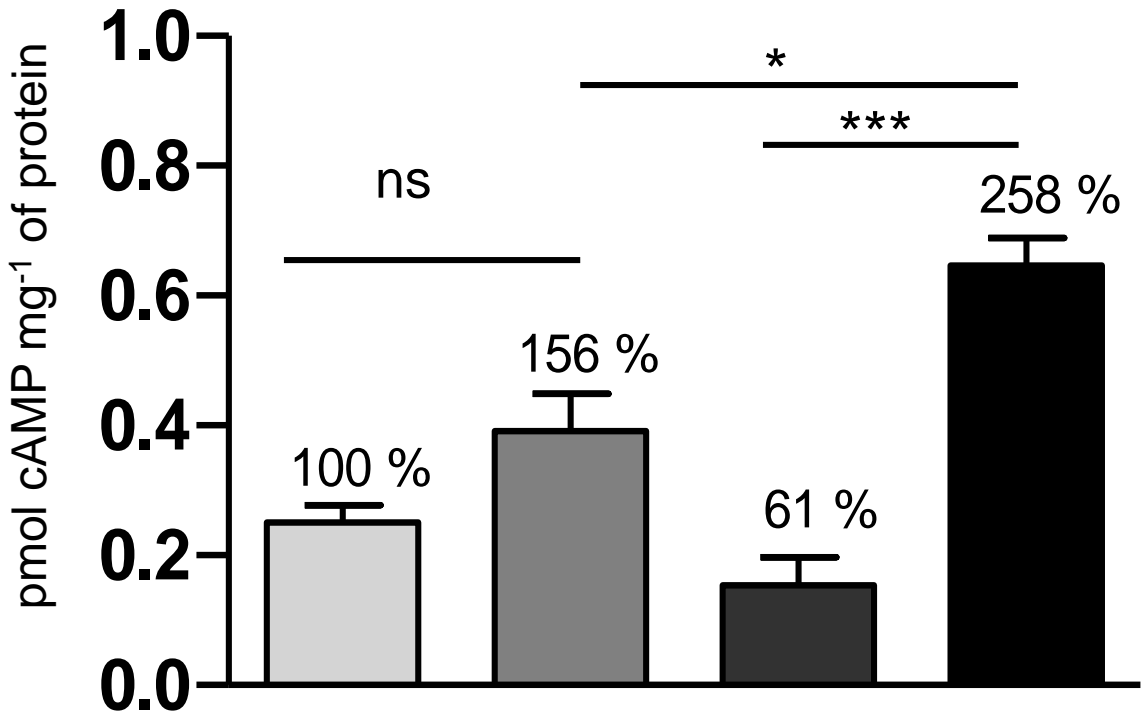


Figure 3

CBA/J+ PBS
  CBA/J+ NCS 613
  MRL/lpr+ PBS
  MRL/lpr+ NCS 613

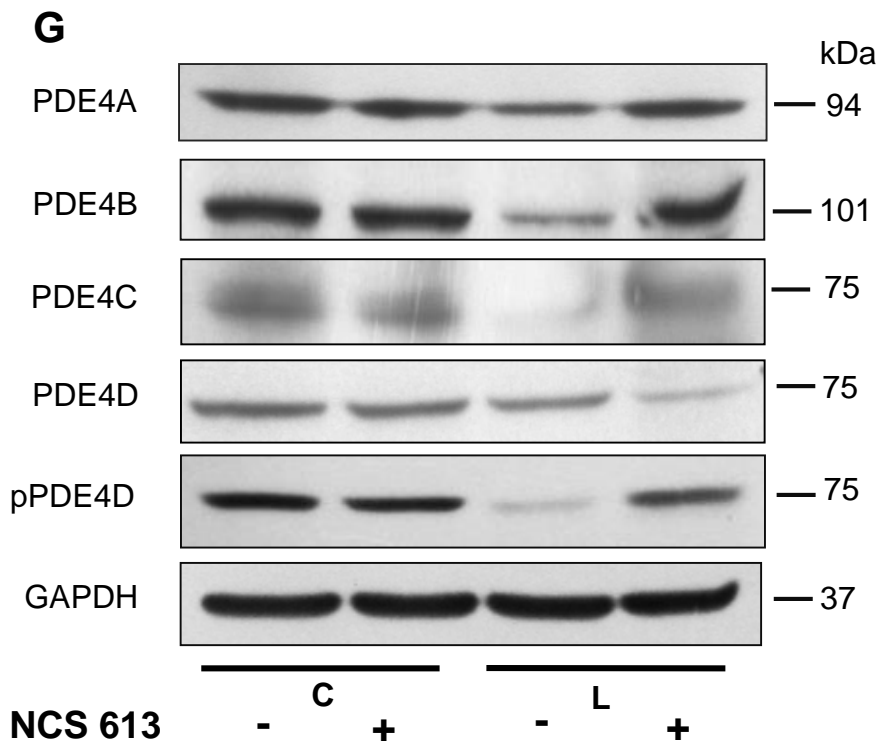
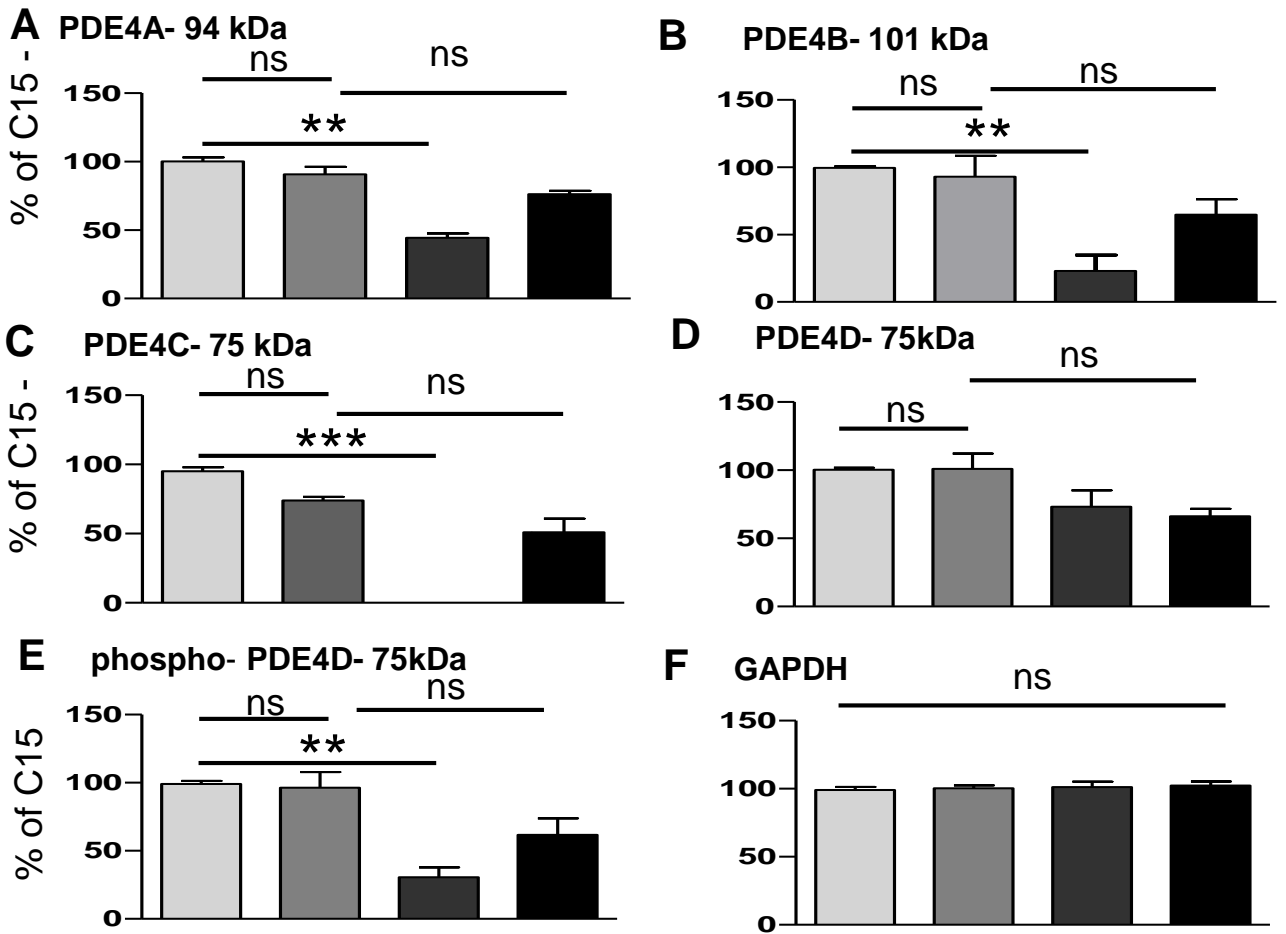


Figure 4

# A Subcellular PDE4 isoforms distribution

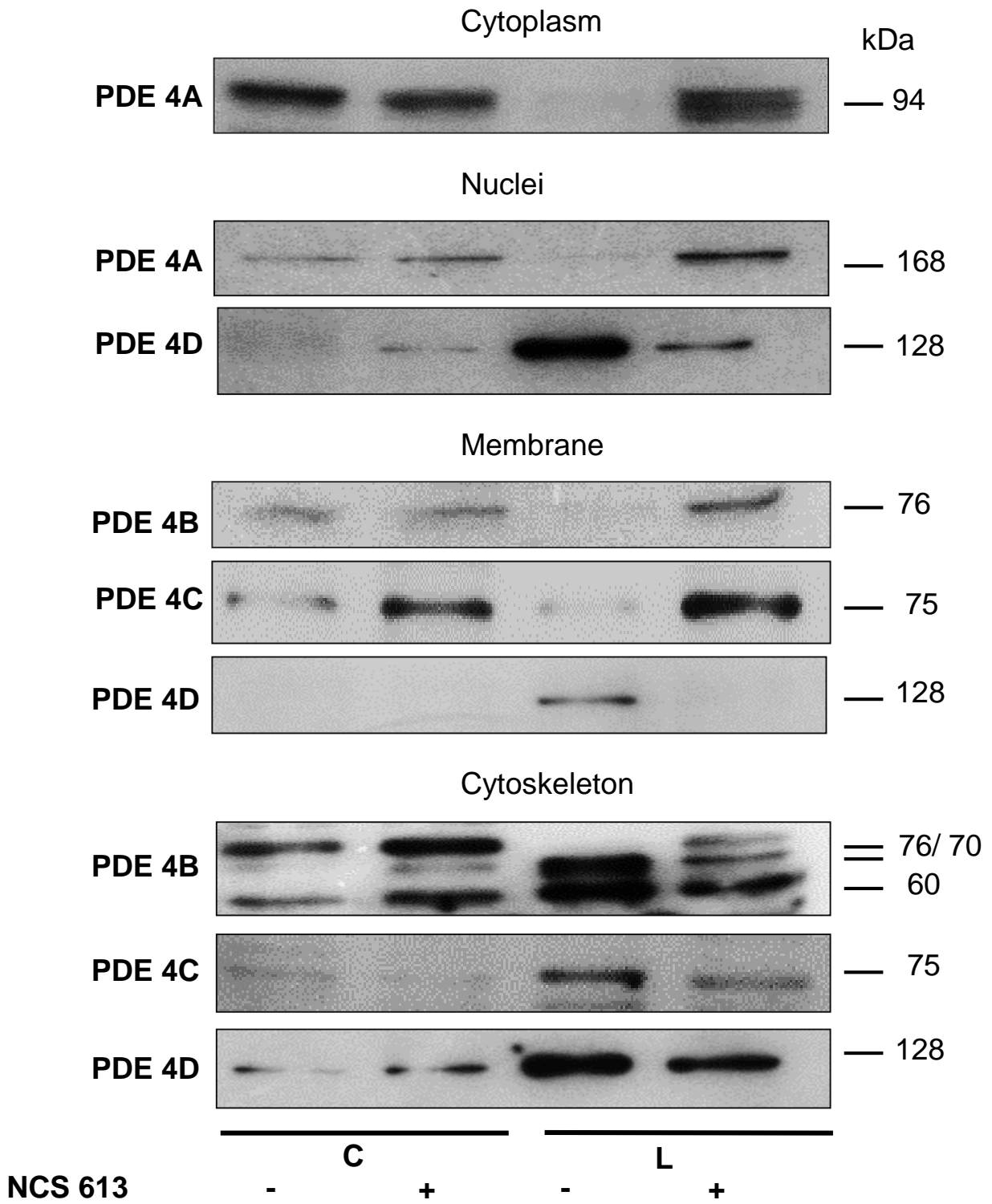
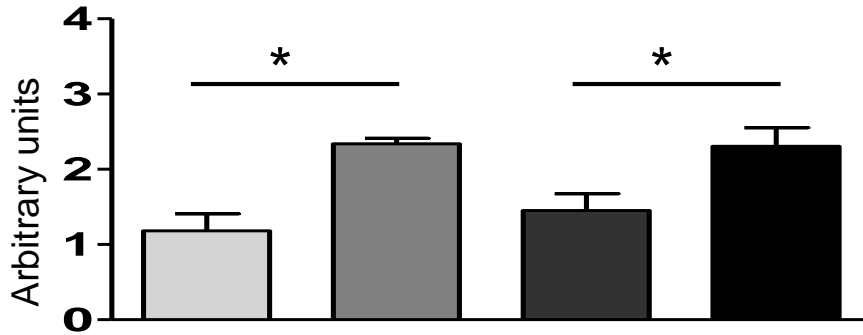


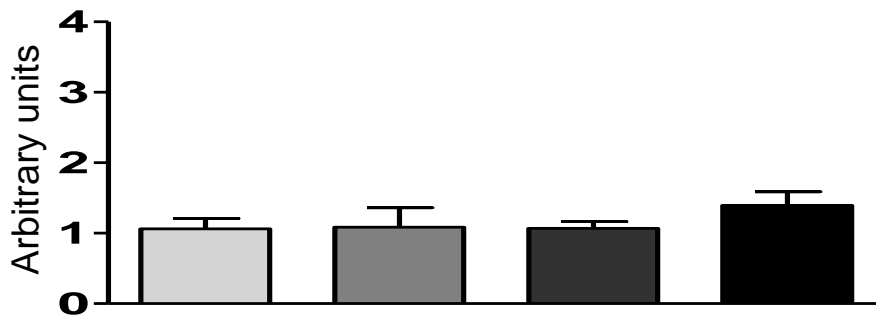
Figure 5

■ CBA/J+ PBS   ■ CBA/J+ NCS 613   ■ MRL/lpr+ PBS   ■ MRL/lpr+ NCS 613

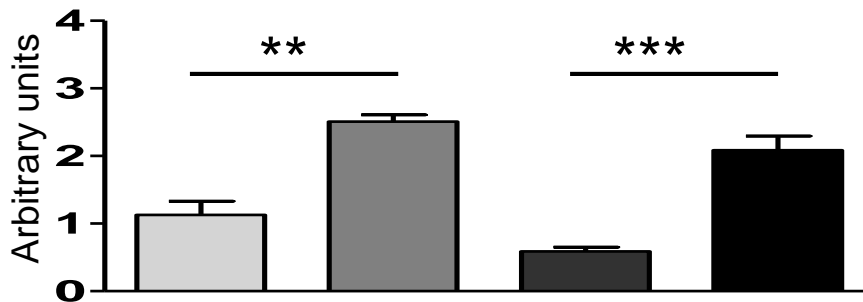
**A PDE4A mRNA**



**B PDE4B mRNA**



**C PDE4C mRNA**



**D PDE4D mRNA**

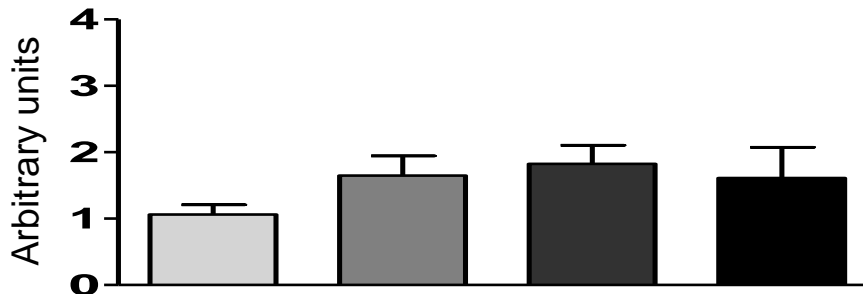
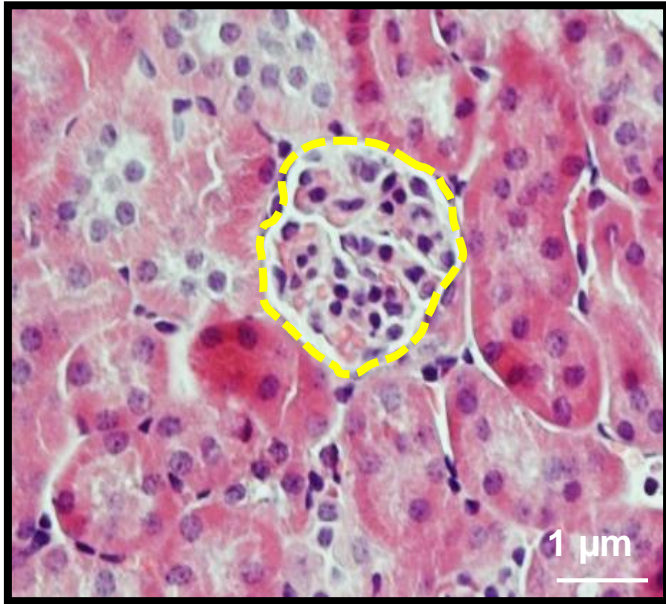
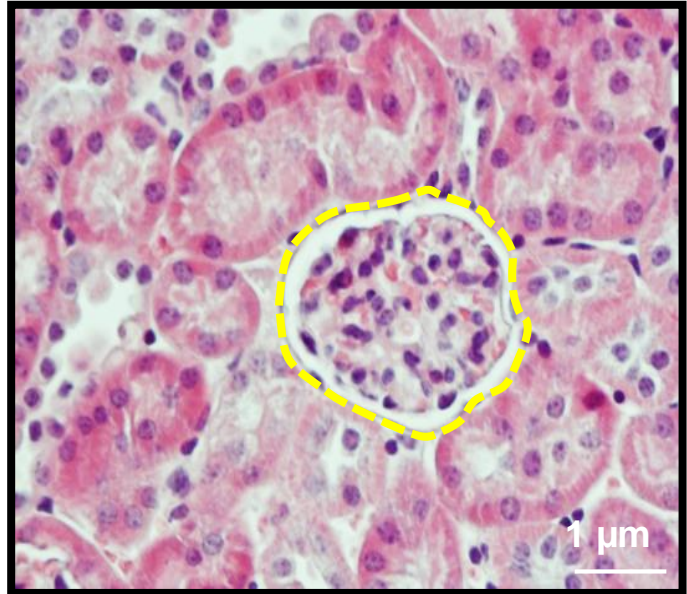


Figure 6

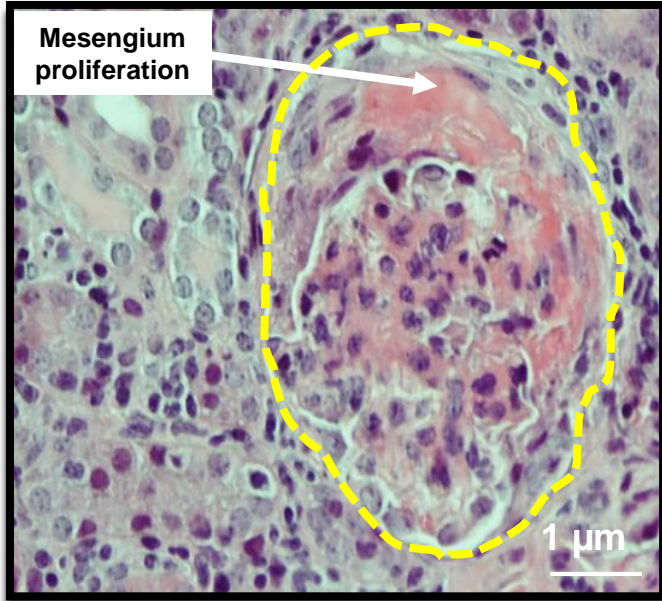
**A) CBA/J PBS**



**B) CBA/J NCS 613**



**C) MRL/lpr PBS**



**D) MRL/lpr NCS 613**

