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Immunization with pentraxin 3 (PTX3) leads to anti-PTX3 antibody production and delayed lupus-like nephritis in NZB/NZW F1 mice

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ABSTRACT

Background: Anti-pentraxin 3 (PTX3) antibodies were associated with the absence of lupus glomerulonephritis in humans.
Aim: To explore the effects of anti-PTX3 antibodies in New Zealand Black/White (NZB/NZW F1) mice and their inherent mechanisms of action.
Materials and methods: 30 NZB/NZW F1 mice were subdivided into 3 groups of 10 mice each and subcutaneously injected with PTX3, alum and PBS (group 1), alum and PBS (group 2) or PBS alone (group 3), 3 times 3 weeks apart, before development of renal disease. Mice were followed until natural death. Histological analysis and immunohistochemistry were performed on harvested kidneys. Effects of anti-PTX3 antibodies on C1q binding to immobilized PTX3-anti-PTX3 immune complexes were evaluated *in vitro* using human SLE sera. Qualitative characterization of human IgG anti-PTX3 was performed.
Results: Only group 1 mice developed anti-PTX3 antibodies. Anti-dsDNA and anti-C1q antibodies appeared significantly later and at lower levels in group 1 mice vs. controls ($p < 0.0001$). Proteinuria-free and overall survival were significantly increased in group 1 mice vs. controls ($p < 0.05$ and $p = 0.03$, respectively). Histopathological analysis showed that glomerular and tubular PTX3 staining and renal lesions were increased in controls compared with immunized mice.
Addition of human SLE sera positive for anti-PTX3 antibodies to C1q and fixed PTX3 interfered with C1q binding to PTX3-anti-PTX3 immune complexes. Qualitative characterization of human IgG anti-PTX3 showed an increased proportion of IgG4.
Conclusions: Anti-PTX3 antibodies delay lupus-like nephritis and prolong survival of NZB/NZW F1 mice. *In vitro* observations suggest anti-PTX3 antibodies may dampen complement activation *via* their Fc fragment, likely hindering renal inflammation.

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1. Introduction

Lupus glomerulonephritis (LGN) is one of the most threatening manifestations of systemic lupus erythematosus (SLE), leading to increased morbidity and mortality, decreased quality of life and increased costs in disease management [1–3]. Renal manifestations may range from asymptomatic urinary abnormalities to renal failure and end-stage renal disease [4], and currently it is still hard to predict which patients will develop any manifestation.

It has been shown that autoantibodies with different specificities may contribute either to progression or attenuation of renal inflammation [5–8]. Particularly, IgG anti-chromatin antibodies, i.e. anti-double-stranded(ds)DNA and anti-nucleosome antibodies, are known to promote initiation of inflammation through binding to

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exposed chromatin fragments and deposition on the glomerular basement membrane. Subsequently, anti-complement fragment 1 (C1q) antibodies are likely to amplify and perpetuate kidney inflammation through enhanced activation of the classical complement pathway [7], while autoantibodies with different specificities and/or different isotypes may dampen inflammation, including IgM anti-dsDNA [5,9].

PTX3 is secreted by immune and non-immune cells and its expression is increased under inflammatory conditions [10–13]. It is likely to modulate the inflammatory response according to a double-faced mechanism. Indeed, membrane-bound PTX3 would exert a pro-inflammatory function, favouring C1q binding to apoptotic debris and subsequent activation of the classical complement pathway [14]; by contrast, soluble PTX3 may dampen inflammation by retaining anti-C1q and preventing fixation of C1q [10]. Actually, the role of PTX3 is even more blurry, depending on the rate of glycosylation of surface moieties that interact with complement fragments [15].

The long PTX3 displays a highly conserved structure from mice to humans [16], allowing inference from murine models to man.

We have recently demonstrated that anti-PTX3 antibodies are more prevalent in SLE patients in respect to patients affected with other autoimmune rheumatic disease or healthy controls, and they are present at higher levels in patients without LGN, suggesting they may exert a protective effect against renal inflammation [17,18].

In this paper, we go over potential LGN-modifying effects of anti-PTX3 antibodies in New Zealand Black/New Zealand White (NZB/NZW F1) mice, a well-characterized model of lethal lupus-like nephritis, and explore their potential mechanisms of action.

2. Materials and methods

2.1. Mice

Thirty 8-week-old NZB/NZW F1 female mice (Harlan Laboratories, Envigo RMS, UD, Italy) were subdivided into 3 groups of 10 mice each and were subcutaneously injected with a total volume of 200 μl which consisted of 100 μg of recombinant human PTX3 [19] in 100 μl of phosphate buffered saline (PBS) and 100 μl of alum in group 1, 100 μl of alum and 100 μl of PBS in group 2 and 200 μl of PBS in group 3. Mice were injected 3 times 3 weeks apart, starting from the 11th to the 17th week of age, and bred until natural death occurred. No difference in mice basal weight among the groups was observed.

This study was approved by the National Institutional Animal Care and Use Committee.

2.1.1. Evaluation of proteinuria and serum creatinine

Disease progression was monitored by weekly urine sampling, in order to evaluate proteinuria levels, using multireactive strips (Siemens) and expressed as mg/dl. Proteinuria was evaluated according to the manufacturer: negative, slight positive, positive: + = 30 mg/dl, ++ = 100 mg/dl, +++ = 300 mg/dl and ++++ = ≥2000 albumin. Traces of proteinuria were defined as 15 mg/dl.

Renal function was monitored through serum creatinine levels that were evaluated on blood samples withdrawn every three weeks starting from week 11 in mice from all groups. The creatinine measurement has been carried out on Cobas 8000 (Roche Diagnostics) using an enzymatic method, traceable to Isotope Dilution Mass Spectrometry (IDMS) reference procedure.

2.1.2. Measurement of serum autoantibodies

Blood samples were collected from the caudal vein before every injection (at the 11th, 14th, and 17th week of age), at the 22nd, 28th, and 35th week of age, and at death. Serum levels of mouse anti-PTX3, anti-dsDNA and anti-C1q antibodies were evaluated by standardized home-made ELISA tests.

Serum levels of anti-PTX3 antibodies were determined as previously described [17,18], with the exception of the secondary antibodies that was an alkaline phosphatase-conjugated goat anti-mouse IgG.

Briefly, for anti-C1q antibodies, plates were coated with C1q at a concentration of 5 μg/ml. Sera were added in duplicate diluted 1:100 in 1% BSA/PBS with 1M NaCl, to prevent immune complex formation. Alkaline phosphatase-conjugated goat anti-mouse IgG was added at the concentration of 100 ng/ml in 1% BSA/PBS with 1M NaCl. Finally, p-nitrophenyl phosphate was added. The plates were read at 405 nm.

For anti-dsDNA antibodies, plates were coated following 3 steps: addition of poly-L-lysin at a concentration of 10 μg/ml, in order to catch the DNA; then addition of calf foetal dsDNA at a concentration of 25 μg/ml; finally, addition of poly-L-glutamate at a concentration of 5 μg/ml in order to neutralize the free negative charges of DNA. Sera were added in duplicate diluted 1:200 in 1% BSA/TBS. Alkaline phosphatase-conjugated goat anti-mouse IgG was added at the concentration of 100 ng/ml in 1% BSA/PBS. Finally, p-nitrophenyl phosphate was added. The plates were read at 405 nm.

ELISA reagents were purchased from Sigma, St Louis, USA; ELISA plates were purchased by Nalgene Nunc, New York, USA.

2.1.3. Histopathological analyses and immunoistochemistry

Kidneys were harvested from each mouse at death for histological analyses.

Histopathological evaluations on kidney was performed by optical microscopy. Each biopsy was stained with Hematoxylin and Eosin and Periodic-Acid Schiff (PAS). Renal sections were screened in a blinded fashion for presence of glomerulosclerosis, mesangial hyperplasia and lymphocytic infiltrates. Semiquantitative score from 0 (no involvement) to 5 (very high involvement) was applied.

Kidney sections further underwent immunoistochemistry using a commercially available rabbit polyclonal IgG anti-PTX3 (Alexis, Enzo Life Sciences, Lausen, Switzerland, 1 mg/ml). Immunostaining for detection of PTX3 glomerular deposits was performed on paraffin embedded kidney tissue, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibody. Microwave and citrate as retrieval method at 1/1000 dilution was done. PTX3 staining was evaluated through a semiquantitative evaluation by selecting 5 high power field (HPF) (40x); for each field we evaluated 4 glomeruli (20 glomeruli in each kidney) and counted the positivity of PTX3 staining. PTX3 immunostaining was expressed as percentage of extension area of PTX3 positivity on the total area of glomeruli for each kidney.

Complement deposition was also evaluated using a polyclonal rabbit anti-human C3d antibody (Dako, 0063, lot 017, 4.8 g/l). Microwave and citrate as retrieval method at 1/1500 dilution was done.

2.1.4. Gene expression analyses

PTX3, Collagen (Coll) IV, Transforming Growth Factor (TGF)-β, Alpha Smooth Muscle Actine (αSMA) and Interferon γ (IFNγ) mRNA expression analyses were performed in kidneys of each mouse.

Cut tissue samples from fresh mouse kidney were submerged in 5 vol of RNA-later and incubated at 2–8 °C overnight. The samples were removed from the RNA-later solution and then stored at −20 °C until used or directly processed to RNA extraction.

Ten mg of kidney tissue was disrupted and homogenized in 350 μl of lysis buffer (Buffer RLT+β-Mercaptoethanol) by Gentle Macs Dissociator (Miltenyl Biotec, CA, USA) until it was uniformly

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