Journal of Autoimmunity 54 (2014) 118–126

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Anti-ribosomal-P antibodies accelerate lupus glomerulonephritis and induce lupus nephritis in naïve mice

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ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form
18 February 2014
Accepted 26 February 2014
Available online 21 March 2014

Keywords:
Anti-ribosomal-P
Systemic lupus erythematosus
Glomerulonephritis

ABSTRACT

Background: Lupus nephritis is known to be associated with several antibodies including autoantibodies that target the DNA, C1q and histone, α-actinin, and the nucleosome. In addition, circulating anti-phosphoribosomal protein antibodies (anti-Ribos.P) were found to be associated with lupus nephritis.
Study objective: We have assessed the direct role of anti-Ribos.P in the development of glomerulonephritis in-vitro and in animal models.
Study design: NZBxW/F1 lupus prone mice were immunized with recombinant Ribos.P0 (rRibos.P). Evaluation of renal disease included mice evaluation for proteinuria and histologic analysis of the kidneys. Anti-Ribos.P monoclonal Ab was prepared from the rRibos.P immunized NZBxW/F1 mice by hybridoma technology. MAPKs expression was analyzed by MAPKs protein array and confirmed by real-time PCR and western blot. To elucidate whether anti-Ribos.P induce glomerulonephritis, naïve C3H mice were immunized with recombinant rRibos.P and the glomerulonephritis was followed up as described above.
Results: The immunized NZBxW/F1 lupus prone mice developed anti-Ribos.P which was cross reactive with Sm and not dsDNA. The mice developed accelerated glomerulonephritis manifested by early proteinuria and immunoglobulin deposites in the mesangium of the kidneys. Anti-Ribos.P deposited in the glomerular mesangium were eluted from the kidney. The Ribos.P immunized naïve C3H/Hen mice developed glomerulonephritis manifested by circulating autoantibodies directed to Ribos.P, dsDNA and Sm. The anti Ribos.P were cross reactive with Sm but not with dsDNA, and were deposited in the glomeruli. Interestingly these mice developed alopecia. In vitro. Primary mesangial cells exposed to mouse anti-Ribos.P mAb originated from the immunized lupus mice and to human anti-Ribos.P Abs, induced activation of mesangial cells via p38α, JNK, AKT and HSP27 MAPKs expression pathway.
Conclusions: Our data show for the first time that anti-Ribos.P are nephritogenic autoantibodies, as illustrated by in-vitro and in-vivo experiments: a) They accelerate the development of glomerulonephritis in lupus prone mice; b) They induce nephritis in naïve mice. c) Anti-Ribos.P Abs trigger MAPKs expression in primary mesangial cells. These data contribute a direct mechanistic link between anti-Ribos.P and nephritis in lupus mice.

1. Introduction

Among the wide spectrum of clinical manifestations in systemic lupus erythematosus (SLE), nephritis occurs in 40–60% of SLE patients. Because lupus nephritis progress into renal failure, it is a principal cause of death among SLE patients [1]. Glomerulonephritis classification in SLE according to the World Health Organization (WHO) comprises six histological classes. The International Society of Nephrology (ISN) and the Renal Pathology Society (RPS), have published a newer, similar classification with the addition of "a" for active and "c" for chronic changes. The advantage of the ISN/RPS classification is that it supplies the information regarding the potential reversibility of the disease [2]. Renal fibrosis and subsequent renal failure are caused by recurrent inflammation

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http://dx.doi.org/10.1016/j.jaut.2014.02.013
0896-8411/

and augmentation of local inflammatory responses, including the deposition of immunoglobulins within the glomerular and tubular basement membranes [3]. The lupus nephritis enigma for several decades. In the late 1960s, using immunofluorescence, Koffler et al. showed that immune complexes are involved in lupus nephritis pathogenesis and they found anti-dsDNA autoantibodies in the kidneys of patients who presented with lupus nephritis [4].

Lupus nephritis is known to be associated with several antibodies, including autoantibodies that target the DNA (4), C1q and histone, α-actinin [5–7], and the nucleosome [8]. dsDNA reactive antibodies are believed to play a critical role in the pathogenesis of lupus nephritis through three main mechanisms: a) tissue damage due to the *in-vitro* mesangial cell penetration of anti-dsDNA antibodies which cross react with kidney antigens; b) the formation of anti-dsDNA antibodies immune complexes with DNA/nucleosomes, which are released from apoptotic cells; and c) *in situ* formation of anti-dsDNA antibodies and nucleosomes immune complexes [6,9]. Additionally, in a more recent study, researchers were able to show that glomerular inflammation induction is strongly related to the direct binding of anti-dsDNA antibodies to antigens in the glomerular basement membrane or mesangial matrix [10].

In 1965, Sturgill and Carpenter [11] were the first to identify anti-Ribos.P Abs which target the intracellular ribosomal phosphoprotein. Anti-Ribos.P Abs bind to three main ribosomal proteins: P0, P1 and P2 (38 kd, 19 kd and 17 kd), respectively [12]. These antibodies targets mainly the22 amino acids region, which is located at the carboxyl terminal end (C-22) of the ribosomal S60 subunit, This complex includes two copies each of P1 and P2 and a single copy of P0. The interaction of the pentameric complex with the 28S rRNA molecule, creates the GTPase domain, which in turn plays a part in the elongation process of protein translation [13].

Among lupus patients, the reported prevalence of anti-Ribos.P Abs is 10–40%, with a high specificity of the Abs with neuropsychiatric SLE (NPSLE) [14,15]. Correlations between anti-Ribos.P Abs and neuropsychiatric clinical expressions include psychosis, delirium, cognitive impairment, mood disorders, and anxiety [16]. Additionally, intracerebroventricular administration of human anti-Ribos.P induced depression in naïve mice [17].

A link between anti-Ribos.P autoantibodies and nephritis, hepatitis and skin manifestations in lupus patients was reported previously [18,19]. Anti-Ribos.P Abs have high specificity for SLE [20] with clear correlation to SLE disease activity. Moreover, the presence of these Abs was more prevalent in patients in which SLE appeared during adolescence compared to patients in whom the disease developed at a later age [21].

Meroni PL et al. first reported the association between anti-Ribos.P Abs and renal injury in patients with lupus in 1984 [22] Reichlin's group showed that in patients demonstrating anti-Ribos.P Abs, lupus nephritis was significantly more prevalent than in those who were negative for these Abs [23]. This group also demonstrated anti-Ribos.P Abs titer fluctuations, based on exacerbation and remission intervals of the disease [24]. Several another studies further showed that the simultaneous expression of anti-dsDNA Abs and anti-Ribos.P strongly correlate with lupus nephritis [21,25,26]. Furthermore, Bonfa et al. [27] proposed that anti-Ribos-P Abs may have a potential serologic marker for membranous glomerulonephritis (class V) in SLE patients, while de Macedo et al. [28] suggested that during nephritis flares, in the absence of anti-dsDNA, anti-Ribos.P Abs predict a better renal prognosis. Another group showed a high (but not significant) co-predominance in lupus nephritis class IV and V of anti-dsDNA Abs and anti-Ribos.P [29]. However, others showed no correlation between lupus nephritis and anti-Ribos.P Abs [30,31].

In our study, we report that anti-Ribos.P accelerate lupus nephritis in lupus prone mice and *de novo* induction of anti-Ribos.P Abs cause renal injury and lupus skin manifestations. We demonstrate the ability of anti-Ribos.P mAbs to bind mesangial cells and induce MAPKs, which can serve as an important mechanism for mediating renal tissue injury.

2. Materials and methods

2.1. Antibodies

The following Abs were used in these studies:

Human anti-Ribos.P IgG Abs were affinity purified on a ribosomal-P column from sera originated from a patient with NPSLE accompanied with nephritis as previously described [32]. Control human IgG (h.cIgG) or mouse Ig1 were of commercial origin (m.cIgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)).

Generation of mouse anti-Ribos. mAbs: The mouse anti-Ribos.P mAbs were prepared as previously described by us (33). Spleen cells were isolated form three NZBxW/F1 rRibos.P immunized, 3 months after the boost. The splenocytes were fused with the mouse plasmacytoma cell line ×63.653 at a ratio of 10:1 in the presence of PEG (polyethylene glycol) 1500, and seeded in 96 wells in HAT medium using standard techniques as previously described by us [33]. The positive clones were subjected three times to a limiting dilution procedure. The anti-Ribos.P specific mAb detection was performed by screening culture fluids on Ribos.P coated plates (AESKU) by ELISA, and on ELISA plates coated with synthetic peptide RIBOSPEP 50 μg/ml in PBS overnight at 4 °C. Scrambled RIBOSPEP peptide coated ELISA plates were used as control. The plates were blocked with 3%BSA in PBS 1 h at 37C. Following 4 h incubation time with the tested culture fluid, the immunoglobulin binding was probed with a goat-anti-mouse IgG-alkaline-phosphatase and later the appropriate substrate was added. Data were read at OD 405 nm ref.650 nm. The selected mouse anti-Ribos.P mAb M7/3 IgG1 bound specifically to Ribos.P in a dose dependent manner and the RIBOSPEP synthetic peptide (carboxy-terminal 22-amino acid of the ribosome.P as described below) was able to inhibit M7/3 mAb binding to Ribos.P by 97 ± 8% at a concentration of 12 μg/ml in comparison to 3 ± 2% in the presence of scRIBOPEP, $P < 0.001$ (Fig. 1).Commercial mouse IgG1 isotype (cmIgG) (Jackson) was used as negative control.

Generation of mouse anti-anti-Ribos. mAbs: C3H mice were immunized with Ribos.P in CFA, The mice were boosted twice with 2 weeks intervals with Ribos.P in incomplete Freuds adjuvant. Two weeks later the mice developed high titers of anti-anti-Ribos.P. IgG. The sera was loaded on anti-Ribos.P mAb column and the eluted IgG was anti-anti-Ribos.P IgG.

2.2. Synthetic peptides

The synthetic peptide is composed of the carboxy-terminal 22-amino acid of the ribosome-P protein and has the following sequence: Lys-Lys-Glu-Glu-Lys-Lys-Glu-Glu-Ser-Glu-Glu-Glu-Asp-Glu-Asp-Met-Gly-Phe-Gly-Phe-Leu-Phe-OH named RIBOSPEP. The control scrambled form of this peptide is: Met-Lys-Glu-Lys-Glu-Glu-Ser-Lys-Phe-Asp-Gly-Glu-Asp-Glu-Glu-Leu-Phe -Glu-Phe-Lys-Gly-Glu-OH. The peptides were synthesized by GL-Biochem, Shanghai Ltd, Shanghai, China.

2.3. Immunization

Female NZBxW/F1 lupus prone mice at 12 weeks of age were obtained from The Harlan Laboratories (Bicester, UK). Female C3H/Hen mice at 12 weeks of age were purchased from Harlan Laboratories (Rehovot, Israel). Mice were handled under protocols

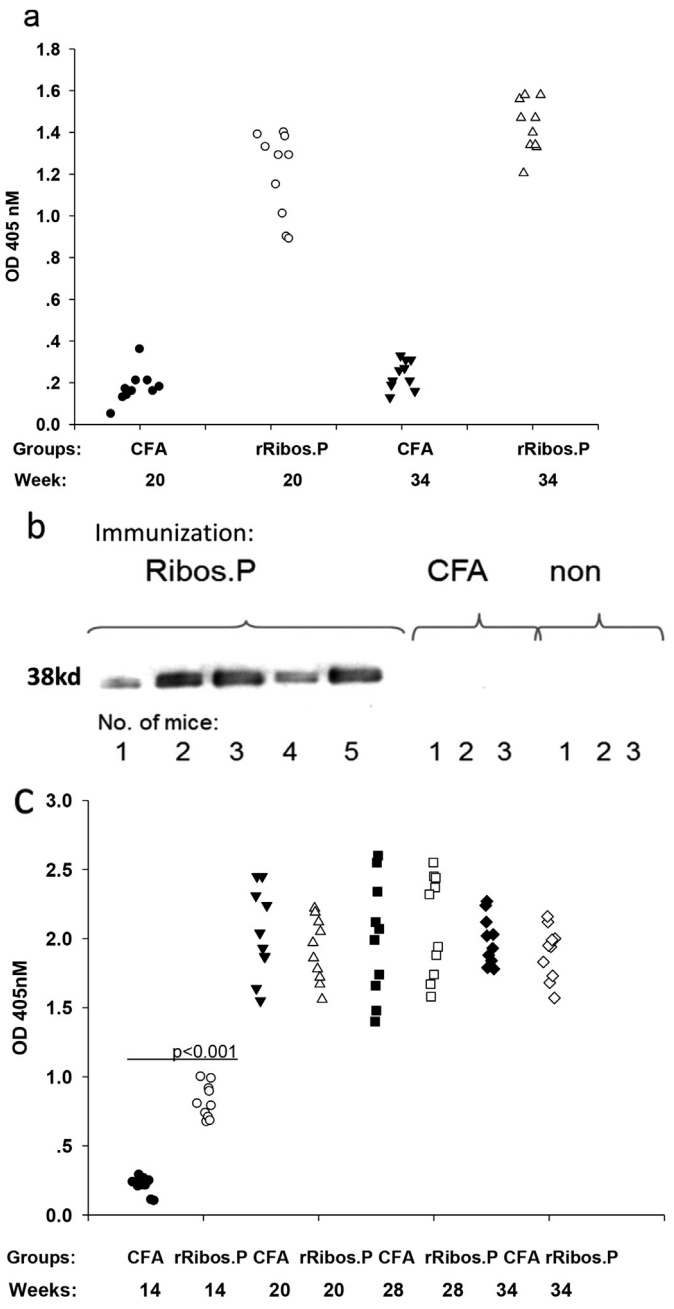

Fig. 1. Autoantibodies profile in the sera of rRibos.P0 immunized NZBxW/F1 mice: **1a**: levels of circulating anti-Ribos.P IgG at the age of 20 weeks in comparison to 34 weeks, when the mice already have proteinuria in NZBxW/F1 rRibos.P immunized mice and CFA injected mice. **1b**: Example of anti-Ribos.P0 binding by immunoblot. **1c**: Titers of anti-dsDNA in NZBxW/F1 rRibos.P immunized mice and CFA injected mice, at immunized with 14, 20, 28, 34 weeks of age.

approved by the Sheba Medical Center Animal Care and The Israel Ministry of Health Committee according to international guidelines.

NZBxW/F1 and C3H/Hen mice were immunized with recombinant ribosomal-P0 (rRibos.P0)(AESKU, Wendelsheim, Germany), 10 μg/0.1 ml/mouse emulsion in Complete Freunds' Adjuvant (CFA) into the hind footpads. Three weeks later a booster injection was given in PBS at the same location. NZBxW/F1 ($n = 20$) mice were immunized with rRibos.P at the age of 12 weeks, boosted at the age of 15 weeks. Five mice were immunized with CFA and one group of NZBxW/F1 mice were not immunized. The C3H/Hen ($n = 20$) mice were immunized at the age of 12 weeks using the same protocol and boosted at 15 weeks. C3H/Hen mice ($n = 20$) were immunized with CFA.

2.4. Detection of autoantibodies in mice sera

Mouse anti-dsDNA: Anti-dsDNA Abs binding in the mice sera were detected by home-made ELISA as previously described by us [34]. Briefly, mice sera diluted 1:200 were applied to the dsDNA coated ELISA plates, incubated for 60 min at room temperature. Bound antibodies were probed by using alkaline phosphatase conjugated goat anti-mouse IgG (H + L) (Jackson) diluted 1:5000 followed by appropriate substrate (Sigma, St Louis, MO, USA). Data were read by a Titertrek ELISA reader at 405 nm filter/620 nm reference filter.

Mouse anti-Ribos.P Abs were evaluated in the mice sera by employing Anti-ribos.P kits (AESKU). Mice sera at a dilution of 1:200 were added to the dilution buffer for 30 min at room temperature. The binding was probed with a goat anti-mouse IgG-peroxidase (Jackson) at a dilution 1:10,000, followed by an appropriate substrate according to the manufacturer instructions. The data were read at OD of 450 nm.

Mouse anti-Sm Abs were evaluated in the mice sera by using ELISA coated and blocked Sm plates (INOVA, Diagnostic Inc. San-Diego, CA, USA) as described above for anti-Ribos.P.

Inhibition assays: Mouse anti-dsDNA Abs were affinity purified from sera of NZBxW/F1 mice and C3H/Hen mice, immunized with rRibos.P, on a cellulose column as previously described by us (34). Inhibition assays were performed using affinity purified anti-dsDNA Abs, IgG from NZBxW/F1 mice immunized with Ribos.P, after depletion of anti-dsDNA on a cellulose column, followed by protein affinity purification and anti-Ribos.P mAb, all at 50% binding to the specific plate. dsDNA, and Ribosomal-P were used as inhibitors at different concentrations (5–50 μg/ml), for dsDNA binding and Sm/RNP binding by ELISA.

2.5. Evaluation of renal disease

Mice were evaluated for proteinuria using Multistix strips (Bayer Diagnostics, Puteau, France). Urine samples were graded 0–4+, corresponding to the following approximate protein concentrations: 0 = negative or trace, 30 mg/dl, 100 mg/dl, 300 mg/dl and ≥2000 mg/dl. Mice with a significant proteinuria (≥300) on two consecutive examinations were designated positive for renal disease. Severely ill and wasted mice with a significant proteinuria were sacrificed when they appeared moribund. These mice were considered as dead at the time they were killed and were included in the survival evaluation.

Histologic analysis of the kidneys: Mice perfused with PBS were paraffin embedded. Five micron sections were stained for H&E. For detection of Ig deposits, paraffin sections (4–5 μm) were incubated with an anti-mouse Fc IgG FITC-conjugated (Jackson). Evaluation was performed by two pathologists that were blinded to the diagnosis.

2.6. Immunoglobulin elution from the kidneys

Lysate from the kidneys were prepared following perfusion with PBS. The kidneys were homogenized with Glycine-HCl O.2M pH2.5. The eluate was dialyzed against PBS. Anti-ribos.P binding was detected by ELISA.

2.7. Preparation of primary mouse mesangial cells (MCs)

MCs from C3H/Hen mice kidneys were isolated according to a protocol described by Deocharan B et al. [7]. The outer cortexes of

10 kidneys were minced with a razor, and the tissue was forced through progressively smaller stainless steel sieves (180, 150, and 90 mm, Sigma). Glomeruli were then caught on a 75-μm sieve, washed twice with PBS, and centrifuged for 5 min at 220 g. The washed glomeruli were treated with collagenase for 20 min at 24 °C and washed again as above. Cells were plated out in RPMI 1640 supplemented with non-essential amino acids, sodium pyruvate, insulin, and 20% FCS, and maintained at 37 °C/5% CO_2. The media was changed every 4 days. After 30 days, the cultures consist of virtually pure MCs.

2.8. MCs Treatments, lysate preparation, immunoblotting and real-time-PCR

MCs derived from C3H mice kidneys, were exposed to human anti-Ribos.P Abs, mouse anti-Ribos.P mAbs control human IgG and control mouse IgG1 isotype control (20 μg/ml) for 4 h. Subsequently, the cells were lysed in lysis buffer (20 mM TrisHCl (pH 7.5), 1%SDS, protease inhibitor cocktail, and 0.2 mM PMSF, all the reagents were purchased from Sigma), incubated 30 min at 4 °C, and centrifuged to remove debris. The protein content of the lysate was measured using the BCA™ protein assay kit (Thermo Scientific, Rockford, IL, USA). Phospho-MAPKs membrane arrays (R&D Systems Inc, Minneapolis, MN, USA) were exposed to the different lysates originated from MCs exposed to anti-Ribos.P Abs and the relevant controls, according to the manufacturer's protocol [35].

Total mRNA was isolated using the Qiagen RNA isolation kit according to the manufacturer's instructions. RNA was then reverse transcribed into cDNA by MMLV reverse transcriptase. Five micrograms of total RNA was transcribed to cDNA in a 30-μl reaction volume. For transcript quantification by real-time PCR, the SYBR Green Mix containing Thermo-Start DNA Polymerase (InVitroGene, CA, USA) was used according to the manufacturer's instructions. cDNA samples were amplified using specific primers. PCR amplification was performed with specific forward and reverse primers for p38α (forward, 5′-^{58}GTGCCCGAGCGTTACCAGACC^{78}-3′; reverse, 5′-^{370}CTGTAAGCTTCTGACATTTC^{351}-3′), The levels of p38α mRNA were adjusted to the levels of αactin. a5' – primer (5′- TCATG AAGTGTGACGTTGACATCCGTA-AAG-3′) and 3′ – primer (5′- CCTAG AAGCATTTGCGGTGCACGATGGAGG – 3′). αactine primers were as follows: forward: 5′-cagcctcaagatcatcagca; reverse-3′: reverse: 5′-gtcttctgggtggcagtgat-3′; accession number: M33197. Amplification was monitored by real-time PCR analysis using the ABI/Prism 7700 Sequence Detector System (Applied Biosystems).

3. Results

3.1. rRibos.P0 NZBxW/F1 immunized mice developed accelerated glomerulonephritis

We studied the potential of *de-novo* generated mouse anti-Ribos.P Abs by immunizing NZBxW/F1 lupus prone mice with rRibos.P0, on the development of glomerulonephritis. We analyzed the titers of autoantibodies, proteinuria, immunoglobulin deposition in the glomeruli and we eluted mouse anti-Ribos.P0 from the kidneys of the rRibos.P0 immunized mice. NZBxW/F1 lupus prone mice, immunized with rRibos.P0, developed an earlier glomerulonephritis than the adjuvant immunized mice week 18–22 in comparison to the adjuvant injected mice which developed glomerulonephritis at week 32–34. These mice developed high titers of anti-Ribos.P Abs which remained steadily high until the time when the mice developed glomerulonephritis, at the age of 34 weeks (Fig. 1a), $P < 0.001$. The data were confirmed by an immunoblot (Fig. 1b). Whereas the CFA immunized mice, did not develop anti-Ribos.P Abs. However, the titers of anti-dsDNA Abs in the sera were elevated in both, in the rRibos.P0 and the CFA immunized mice (Fig. 1c). The titers of anti-dsDNA were not affected by the immunization procedure with rRibos.P, $p > 0.05$, (Fig. 1c).

As depicted in Table 1, anti-Ribos.P Abs were not cross reactive with dsDNA since anti-dsDNA affinity purified from the sera of NZBxW/F1 mice, did not bind Ribos.P ELISA plate and was not inhibited by Ribos.P on dsDNA ELISA plate. Moreover, the data were confirmed by using anti-Ribos.P mAb derived from these mice. The anti-Ribos.P mAb binding to Ribos.P was not inhibited by dsDNA and no anti-dsDNA binding was observed (Table 1).Interestingly, a cross reactivity between Ribos.P and Sm antigen was detected (Table 1).Anti-Sm binding was tested by using polyclonal IgG from the lupus mice which were anti-dsDNA depleted, anti-Ribos.P mAb, and Ribos.P as inhibitor. The data show that Ribos.P inhibited anti-Ribos.P binding by 40%. $p < 0.004$.

Table 1
Percent inhibition of binding of affinity purified anti-dsDNA, mouse-IgG depleted of anti-dsDNA and anti-Ribos.P mAb to dsDNA, Ribos.P and Sm by ELISA.

ELISA															
Ribos.P							Sm				dsDNA				
Tested Ab:		Anti-dsDNA		[b]IgG, anti dsDNA depleted	Anti-Ribos.P mAb		Anti-dsDNA	IgG anti-dsDNA depleted	Anti-Ribos.P mAb		Anti-dsDNA		IgG anti-dsDNA depleted	Anti-Ribos.P mAb	
Inhibitor:		dsDNA	Ribos.P	Ribos.P	dsDNA	Ribos.P	Ribos.P	Ribos.P	dsDNA	Ribos.P	dsDNA	Ribos.P	Ribos.P	dsDNA	Ribos.P
Concentration μg/ml		50		50	50		50	50	50		50		50	50	
NZBxW/F1 immunized with Ribos.P	1[a]	3[c]	4	98 ± 5	4 ± 2	97 ± 4	15	45 ± 3	7 ± 2	47 ± 4	97	3	6 ± 1	5 ± 2	3 ± 1
	2	5	2	95 ± 3			9	36 ± 4			99	5	7 ± 1		
	3	4	3	99 ± 7			11	54 ± 6			98	6	8 ± 7		
	4	6	5	97 ± 9			14	39 ± 4			96	4	9 ± 2		
	5	2	4	98 ± 4			12	45 ± 3			95	5	7 ± 3		
C3H/Hen immunized with Ribos.P	1	2	9	96 ± 6			16	49 ± 4			99	6	8 ± 3		
	2	6	8	98 ± 4			12	52 ± 5			97	8	9 ± 2		
	3	7	10	96 ± 5			9	57 ± 6			98	6	6 ± 2		
	4	11	6	99 ± 7			17	46 ± 5			95	9	7 ± 3		
	5	8	7	97 ± 9			11	53 ± 5			99	7	8 ± 2		

Control IgG inhibition of binding by specific antigen to: Ribos.P – 8 ± 2; Sm – 5 ± 2; dsDNA – 11 ± 3.
[a] Mouse number from which IgG was purified. Each number composed of IgG affinity purified from sera pooled from 2 mice.
[b] IgG from NZBxW/F1 mice immunized with Ribos.P, after depletion of anti-dsDNA on a cellulose column, followed by protein affinity purification. The anti-Ribos.P mAb was originated from NZBxW/F1 mice immunized with Ribos.P.
[c] Data are presented as percent of inhibition.

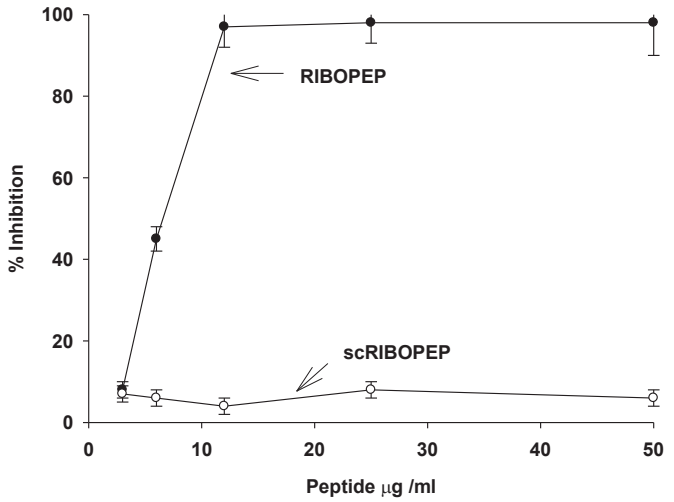

Fig. 2. Inhibition assay of mouse anti-Ribos.P mAb binding to Ribos.P by Ribos.P specific peptide RIBOPEP. The inhibitors were RIBOPEP and its scrambled form scRIBOPEP. The data are mean ± SD of 3 experiments.

Given that the titers of circulating anti-Ribos.P were elevated in the rRibos.P0 immunized mice, we assessed the deposition of these Abs in the kidneys. Mouse anti-Ribos.P IgG Abs were extracted from the kidneys of rRibos.P0 immunized mice. The immunoglobulin extracted from the kidneys of the rRibos.P immunized mice in comparison to the adjuvant immunized mice were analyzed for anti-Ribos.P binding by ELISA. The data revealed 1.065 ± 0.112 OD at 405 nm for the Ig extracted from rRibos.P immunized mice kidneys in comparison to 0.213 ± 0.087 for the Ig extracted from the CFA immunized kidneys. Yet, a direct confirmation for the nephritogenic activity of anti-Ribos.P in the development of glomerulonephritis was needed. Therefore, we have generated a mouse anti-Ribos.P0 mAb from the splenocytes of NZBxW/F1 rRibos.P0 immunized mouse. The anti-Ribos.P0 mAb recognized a synthetic peptide composed of the carboxy-terminal 22-amino acid of the ribosome-P protein (RIBOSPEP) by ELISA. As illustrated in Fig. 2, the RIBOSPEP inhibited the binding of anti-Ribos.P mAb to rRibos.P0 by 98.4 ± 7.1% at the concentration of 10 μg/ml, whereas scRIBOSPEP inhibited by only 2.3 ± 1.2, at the same concentration $p < 0.001$.

Accelerated proteinuria was discerned in the group of mice immunized with rRibos.P0, as illustrated in Fig. 3a, $p < 0.01$. The rRibos.P0 immunized group of mice, showed a higher percentage of mice having >100 mg/dl proteinuria (40% at week 20, 60% at week 24) in comparison with the CFA immunized mice (1% at week 20 and 59% at week 24).At the age of 18 weeks (3 weeks after boost administration), Ribos.P0 immunized mice already began to develop proteinuria whereas the control mice did not (Fig. 3a). As shown in Fig. 3b, renal histopathology at week 24 of age, revealed typical findings of focal and diffuse proliferative damage in glomerulus shown by H&E staining. Immunofluorescence staining illustrate immunoglobulin deposits in the glomeruli with diffuse pattern of diffuse peripheral deposition (Fig. 3b). Whereas, no significant immunoglobulin deposition in the glomeruli was observed in the kidneys of adjuvant immunized mice or in the non-immunized mice of the same age.

3.2. Naïve mice immunized with rRibos.P0, developed glomerulonephritis and experimental lupus

We immunized C3H/Hen female mice with rRibos.P0 emulsified in CFA as an adjuvant. One-month after booster administration, the rRibos.P0 immunized mice developed significantly elevated titers of circulating anti-Ribos.P0 Abs ($p < 0.001$), when compared to control CFA immunized mice (Fig. 4a). In addition, the mice developed high levels of circulating anti-dsDNA Abs which remained constant over the time as depicted in Fig. 4b. Elevated anti-Sm Abs were shown in the rRibos.P0 injected mice (Fig. 4c). As shown in Table 1, anti-Ribos.P Abs were not cross reactive with dsDNA since anti-dsDNA affinity purified from the sera of C3H/Hen mice, did not bind Ribos.P and were not inhibited by Ribos.P on dsDNA ELISA plate. However, a cross reactivity between anti-Ribos.P and Sm antigen was illustrated (Table 1). Polyclonal IgG anti-dsDNA depleted binding to Sm ELISA plates was inhibited by Ribos.P (46% up to 57% inhibition), $p < 0.01$. Clinically, all the rRibos.P0 injected mice exhibited proteinuria at 4 months after the booster administration (e.g 100 mg/dl) whereas the CFA injected mice did not develop proteinuria (0–30 mg/dl). Renal histopathology at 4 months after boost injection revealed a diffuse

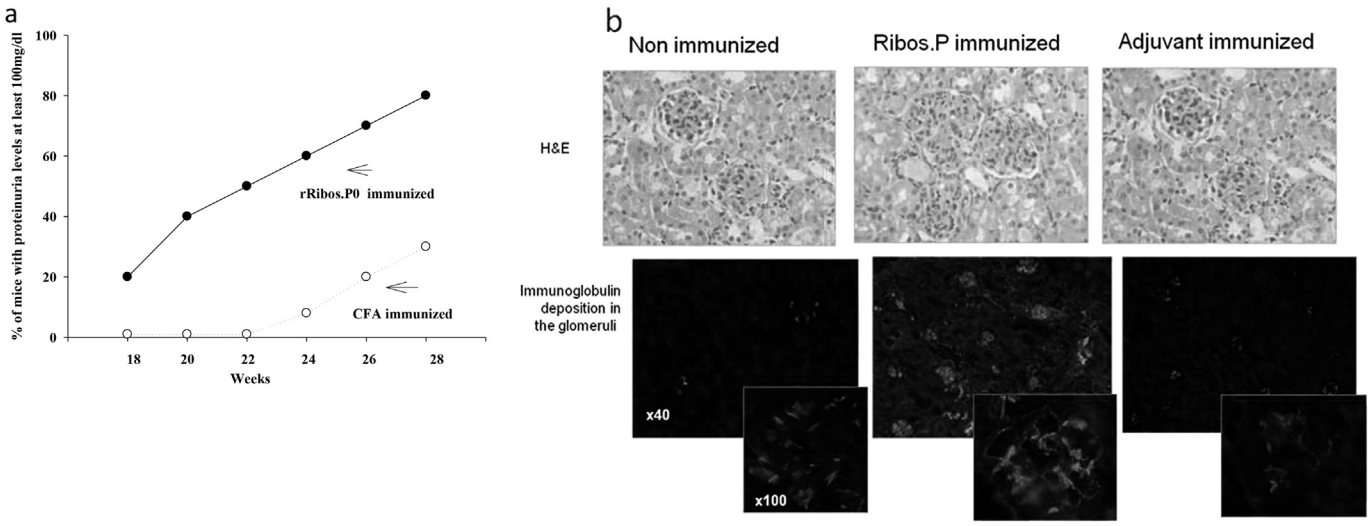

Fig. 3. A link between anti-Ribos.P Abs and glomerulonephritis in lupus prone mice. **3a**: Percent of mice with proteinuria above 100 mg/dl. Comparison between mice immunized with rRibos.P and mice immunized with adjuvant (CFA). **3b**: Histological analyses of kidneys: comparison of glomerulonephritis between mice immunized with rRibos.P, adjuvant and non-immunized mice, exemplified by H&E staining and immunoglobulin deposition in the glomeruli by immunofluoresence.

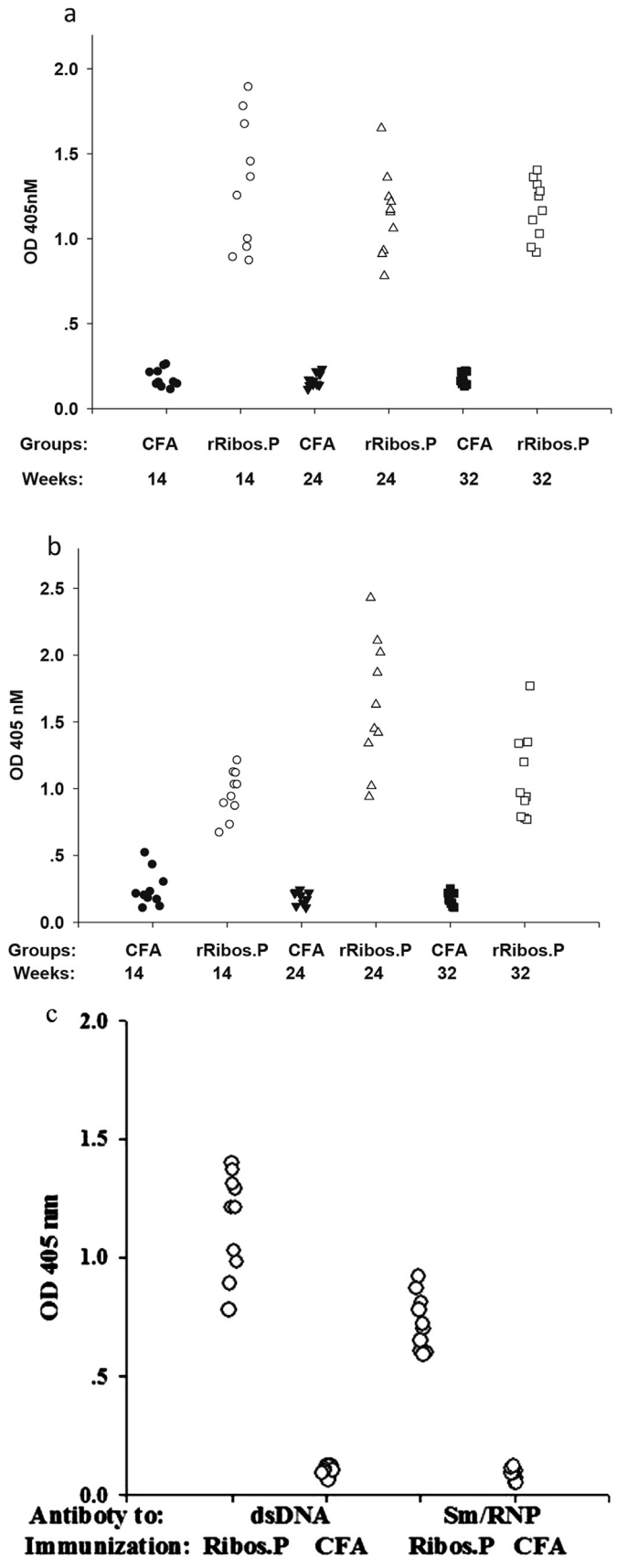

Fig. 4. Autoantibodies profile in the sera of naïve mice immunized with rRibos.P. **4a**: Titers of circulating anti-Ribos.P Abs in the sera of C3H/Hen mice at weeks 14, 24, 32 weeks after boost injection. **4b**: Titers of circulating anti-dsDNA in the sera of C3H/Hen mice at weeks 14, 24, 32 weeks after boost injection. **4c**: Titers of circulating anti-Sm/RNP in the sera of C3H/Hen mice at week 32 after boost injection.

proliferative mesangial damage with diffuse peripheral deposition by immunofluorescence (Fig. 5a). Furthermore, anti-Ribos.P0 was eluted from the kidney. Analyses of the immunoglobulins eluted from the kidneys,showed elevated anti-Ribos.P0 activity by ELISA (OD of 0.893 ± 0.078 in comparison to OD of 0.0930 ± 0.03 in the lysate of kidneys from the CFA injected mice).

In addition all the C3H/Hen immunized mice with rRibos.P developed alopecia on their face (a well-known skin manifestation of lupus), whereas control mice did not (Fig. 5b).

3.3. Anti-Ribos.P activate p38 MAPK in mesangial cells in-vitro

Mouse mesangial cell cultures prepared from healthy C3H/Hen female kidneys. In order to follow the effect of anti-Ribos.P on mesangial cells signaling cascade, a lysate from the mesangial cells was introduced to protein MAPKs protein array. The mesangial cell cultures were pre-exposed for 24 h to mouse anti-Ribos.P mAb and its IgG1 control or to a human anti-Ribos.P IgG and its IgG control. As shown in Fig. 6a, anti-Ribos.P originated either from human lupus patient or from a mouse origin, enhanced significantly the expression of phosphorylated p38α $P < 0.001$ by densitometry and HSP27, $p < 0.02$ in densitometry. Furthermore, as described in Fig. 6b, the expression of p-p38 was confirmed in the level of mRNA by real time PCR, $p < 0.004$ for human anti-Ribos.P and $p < 0.01$ for a mouse anti-rRibos.P in comparison to the relevant control immunoglobulin. The data were confirmed by immunoblot, Fig. 6c. The immunoblot analyses demonstrated that anti-Ribos.P from mouse and human origins, as well as anti-dsDNA IgG affinity purified from the lupus mice, enhanced the expression of p-p38, whereas anti-anti-Ribos.P inhibited the anti-Ribos.P effect on the mesangial cells, which confirm the specificity of the p38 activation by anti-Ribos.P Abs.

4. Discussion

In the present study we demonstrated that recombinant Ribos.P0 (38 kD) immunized NZBxW/F1 lupus prone mice, developed mouse anti-Ribos.P Abs, which did not target dsDNA and share specificity with Sm antigen by 40%. Accelerated proteinuria was detected in the Ribos.P immunized mice associated with renal histopathology at week 24 of age revealed focal and diffuse proliferative damage in glomeruli with a diffuse peripheral deposition demonstrated by immunofluorescence. Non-significant immunoglobulin deposition was observed in the CFA adjuvant immunized mice or non-immunized mice at the same age of 24 weeks. The direct nephritogenic properties of anti-Ribos.P was approved by a) rRibos.P immunized mice developed accelerated glomerulonephritis. Mouse anti-Ribos.P Abs were detected in the kidneys from the NZBxW/F1 mice .b) Anti-Ribos.P mAb originated from rRibos.P0 immunized NZBxW/F1 did not bind dsDNA,share specificity with Sm antigen. Anti-Ribos.P Abs from mouse and human origin activate the phosphorylation of p38α and HSP27 MAPKs. c) C3H/Hen naïve mice immunized with rRibos.P0 developed high levels of circulating anti-Ribos.P, anti-dsDNA and anti-Sm/RNP Abs. The anti-Ribos.P Abs did not bind dsDNA but were cross reactive with Sm antigen. The profile of circulating autoantibodies was associated with sever glomerulonephritis that was illustrated by proteinuria and diffuse proliferative mesangial damage with diffuse peripheral deposition. All C3H/Hen immunized mice with rRibos.P developed alopecia. Control mice did not develop either circulating autoantibodies nor nephritis or alopecia.

The MAPKs cascade is involved in many pathways of immune responses, including cell proliferation, differentiation, and cell death. One of the three major groups of MAPKs in mammalian cells is the p38 MAPK. It is now well recognized that there are multiple

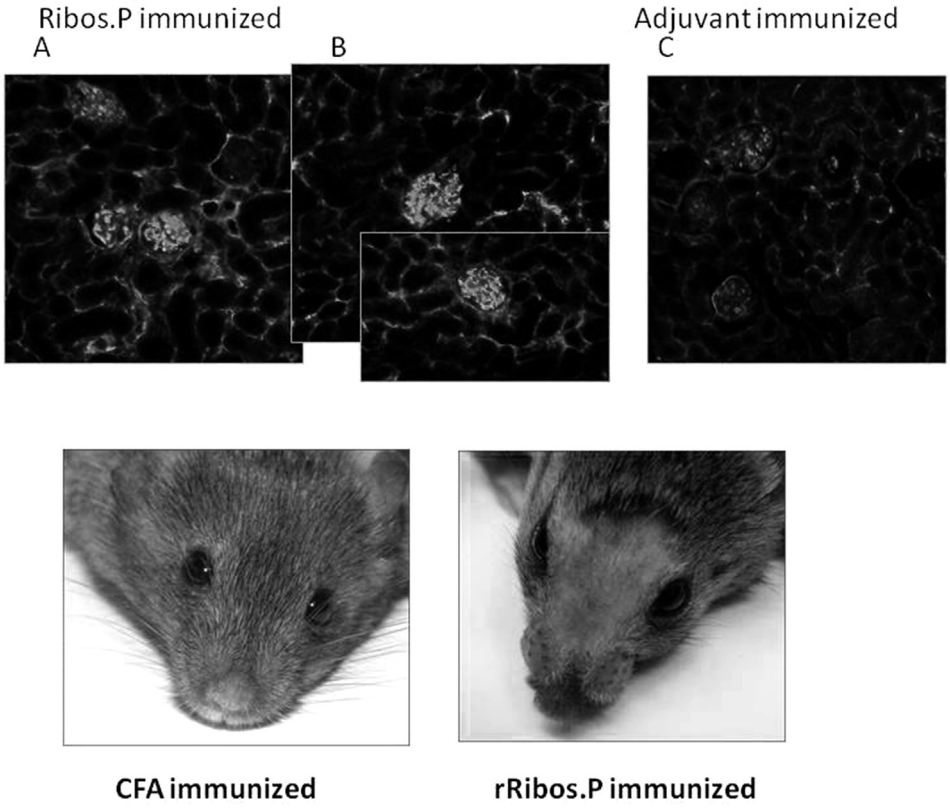

Fig. 5. Glomerulonephritis and alopecia in C3H/Hen naïve mice immunized with rRibos.P. **5a**: Immunoglobulin deposition in the kidneys of rRibos.P (A,B) or adjuvant (C) immunized mice. **5b**: Alopecia on the face of rRibos.P immunized mouse in comparison to adjuvant injected mouse.

mechanisms of p38 activation. In the classical conserved cascade, MAP K 3 (MKK3), MKK4, and MKK6 activates p38 MAPK by a dual phosphorylation at the tripeptide motif Thr-Gly-Tyr [43,44]. Toll-like receptors (TLRs) stimulate inflammatory pathways, ending in the activation of nuclear factor (NF)-κB and activating protein-1 (AP-1). p38 MAPK plays essential roles in mediating AP-1 activation in TLR signaling [45]. It was suggested that MAPs are involved in the development of renal injury and have an important role in the pathogenesis of glomerulonephritis. p38 MAPK is being activated in normal human kidney and its activation is significantly increased in class III and IV lupus glomerulonephritis and ANCA-positive pauci-immune crescentic glomerulonephritis. Activation of p38 MAPK in renal cells correlates with the degree of interstitial inflammation [46]. The expression of vascular cell adhesion molecule-1 (VCAM-1) in human mesangial cells leads to recruitment of leukocytes and monocytes adhesion to the glomerular mesangium. This is mediated, at least partially, by activation of p38 MAPK [47]. Moreover, studies show that p38 MAP K inhibitors have favorable effects by reducing glomerular infiltration and proliferation [48,40]. Inhibition of p38 MAPK reduce glomerular macrophage accumulation and crescent formation *in vivo*, and decrease anti-neutrophil cytoplasmic autoantibodies(ANCA)-activation of neutrophils *in vitro* [49]. The specific deposition and concentration of anti-Ribos.P at the site of tissue injury was previously demonstrated by Reichlin's group, as they discovered a 30-fold enrichment of anti-Ribos.P activity in the renal eluate from a patient with lupus nephritis [50]. In our study, human and mouse originated anti-Ribos.P activated mesangial cells *in-vitro*, resulting in p38α and HSP27 phosphorylation. This finding confirms the pathogenic role of anti-Ribos.P autoantibodies.

Our data contribute additional target epitope to the muliepitope network of lupus related autoantibodies related to the mesangium in lupus. There are several autoantibodies which cross react with dsDNA such as actinin and annexin-II, which also activate mesangial cells *via* p38 [51,52].

SLE has a wide range of clinical manifestations and lupus nephritis is a principal cause of death [1]. The currently used classification of lupus nephritis includes 6 histologic classes as diagnosed by kidney biopsy [3]. During exacerbation of lupus nephritis there is an increase in titer of anti dsDNA autoantibodies and formation of immune complex. Recurrent inflammation cause eventually mesangial fibrosis and tubular atrophy [4].

The nephritogenic properties of anti-Ribos.P in the currenet study goes along with the previously suggested mechanisms which encompass the following: a) Anti-ribos.P Abs-mediated tissue injury. Ribos.P proteins are expressed as intracellular antigens and also as antigens that are displayed on the cell surfaces of different tissues and numerous types of cells, including mesangial cells, astrocytes, neuroblastoma cells, hepatoma cells and fibroblasts [32,36,37,39]. For example, anti-Ribos.P target and penetrate intracellular Jurkat T cells followed by apoptotic cell-death process [38]. Anti-Ribos.P penetrateinot HepG2 hepatocytes *in-vitro* and cause damage apolipoprotein B synthesis, resulting in tripling of the amount of intra-cell cholesterol and lipid droplets (and thereby consistent with what is seen in various chronic liver diseases) [32]. b) Anti-ribos.P Abs substantially inhibit the overall level of protein synthesis *in-vitro,* (as measured by methionine incorporation [^{35}S]) [32,40]. c) Anti-Ribos.P Abs bind a neuronal surface and are capable of inducing *in vivo* and *in vitro* apoptosis in neurons, following the flow of calcium into cells [41]. d) Anti-Ribos.P may interfere with Th17 responses as a result of their activation of the p38 MAPK, as it was shown that the p38α acted in DCs to regulate IL-6 and IL-27 and to initiate Th17 differentiation and autoimmune inflammation [42].

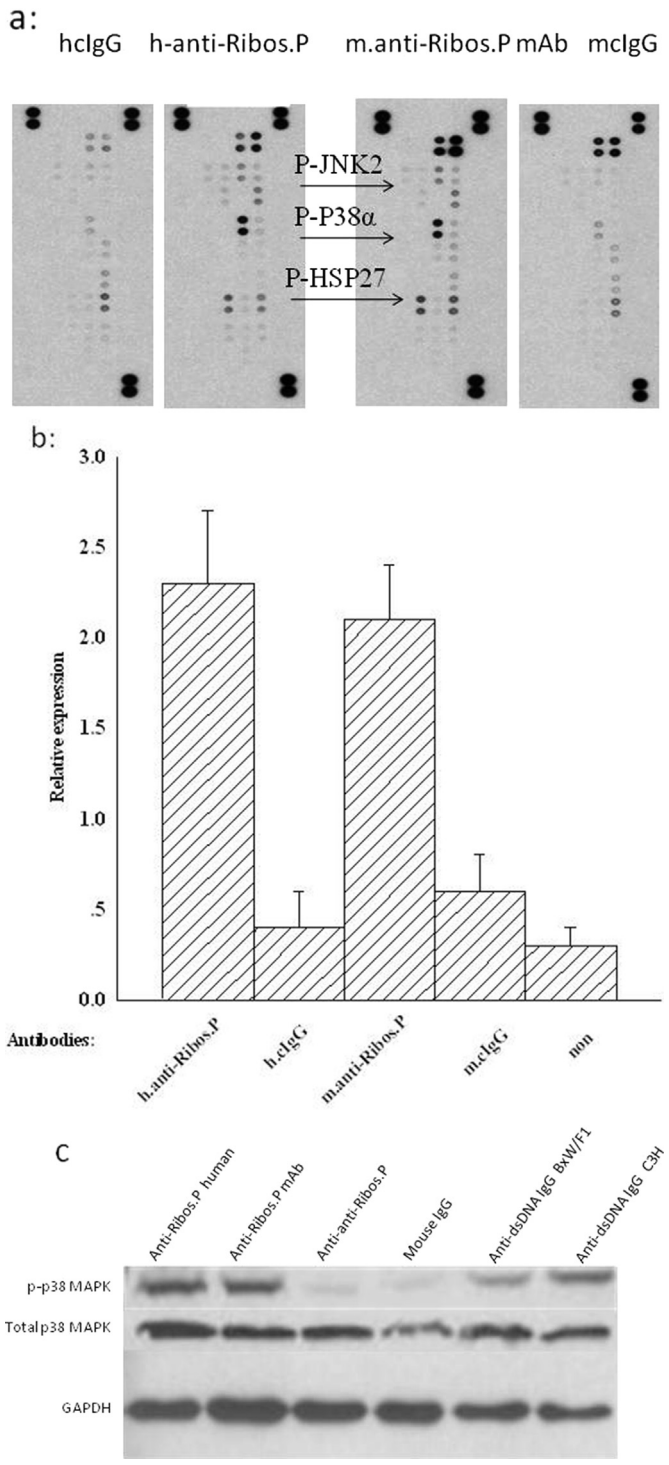

Fig. 6. ***In-vitro*** **MAPKs analyses in primary mesangial cells culture upon exposure to anti-Ribos.P human or mouse mAb and controls IgG. 6a**: MAPKs expression by mouse mesangial cells preexposed to human anti-Ribos.P or mouse anti-Ribos.P mAb and control human and mouse IgG1, for 1 h. **6b**: Relative expression of p38α (real-time PCR) by mesangial cells preexposed to human anti-Ribos.P or mouse anti-Ribos.P mAb and control human and mouse IgG1, for 1 h. **6c**: Immunoblot analysis of mesangial cells preincubated with anti-Ribo.P ± anti-anti-Ribos.P and control IgG.

Finally, the association of glomerulonephritis and elevated titers of anti-Ribos.P Abs in lupus patients was previously reported by several groups [22,23,27]. Our findings demonstrate for the first time the direct nephritogenic properties of anti-Ribos.P autoantibodies and their role in the development of glomerulonephritis in experimental model. In lupus As reported by Bonfa et al. Implication to human: anti-Ribos.P can be considered as a biomarker for lupus nephritis in the sera.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Update

Journal of Autoimmunity

Volume 67, Issue , February 2016, Page 111

DOI: https://doi.org/10.1016/j.jaut.2015.12.001

Journal of Autoimmunity 67 (2016) 111

Contents lists available at ScienceDirect

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Corrigendum

Corrigendum to "Anti-ribosomal-P antibodies accelerate lupus glomerulonephritis and induce lupus nephritis in naïve mice" [J. Autoimmun. 54C (2014) 118–126]

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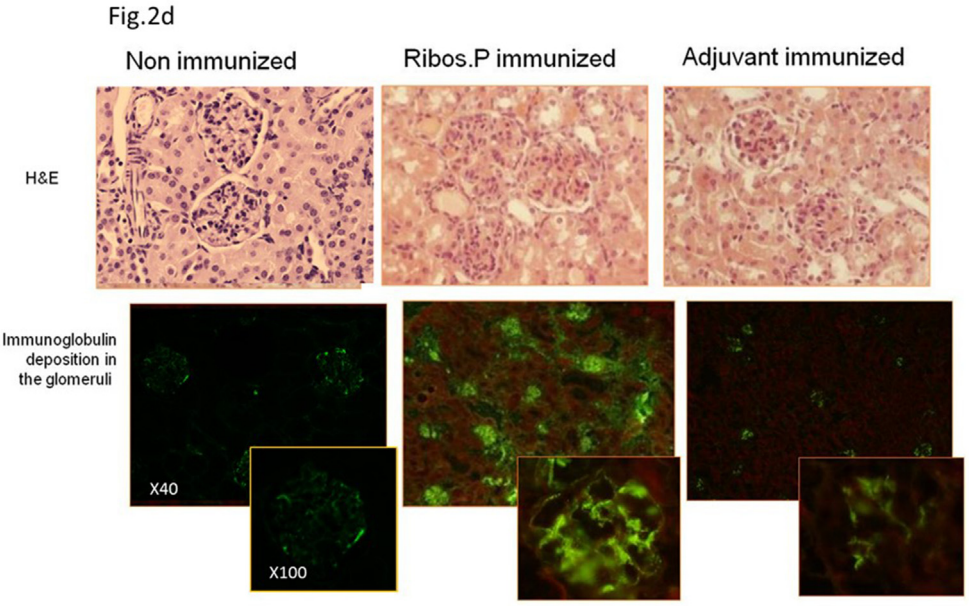
Unfortunately the following error was made for Fig. 3B in the original article:

There were 2 negative pictures, no immunoglobulin deposits in the kidney sections originated from the lupus mice (NZB × W/F1) at an early stage and in the adjuvant injected lupus prone mice.

The mistake was technical; the author had two negative pictures from the 2 groups. The author chosed the negative pictures from each group but uploaded twice the negative picture from the group of adjuvant injected mice.

Therefore, please find the corrected negative picture below.



The authors wish to apologise for any inconvenience caused.

DOI of original article: http://dx.doi.org/10.1016/j.jaut.2014.02.013.
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http://dx.doi.org/10.1016/j.jaut.2015.12.001
0896-8411/