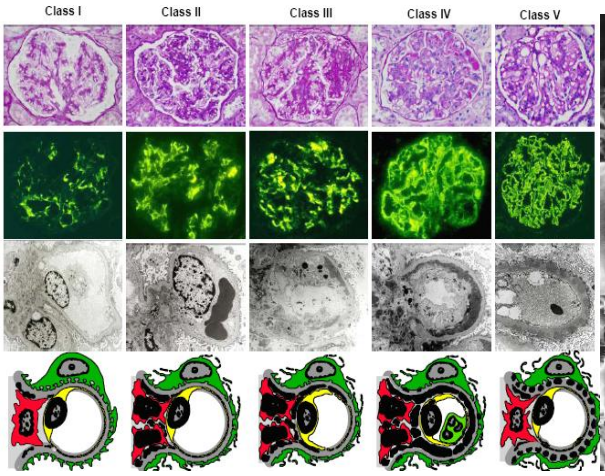
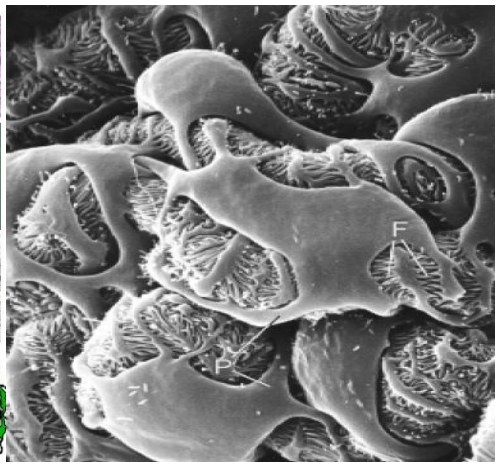
Laboratory of Nephrology
Medical Department
School of Health Sciences – University of Crete

TREATMENT EFFECT
ON THE EXPRESSION OF PODOCYTE PROTEINS
IN SYSTEMIC LUPUS ERYTHEMATOSUS NEPHRITIS





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του ιατρού

Μωυσιάδη Κ. Δημητρίου

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ΠΡΟΛΟΓΟΣ

Η Νεφρολογία ήδη από τα φοιτητικά μου χρόνια με συνάρπαζε, γιατί αποτελεί ένα κομμάτι της επιστήμης της Ιατρικής που συνδυάζει την ενασχόληση με την κλινική ιατρική, αλλά και γνώσεις μοριακής βιολογίας, γενετικής, ανοσολογίας, βιοχημείας, ακόμη και στοιχειώδεις αρχές της χημείας και των μαθηματικών. Η νεφρίτιδα του συστηματικού ερυθηματώδους λύκου αποτελεί πρότυπο σπειραματονεφρίτιδας ανοσολογικής αρχής με μεγάλη ποικιλία εκδηλώσεων, μηχανισμών παθογένειας, αλλά και θεραπευτικών προκλήσεων, πράγμα που εξάπτει πάντα το επιστημονικό και ερευνητικό ενδιαφέρον.

Η εκπόνηση ενός ερευνητικού έργου και πολύ περισσότερο μιας διδακτορικής διατριβής ήταν μια τεράστια ευκαιρία για μένα, καθώς με έφερε σε επαφή με ένα διαφορετικό κόσμο που για πολλούς δεν αποτελεί τίποτε παραπάνω από σελίδες συγγραμάτων. Πιο σπουδαίο όμως ήταν το γεγονός ότι μου δόθηκε η δυνατότητα να αντιληφθώ τη σημασία της ομαδικής εργασίας, της μεθοδικότητας, αλλά και η δύναμη να αντιμετωπίζω τα καθημερινά σχεδόν εμπόδια και να τα αξιοποιώ κριτικά και δημιουργικά. Ήταν τύχη για μένα το γεγονός ότι βρέθηκα στο Πανεπιστήμιο Κρήτης, όπου όλα τα παραπάνω μπορούν να γίνουν πραγματικότητα.

Πρωτα από όλους θα ήθελα να εκφράσω τις ευχαριστίες μου στον καθηγητή Νεφρολογίας κ. Δαφνή που μου εμπιστεύθηκε την εκπόνηση αυτής της διατριβής, αλλά και το στήσιμο του νέου ερευνητικού εργαστηρίου Νεφρολογίας μαζί με την κ. Περυσινάκη. Χωρίς τη συνδρομή, την καθοδήγηση, την αισιοδοξία και την επιμονή του σχεδόν τίποτε δε θα είχε γίνει.

Σημαντική όμως ήταν και η συνδρομή του καθηγητή Παθολογίας κ. Μπούμπα, για τον οποίο θέλω να εκφράσω ιδιαίτερη ευγνωμοσύνη. Η συμβολή του, πέρα από την επιμέλεια και τη συμπαράσταση στην εκπόνηση και ολοκλήρωση αυτής της διατριβής, ήταν πολύ σημαντική μιας και ήταν ο δάσκαλός μας στα πρώτα μας βήματα στην Ιατρική. Μας δίδαξε το επιστημονικό ήθος, την ανθρωπιά, αλλά και τη συνεχή αναζήτηση του καλύτερου.

Θερμές ευχαριστίες επίσης στην καθηγήτρια Παθολογικής Ανατομικής του Πανεπιστημίου Αθηνών κ. Νακοπούλου, τη συνεργάτιδά της κ. Γιαννοπούλου, καθώς και τους τεχνολόγους του εργαστηρίου κ. Σκεμπάρη και Μαβίδη. Χωρίς τις εικόνες που μας παρείχαν δε θα ήταν δυνατή η κατανόηση και η ολοκλήρωση του επιστημονικού μας έργου.

Επίσης σημαντική συμβολή είχε και ο Δρ Κυριάκου, Διευθυντής του τμήματος Ηλεκτρονικού Μικροσκοπίου/Μοριακής Παθολογοανατομίας του Ινστιτούτου Νευρολογίας και Γενετικής της Κύπρου. Η βοήθειά του ήταν καθοριστική για το πειραματικό μας έργο.

Θα ήθελα επίσης να ευχαριστήσω τον επίκουρο καθηγητή Ρευματολογίας κ. Σιδηρόπουλο για τη συμβολή και τις παρατηρήσεις του στην ολοκλήρωση της διατριβής αυτής.

Στο σημείο αυτό θα πρέπει να τονίσω τη σπουδαία βοήθεια της συναδέλφου κ. Περυσινάκη στην οποία χρωστώ τεράστια ευγνωμοσύνη. Δουλέψαμε μαζί ακούραστα τις ατέλειωτες ώρες στο εργαστήριο, αλλά και έξω από αυτό. Πιο σημαντική όμως θεωρώ την καθοδήγησή της και την ηθική της συμπαράσταση, απαραίτητες στην ολοκλήρωση αυτού του έργου.

Δεν θα πρέπει ωστόσο να ξεχάσω τη βοήθεια του συναδέλφου κ. Μπερτσιά. Η καθοδήγηση και η επιμέλεια στη συγγραφή του άρθρου, μαζί με τις εύστοχες παρατηρήσεις του ήταν καθοριστική για να φέρω εις πέρας τη διαδακτορική μου διατριβή. Ευχαριστώ επίσης το συνάδελφο κ. Στρατάκη για τη σπουδαία βοήθεια στο πειραματικό κομμάτι της έρευνας.

Ευχαριστώ ακόμη τον Δρ Rakesh Verma και τον καθηγητή Lawrence B. Holzman από το τμήμα Ιατρικής του Πανεπιστημίου του Michigan που μας δώρισαν το αντίσωμα κατά της νεφρίνης και μας δίδαξαν τις μεθόδους επεξεργασίας των νεφρικών σπειραμάτων.

Ακόμη ευχαριστίες αξίζει το εργαστήριο Ογκολογίας, η υπεύθυνη κ. Περάκη και οι ερευνήτριες κ. Βουτσινά και κ. Σπηλιωτάκη. Περάσαμε ώρες μαζί και η βοήθειά τους στην κατανόηση των πειραματικών τεχνικών ήταν πολύ σημαντική. Επίσης ευχαριστώ το εργαστήριο Ρευματολογίας, Κλινικής Ανοσολογίας και Αλλεργιολογίας του Πανεπιστημίου Κρήτης και κυρίως την ερευνήτρια κ. Χουλάκη που μας δίδαξε τη real time RT-PCR και μας σχεδίασε τους primers της νεφρίνης και GAPDH. Ακόμη ευχαριστώ την κ. Καστρινάκη για το σχεδιασμό των primers της ποδοσίνης.

Τέλος ιδιαιτέρως πρέπει να ευχαριστήσω τη σύζυγό μου παιδίατρο κ. Μαρία Τσούκα για την αγάπη της, την ανοχή και την υποστήριξή της, που μου έδωσε τη δύναμη να ολοκληρώσω το δύσκολο αυτό έργο. Ευχαριστώ επίσης τους γονείς μου Κώστα και Ασήμω και τον αδελφό μου Θανάση για την ηθική, πνευματική και υλική συμπαράσταση και αγάπη τους, χωρίς τα οποία δε θα είχα καταφέρει τίποτε.

CONTENTS

1. ABSTRACT

Renal epithelial cells (podocytes) and their slit diaphragms (SD) that cover the slit pores among adjacent foot processes (FP) of the podocytes are crucial constituent of the primary barrier for ultrafiltration of plasma in renal glomeruli. They ensure the integrity of the glomerular basement membrane (GBM) and prevent the urinary loss of proteins. A putative distraction of the SDs, of its main components, nephrin and podocin is involved in the immune mediated glomerulonephritis.

We have previously reported that decreases of the podocyte SD proteins nephrin and podocin represent early events in the podocytopathy of lupus nephritis (LN). The latter is a prototype of autoimmune disease with a various histological and clinical picture with proteinuria been a major clinical manifestation. We asked whether immunosuppressive agents such as glucocorticoids (GC) and cyclophosphamide (CY), may have direct effects on SD and subsequently on podocytes.

We used NZB/W F1 female mice in our experiments. NZB/W are thought to be a representative model of spontaneous development of LN. Animals were divided in groups of 6 and 9 months old, as well as in groups of: a) untreated (control) mice (n = 24), b) GC–treated mice, which received dexamethasone p.o. for 3 (n = 4) or 6 (n = 5) consecutive months, and c) CY–treated mice, which received CY i.p. for 3 (n = 4) or 6 (n = 4) consecutive months. C57Bl/6 mice (n = *20*) were used as normal controls. Comparisons were made with the parental strains of NZB (n = 5) and NZW (n = 4) as well.

Assessment of 24h proteinuria was made by Bradford assay. After mice were euthanized tissue from the left kidney was used for histologic studies: light microscopy, IgG immunofluorescence and electron microscopy. Right kidney was used for biochemical studies: glomerular expression of nephrin and podocin in the protein level was studied with western blot. Nephrin expression in kidney tissue was also examined with immunofuorescence. To determine whether changes in nephrin and podocin expression in NZB/W treated mice correlate with altered transcription in corresponding genes, we performed quantitative real-time PCR. Finally, anti-dsDNA antibodies in mice serum was examined with ELISA.

Data are expressed as the mean ± standard error of the mean (SEM) values. Comparisons were performed with the Mann-Whitney U test for numerical data. The Spearman's rho (ρ) test was used for correlation analysis.

Treatment of NZB/W mice with GC or CY prevented the development of proteinuria, whereas untreated littermates developed overt proteinuria by the age of 6 months.

Three-month-old NZB/W mice demonstrated normal histology or findings of mild mesangial LN (MMLN) in light microscopy. In contrast, all untreated 6- and 9-month-old mice developed focal or diffuse proliferative glomerulonephritis (FPLN and DPLN, respectively) associated with numerous IgG deposits. In GC– and CY–treated mice, glomerulonephritis was milder with markedly reduced interstitial inflammation and mesangial hyperplasia; seven out of eight

(7/8) NZB/W mice treated for 6 months had normal LM histology. Accordingly, IgG kidney deposition was significantly reduced in treated mice being undetectable in mice treated with CY for 6 months.

Electron microscopy examination of kidney sections from untreated 6- and 9-month-old mice with FPLN or DPLN revealed extensive mesangial, subendothelial, and subepithelial EDD fusion of podocytes and effacement of FPs, and destruction of slit diaphragms. Conversely, in mice treated with GC or CY, there were only few sub-endothelial EDD. Both SD and podocytes FPs and SD were well preserved.

Next the glomerular expression of the main SD proteins was examined with western blot. Nephrin was increased in treated mice compared to untreated littermates both in GC and CY groups ($p<0,05$). Glomerular nephrin expression showed negative association with the histological class of nephritis (ρ=-0.69, $p<0.001$), and positive association with podocin protein levels (ρ=0.53, p=0.007). Treatment of NZB/W mice for 3–6 months resulted also in higher glomerular podocin protein levels compared to untreated aged-matched mice with FPLN or DPLN. Similar to nephrin, podocin levels correlated inversely with the histological class of nephritis (ρ=-0.62, p=0.001). Nephrin expression and localization was further assayed by IF in kidney sections from NZB/W mice. In accordance with the WB results, nephrin IF staining was stronger in treated mice which was reduced in older untreated mice with FPLN or DPLN, and became diminished in 9-month-old diseased mice. Nephrin IF score correlated with the nephritis histological class (ρ=0.55, p=0.008) and nephrin expression assayed by WB (ρ=0.71, p=0.004).

To determine whether changes in nephrin and podocin expression in NZB/W treated mice correlate with altered transcription in corresponding genes, we performed quantitative real-time PCR in total RNA. Nephrin mRNA was significantly increased after 3–6 of GC and CY therapy compared to age-matched untreated littermates ($p<0,05$ in the GC group and $p<0,01$ in the CY group). Podocin mRNA levels was increased in treated mice but differences were statistically significant only after 6 months of treatment. We observed a significant correlation between nephrin and podocin mRNA both in untreated (ρ=0.67, p=0.005, n = 16) and in GC / CY-treated (ρ=0.72, p=0.002, n = 16) mice.

Next, we addressed whether electron microscopy findings in kidney biopsies correlated with differential expression of SD proteins. Indeed, electron dense deposits showed significant inverse correlation with glomerular nephrin (ρ correlation coefficient ranging from -0.75 to -0.85) and podocin (ρ ranging from -0.88 to -0.93) protein expression. Similarly, podocyte FP effacement showed negative association with both nephrin (ρ = -0.77, p = 0.009) and podocin (ρ= -0.93, $p < 0.001$). With respect to mRNA levels, deposits of any type and podocyte FP effacement showed also inverse association with nephrin – but not podocin – expression.

We also examined the SD protein expression and their mRNA levels in treated mice compared to normal controls and their parental strains.

Our results present the effect of early treatment of LN on podocytes SD proteins. Treatment with GC or CY prevented the development of proteinuria, and halted the histologic alterations

in light and electron microscopy, where deposits of any type were diminished and podocytes structure was preserved. Glomerular nephrin and podocin expression significantly increased in both treated groups up to normal levels. Nephrin immunofluorescence confirmed these results. The findings above and the increased mRNA levels may imply that the SD alterations at the protein level precede the alterations in the mRNA level.

The previous results are confirmed by the correlation of SD proteins with the histologic findings.

Podocytopathy may be due to immunological mechanisms that involve elements of both the innate and adaptive immunity (CD8+ T cells, anti-dsDNA antibodies, co-stimulatory molecules such as B7-1 and Toll-like receptors).

Immunosuppressive agents, in addition to the effect on the immune system, may directly influence the unique structure and function of podocytes. Our findings further emphasize the role of podocytes and the slit diaphragm in the pathogenesis of immune-mediated glomerulonephritis, and point toward the potential benefits of development of selective, antiproteinuric, and podocyte-protective drugs.

2. ΠΕΡΙΛΗΨΗ

Τα επιθηλιακά κύτταρα των νεφρικών σπειραμάτων (ποδοκύτταρα) και οι λεπτές μεμβράνες των σχισμών διήθησης (ΛΜ) που γεφυρώνουν τους πόρους μεταξύ των γειτονικών ποδοειδών προσεκβολών (ΠΠ) των ποδοκυττάρων αποτελούν σημαντικά στοιχεία του φραγμού της υπερδιήθησης του πλάσματος στα νεφρικά σπειράματα. Διασφαλίζουν έτσι την ακεραιότητα της βασικής μεμβράνης και εμποδίζουν την απώλεια πρωτεϊνών στα ούρα. Βλάβη των ΛΜ και κυρίως των σημαντικότερων συστατικών τους, νεφρίνη και ποδοσίνη, εμπλέκεται σε σπειραματονεφρίτιδες ανοσολογικής αρχής. Έχουμε πρόσφατα αναφέρει ότι ελάττωση αυτών των πρωτεϊνών των ΛΜ αποτελεί πρώιμο γεγονός στην «ποδοκυττοπάθεια» της νεφρίτιδας του λύκου (ΝΛ). Η τελευταία αποτελεί πρότυπο αυτοάνοσου νοσήματος με ποικίλη ιστολογική και κλινική εικόνα με την πρωτεϊνουρία να αποτελεί την κυριότερη κλινική εκδήλωση. Διενεργήσαμε μία μελέτη με πειραματόζωα προκειμένου να εξετάσουμε κατά πόσο η ανοσοκατασταλτική θεραπεία με κορτικοειδή και κυκλοφωσφαμίδη, μπορεί να έχει άμεση επίδραση στις πρωτεϊνες των ΛΜ και κατ'επέκταση στα ποδοκύτταρα.

Ως πειραματόζωα χρησιμοποιήθηκαν θηλυκά ποντίκια NZB/W F1. Τα NZB/W αναφέρθηκε προηγούμενα ότι θεωρούνται το πιο αντιπροσωπευτικό μοντέλο αυθόρμητης εκδήλωσης της ΝΛ. Τα πειραματόζωα χωρίστηκαν σε ομάδες ηλικίας έξι και εννέα μηνών και επίσης σε ομάδες ανάλογα με το αν έλαβαν ή όχι κορτικοειδή p.o ή ώσεις κυκλοφωσφαμίδη i.p. για 3 ή 6 μήνες αντίστοιχα. Επίσης χρησιμοποιήθηκαν ποντίκια C57Bl/6 ως φυσιλογικοί μάρτυρες. Τέλος συγκρίσεις έγιναν και με τους προγόνους των NZB/W , δηλ. τα ποντίκια NZB και NZW.

Η πρωτεϊνουρία 24ώρου προσδιορίσθηκε με τη μέθοδο Bradford. Μετά την ευθανασία των πειραματόζωων απομονώθηκε ο αριστερός νεφρός για ιστολογική μελέτη: φωτονικό μικροσκόπιο, ανοσοφθορισμός IgG και ηλεκτρονικό μικροσκόπιο. Αντίθετα ο δεξιός νεφρός απομονώθηκε για μοριακή και βιοχημική μελέτη: η σπειραματική έκφραση της νεφρίνης και ποδοσίνης σε πρωτεϊνικό επίπεδο μελετήθηκε με western blot, ενώ η έκφραση της νεφρίνης εξετάστηκε επίσης με ανοσοφθορισμό στα παρασκευάσματα νεφρικού ιστού. Για να προσδιοριστεί αν οι μεταβολές της νεφρίνης και ποδοσίνης οφείλονται σε μεταβολή της μεταγραφής των αντίστοιχων γονιδίων προσδιορίσαμε το mRNA τους με ποσοτική αντίστροφη real time PCR στα ίδια δείγματα. Τέλος με ELISA μετρήθηκαν και τα επίπεδα anti-DNA του ορού των ποντικών.

Τα αποτελέσματα εκφράζονται ως μέση τιμή ± τυπικό σφάλμα της μέσης τιμής (SEM). Για τις συγκρίσεις χρησιμοποιήθηκαν οι έλεγχοι Mann-Whitney U-test (αριθμητικές μεταβλητές) και το Fischer's exact test (κατηγορικές μεταβλητές).Το Spearman's rho (ρ) test χρησιμοποιήθηκε για την ανάλυση συσχετίσεων.

Η θεραπεία με κορτικοειδή ή κυκλοφωσφαμίδη εμπόδισε την ανάπτυξη πρωτεϊνουρίας στα NZB/W ποντίκια, ενώ τα αντίστοιχα μη θεραπευμένα εμφάνισαν από την ηλικία των 6 μηνών νεφρωσικού επιπέδου πρωτεϊνουρία.

Στο επίπεδο του φωτονικού μικροσκοπίου τα ζώα μάρτυρες 3 μηνών είχαν φυσιολογική ιστολογική εικόνα ή ήπια μεσαγγειακή υπερπλασία (ΗΜΝΛ), ενώ τα 6-9 μηνών εμφάνισαν όλα εστιακή ή διάχυτη υπερπλαστική ΝΛ (ΕΥΝΛ και ΔΥΝΛ αντίστοιχα) με άφθονες IgG εναποθέσεις. Τα θεραπευμένα είχαν ηπιότερη εικόνα από τους 3 μήνες θεραπείας (ηλικίας 6 μηνών), ενώ στους 6 μήνες θεραπείας (9μηνών) 7/8 είχαν φυσιολογική ιστολογική εικόνα. Οι εναποθέσεις IgG μειώθηκαν από τους 3 μήνες θεραπείας μέχρις μη ανίχνευσή τους στους 6 μήνες θεραπείας.

Η εξέταση με ηλεκτρονικό μικροσκόπιο των ποντικών-μαρτύρων με ΕΥΝΛ και ΔΥΝΛ κατέδειξε εκτεταμένες μεσαγγειακές, υπενδοθηλιακές και υποεπιθηλιακές εναποθέσεις με συνοδό ποδοκυττοπάθεια (επιπέδωση των ποδοκυττάρων και εξάλειψη των ποδικών εκβλαστήσεων). Αντίθετα τα πειραματόζωα που έλαβαν θεραπεία είχαν ελάχιστες υπενδοθηλιακές εναποθέσεις, ενώ τα ποδοκύτταρα και οι ΛΜ διατηρούνταν φυσιολογικές.

Η μελέτη της σπειραματικής έκφρασης των κύριων πρωτεϊνών των ΛΜ με western blot έδειξε αύξηση των επιπέδων νεφρίνης στα ζώα με θεραπεία σε σχέση με τους μάρτυρες χωρίς θεραπεία ($p<0,05$) και για την ομάδα με κορτικοειδή και για αυτή με κυκλοφωσφαμίδη. Επίσης η έκφραση της νεφρίνης είχε αρνητική συσχέτιση με την ιστολογική τάξη της ΝΛ (ρ= -0,69, $p<0,001$) και θετική συσχέτιση με τα επίπεδα της ποδοσίνης (ρ= 0,53, p=0,007). Η θεραπεία με κορτικοειδή ή κυκλοφωσφαμίδη για 3-6 μήνες εμφάνισε επίσης υψηλότερα επίπεδα σπειραματικής έκφρασης ποδοσίνης σε σχέση με ζώα ίδιας ηλικίας με ΕΥΝΛ και ΔΥΝΛ που δεν έλαβαν θεραπεία. Αντίστοιχα με τη νεφρίνη τα επίπεδα της ποδοσίνης συσχετίστηκαν αντίστροφα με την ιστολογική κατάταξη της ΝΛ (ρ= -0,62, $p<0,001$).

Η νεφρίνη περαιτέρω εκτιμήθηκε με ανοσοφθορισμό νεφρικών ιστών των NZB/W ποντικών. Σύμφωνα και με τα αποτελέσματα των western blot η ένταση της χρώσης της νεφρίνης αυξήθηκε στα ζώα με θεραπεία, ενώ στα μη θεραπευμένα ζώα με ΕΥΝΛ και ΔΥΝΛ 6 μηνών ήταν μειωμένη και εντελώς απούσα στα ζώα ηλικίας 9 μηνών. Επιπλέον η ένταση της χρώσης της νεφρίνης συσχετίστηκε με την ιστολογική τάξη (ρ=0,55, p=0,008) και με την western blot (ρ= 0,71, p=0,004).

Για να καταδειχθεί αν η έκφραση των πρωτεϊνών σχετίζεται με μεταβολή της μεταγραφής των αντίστοιχων γονιδίων προσδιορίσθηκε το mRNA νεφρίνης των θεραπευμένων ποντικών για 3-6 μήνες και ήταν σημαντικά αυξημένο σε σχέση με τους μάρτυρες ($p<0,05$ για την ομάδα των κορτικοειδών και $p<0,01$ για την ομάδα της κυκλοφωσφαμίδης). Το mRNA της ποδοσίνης ήταν αυξημένο ήδη από τους 3 μήνες θεραπείας αλλά στατιστικά σημαντικά μόνο μετά από 6 μήνες θεραπείας. Παρατηρήθηκε επίσης συσχέτιση μεταξύ του mRNA της νεφρίνης και της ποδοσίνης και στους μάρτυρες (ρ=0.67, p=0.005, n = 16) αλλά και στα θεραπευμένα ζώα (ρ=0.72, p=0.002, n = 16).

Έπειτα προσδιορίσθηκαν πιθανές συσχετίσεις των ευρημάτων του ηλεκτρονικού με την έκφραση των πρωτεϊνών. Πράγματι, βρέθηκε αρνητική συσχέτιση των εναποθέσεων με τη νεφρίνη (ρ από -0.75 ως -0.85, $p<0,013$) και την ποδοσίνη (ρ από -0.88 ως -0.93, $p<0,013$). Ομοίως η εξάλειψη των ποδικών εκβλαστήσεων συσχετίστηκε αρνητικά και με τη νεφρίνη (ρ = -0.77, p = 0.009) και με την ποδοσίνη (ρ= -0.93, $p<0,001$). Σχετικά με τα επίπεδα mRNA οι

εναποθέσεις κάθε τύπου και η εξάλειψη των ποδικών εκβλαστήσεων είχαν αρνητική συσχέτιση με τη νεφρίνη, αλλά όχι με την ποδοσίνη.

Επίσης εξετάστηκε η έκφραση των πρωτεϊνών των ΛΜ καθώς και τα επίπεδα mRNA στα θεραπευμένα ζώα σε σχέση με φυσιολογικούς μάρτυρες, αλλά και με τα προγονικά στελέχη τους.

Τα αποτελέσματά μας καταδεικνύουν την επίδραση της πρώιμης θεραπείας της ΝΛ στις κύριες πρωτεΐνες των ΛΜ των ποδοκυττάρων. Η θεραπεία με κορτικοειδή ή κυκλοφωσφαμίδη εμπόδισε την ανάπτυξη πρωτεϊνουρίας, αλλά και τις ιστολογικές αλλοιώσεις τόσο με το φωτονικό, όσο και με το ηλεκτρονικό μικροσκόπιο όπου μειώθηκαν οι κάθε τύπου ανοσοεναποθέσεις και διατηρήθηκε η δομή των ποδοκυττάρων. Η σπειραματική έκφραση της νεφρίνης και της ποδοσίνης αυξήθηκαν σημαντικά στα ζώα με θεραπεία ως το επίπεδο των φυσιολογικών μαρτύρων, ενώ το ίδιο επιβεβαίωσε για τη νεφρίνη η μελέτη με ανοσοφθορισμό. Τα παραπάνω ευρήματα σε συνδυασμό με την αύξηση των επιπέδων mRNA των πρωτεϊνών κυρίως στη μεγαλύτερη διάρκεια θεραπείας πιθανά υποδηλώνουν ότι οι αλλαγές στις ΛΜ συμβαίνουν πρώτα σε επίπεδο πρωτεΐνης και ακολούθως σε επίπεδο μεταγραφής της. Τα παραπάνω επιβεβαιώνονται και από την συσχέτιση των πρωτεϊνών με τα ιστολογικά ευρήματα. Η ποδοκυττοπάθεια πιθανά οφείλεται σε ανοσολογικούς μηχανισμούς όπου εμπλέκονται στοιχεία της φυσικής, αλλά και της επίκτητης ανοσίας (CD8+ Τ-κύτταρα, αντι-DNA αντισώματα, συνδιεγερτικά μόρια όπως το B7-1 και οι υποδοχείς TLR). Η ανοσοκατασταλτική θεραπεία που χρησιμοποιήθηκε πέρα από την επίδραση στο ανοσολογικό σύστημα πιθανά επιδρά άμεσα στη δομή και λειτουργία των ποδοκυττάρων. Τα αποτελέσματά μας τονίζουν περαιτέρω το ρόλο των ποδοκυττάρων και των πρωτεϊνών των ΛΜ σε ανοσολογικής αρχής σπειραματονεφρίτιδες και αναδεικνύουν τα πιθανά οφέλη της ανάπτυξης εκλεκτικών, προστατευτικών για τα ποδοκύτταρα θεραπειών.

3. ABBREVIATIONS

ANCA: Antineutrophil cytoplasmic antigen autoantibodies
AP-1: activator protein 1
APC: antigen presenting cells
AT: angiotensin II receptors type
BAFF: B-cell–activating factor
CASK: calcium/calmodulin-dependent serine protein kinase
CD2AP: CD2 associated protein
Crry: CR1-related gene/protein y
CY: cyclophosphamide
DPLN: diffuse proliferative lupus nephritis
EDD: electron dense deposits
EM: electron microscopy
EMT: epithelial-mesenchymal transition
FP: foot processes
FPLN: focal proliferative lupus nephritis
FSGS: focal and segmental glomerulosclerosis
GBM: glomerular basement membrane
GC: glucocorticosteroids
GM-CSF: granulocyte-macrophage-colony stimulating factor
GN: glomerulonephritis
IC: immunocomplexes
IF: immunofluorescence
IL: interleukin
IQGAP1: IQ motif-containing GTPase activating protein 1
LM: light microscopy
LN: lupus nephritis
MAC: membrane attack complex
MAGI2: membrane-associated guanylate cyclase inverted 2
MCD: minimal change disease
MMF: mycophenolate mofetil
MMLN: mild mesangial lupus nephritis
MN: membranous nephropathy
NF-κB: nuclear factor κB
ROS: reactive oxygen species
SD: slit diaphragm
SLE: systemic lupus erythematosus
TLR: Toll-like receptors
TNF: tumor necrosis factor
WB: western blot

4. INTRODUCTION

4.1 Overview of kidney anatomy and function.

The kidneys are two organs lying in the retroperitoneal space, each weighing about 150g. They consist of highly specialized cells. The outer layer of the kidney is called cortex. The inner section is called medulla. The specific components of the kidney are the nephrons, the collecting ducts, and a unique microvasculature. The multipapillary kidney of humans contains roughly one million nephrons; however, the number is quite variable. A nephron consists of a renal corpuscle (glomerulus) connected to a complicated and twisted tubule that finally drains into a collecting duct. By the location of renal corpuscles within the cortex, three types of nephron can be distinguished: superficial, midcortical, and juxtamedullary nephrons. The free wall of glomerular capillaries across which filtration takes place, consists of a basement membrane covered by visceral epithelial cells (podocytes) with individual foot processes (FP) and lined by endothelial cells. The blood is filtrated through the glomerular capillary wall which is consisted of three layers: the fenestrated endothelial cell, the glomerular basement membrane (GBM) and the slit diaphragm (SD) membranes that bridge the slit pores between the foot processes of adjacent podocytes. Each glomerulus is enclosed within an epithelial cell capsule (Bowman's capsule) that is continuous with the proximal convoluted tubule.The tubular part of the nephron consists of a proximal tubule and a distal tubule connected by Henle's loop. There are two types of nephron, those with long Henle's loops and those with short loops. Long loops turn back at successive levels of the inner medulla. A collecting duct is formed in the renal cortex where several nephrons join. A connecting tubule is interposed between a nephron and a cortical collecting duct. Cortical collecting ducts descend within the medullary rays of the cortex. They traverse the outer medulla as unbranched tubes. On entering the inner medulla, they fuse successively and open finally as papillary ducts into the renal pelvis. The microvascular pattern of the kidney is also similarly organized in mammalian species.

The renal artery, after entering the renal sinus, finally divides into the interlobar arteries. At the junction between cortex and medulla, they divide and pass over into the arcuate arteries, which also branch. They give rise to the cortical radial arteries (interlobular arteries) that ascend radially through the cortex. No arteries penetrate the medulla. Afferent arterioles generally arise from cortical radial arteries; they supply the glomerular tufts. Glomeruli are drained by efferent arterioles. The intrarenal veins accompany the arteries. Central to the renal drainage of the kidney are the arcuate veins. They accept the veins from the cortex and the renal medulla. The arcuate veins join to form interlobar veins, which run alongside the corresponding arteries.

The main function of the kidneys is the maintenance of body composition and homeostasis (e.g. volume of body fluid, its osmolarity, electrolyte content, restriction the passage of large molecules such as albumin). In addition kidneys clear metabolic products and substances that can be toxic for the organism (e.g. urea, toxins, drugs). Finally the renal production of enzymes and hormones such as renin, angiotensin, erythropoietin or 1,25-dihydroxyvitamin-D_3 are essential for the control of blood pressure and body fluids, the erythrocyte production and the homeostasis of calcium and phosphorus.

The kidney diseases are divided according to the structures affected. For example when glomeruli are affected the disease is called glomerulonephritis, while it is called tubulointerstitial nephritis when the tubules are the affected structure. They are also categorized according to their etiology (e.g. minimal change disease is a primary glomerulonephritis, but lupus nephritis is a secondary one caused by systemic lupus erythematosus).

We will focus on kidney disease affecting the glomerulus, lupus nephritis (LN) with special interest for the podocyte slit diaphragm [1, 2].

4.2 Glomerulonephritis (GN)

4.2.1. Pathogenesis of GN

Glomerular disease may have a wide variety of causes and clinical presentations. Some glomerular diseases are given the generic title of glomerulonephritis (GN), which implies an immune or inflammatory pathogenesis. Although there are some situations in which specific diagnosis can be made on the basis of clinical presentation and laboratory tests, a renal biopsy is useful for both classification and prognosis in most cases. According to the cells of the glomerulus involved the GNs are categorized as mesangial-cell diseases (e.g. IgA nephropathy, diabetic nephropathy mesangioproliferative GN, class II LN) diseases with endothelial-cell injury (vasculitis, mesangiocapillary GN, anti-GBM GN, class III and IV LN) and diseases with epithelial-cell injury [membranous nephropathy, minimal change disease (MCD), class V lupus nephritis, focal and segmental glomerulosclerosis (FSGS), diabetic nephropathy [3-5]. Injury to the kidney may be caused by the deposition of immune complexes within the glomeruli or by autoantibodies directed against antigens present within the kidney (**Figure 4.1**). Additionally, in pauci-immune GNs lymphocyte and macrophage infiltrations are observed. Thus, both innate and adaptive immunity mechanisms are involved.

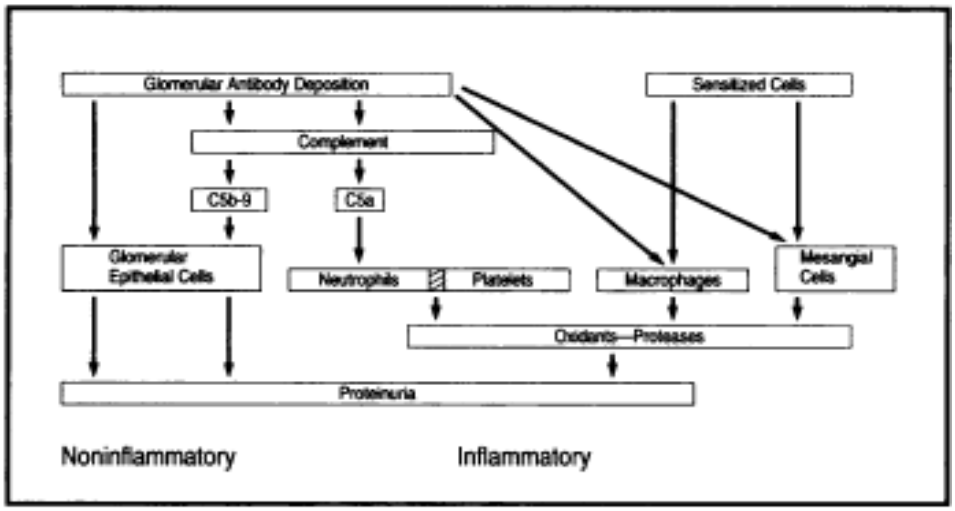


Figure 4.1 Mechanisms of immune glomerular injury.

The emergence of autoimmunity in GNs may be due to a breakdown in central and peripheral tolerance, as well as in genetic and environmental factors. Before clinical presentation of an autoimmune disease, several breaks in checkpoints in both the innate and adaptive immune system have occurred. Autoreactive T- and B-cell clones must become activated and have the ability to sustain a self-directed immune response. The autoantibodies have to be present in sufficiently high quantities to bind antigen with necessary avidity and elicit appropriate co-stimulatory molecule expression to product inflammatory cytokines. In addition, components of the innate immune system must also be dysregulated, so that antigen-presenting cells (APC) present autoantigen inappropriately and complement proteins escape multiple well-regulated control mechanisms. Thus, there must be a failure of multiple overlapping central and peripheral regulatory mechanisms to begin an abnormal self-directed immune response, before there is emergence of autoimmune kidney disease [6,7,8**]** In the following paragraphs we shall describe the main immunological pathways of GNs, with emphasis to the podocyte and slit diaphragm biology.

4.2.1.1. Immune complexes

Most human glomerular diseases are characterized by glomerular immunoglobulin deposition along with complement components. Examples include post-infectious GN, IgA nephropathy, anti GBM-antibody disease, LN, membranous nephropathy, type 1 membranoproliferative GN, and some forms of rapidly progressive GN.

Immune deposits form in the glomerulus either actively, because the target antigen(s) is localized predominantly in this structure, or passively because of the role of the glomerulus in filtration. Antibodies which induce glomerular immune deposits may be directed against the following antigens: a. Normal constituents of the glomerulus, such as the Goodpasture antigen on the non-collagenous domain of the alpha-III chain of type IV collagen [9]. b. Non-renal self antigens localized in glomeruli, such

as DNA-nucleosome complexes in systemic lupus erythematosus (SLE) or abnormally glycosylated IgA in IgA nephropathy [10]. Antigens may be localized alone followed by antibody binding to form immune complexes (IC) (in situ IC formation), or they may be passively trapped as components of IC formed in the circulation (**Figure 4.2**). Exogenous antigens or immune aggregates that localize in glomerular capillaries via charge affinity for glomerular structures, passive trapping, or local precipitation of macromolecular aggregates (eg, HCV antigen-containing cryoglobulins in hepatitis C virus-associated MPGN, and abnormally glycosylated IgA that self-aggregates or binds to fibronection in IgA nephropathy). Megalin and neutral endopeptidase both podocyte sole membrane proteins have been recognized as autoantigenic target in Heymann nephritis and neonatal MN respectively [11-13] of a circulating antibody [14, 15]. Only recently a conformation-dependent epitope in M-type phospholipase 2 receptor (PLA2R) has been recognized as the target of IgG4 in idiopathic human MN [16,17].

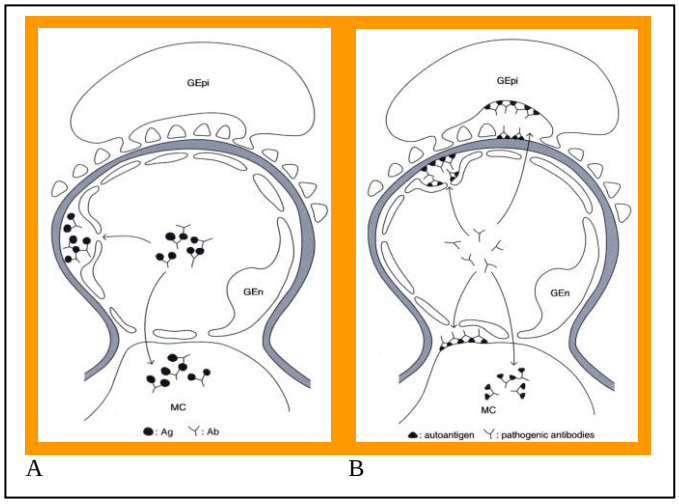


Figure 4.2 Immune complex deposition within the kidney. Panel A depicts circulating IC, while in panel B in situ formation

4.2.1.2. Complement

Complement is a part of the body's innate immune system. The kidney appears to be particularly vulnerable to complement-mediated inflammatory injury and many kidney pathologies have been linked to abnormal complement activation. Complement causes injury through the generation of anaphylatoxins (C3a, C5a), opsonins (C3b)

which facilitates transport of target cells and IC, and the Membraner Attack Complex (MAC) which consists of various complement proteins (C5b-9) and activates the resident glomerular cells (**Figure 4.3)**. To prevent host injury several inhibitory proteins have been developed to control complement activation. Membrane-bound complement regulators include decay-accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46), complement receptor 1 (CR1/CD35) and its rodent analogue CR1-related gene/protein y (Crry), and CD59. DAF is likewise ubiquitously expressed in the human kidney, but seems to be particularly abundant in the juxtaglomerular apparatus [18] while in mice DAF is mostly found on podocytes and endothelial cells.18 MCP is expressed throughout human renal tissues, but rodents do not normally express MCP besides spermatozoa [19, 20]. In humans, CR1 is mostly restricted to erythrocytes and podocytes but like MCP, rodents only have limited expression of CR1 that is generated by alternative splicing from the Cr1/2 gene.21 In place of MCP, the rodent-specific complement regulator Crry is expressed ubiquitously in mice [19, 20] and is considered a functional homolog of human MCP [21, 22].

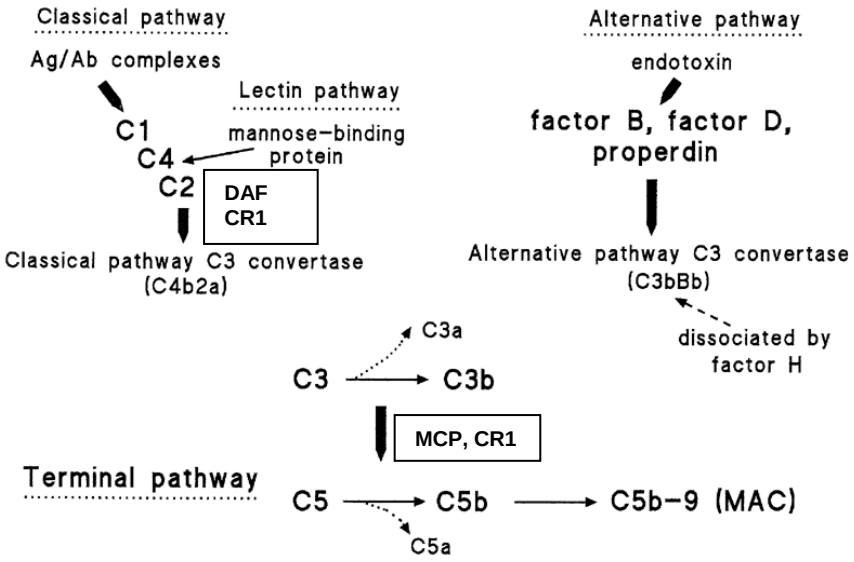


Figure 4.3 Schematic view of complement activation during GN.

4.2.1.3 Leucocytes

Macrophages, lymphocytes and neutrophils are observed in most forms of GN and play a crucial role in promoting inflammation and consequently functional and structural damage.

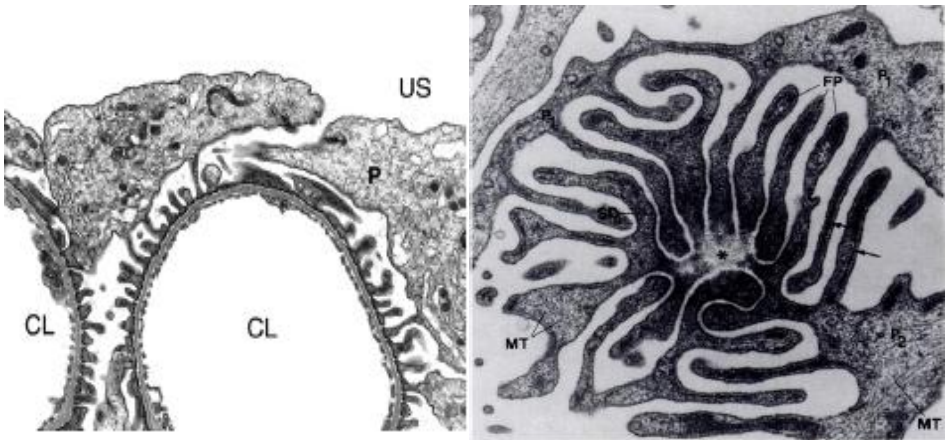
Macrophages have been considered a major cellular mediator in proliferative and crescentic forms of GN. The importance of macrophages in mediating glomerular injury is well-documented by studies, such as macrophage depletion and inhibition of MIF [23, 24]. Macrophages localize to glomeruli via interactions with both deposited immunoglobulins and several chemokines, such as macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1-alpha (MIP-1alpha) and RANTES [23-25]. They are also recruited by lymphocyte-derived molecules, such as macrophage inhibitory factor (MIF) and with leukocyte adhesion molecules, such as ICAM-1, VCAM-1, and osteopontin [26, 27]. They release tumor necrosis factor α (TNFα), chemotactic molecules (e.g. macrophage chemotactic molecule 1), reactive oxygen species (ROS) and nitrogen oxide (NO) resulting in glomerular inflammation promotion, other leukocyte recruitment and cell death respectively.

T cells are also detected particularly in diseases primarily mediated by macrophages such as crescentic GN and have been studied in Heymann nephritis models and from a model of anti-GBM [28, 29] T-cell mediated injury occurs primarily via the release of chemokines and recruitment of macrophages, which subsequently function as effector cells. In addition, T cells may be the source of permeability factors that contribute to noninflammatory glomerular injury, such as FSGS and MCD [30].

ANCA associated pauci-immune necrotizing GN and anti-GBM disease animal models have shown the importance of neutrophils in GN. In addition to that neutrophils are present in post infectious, LN, membranoproliferative (MPGN), and IgA GN, localized at sites with necrosis. A primary role in the development of MPO positive glomerulonephritis has been illustrated [31, 32]. Myeloperoxidase and proteinase 3, both cationic protease in granules of neutrophils contribute in the ANCA

associated glomerulonephritis pathogenesis [33]. There is increasing evidence that neutrophils release ROS which contribute to the development of proteinuria, alter glomerular filtration rate, and induce morphological changes in glomerular cells. In addition, neutrophil-derived phospholipase products such as leukotrienes and platelet-activating factor contribute to vascular changes in acute inflammation and amplify tissue damage. Increasing evidence suggests that neutrophils release chemoattractants (eg, interleukin 8), which further promote neutrophil migration to the kidney, activate neutrophils, and increase glomerular injury. Also, the expression of adhesion molecules (eg, ICAM-1 on kidney-specific cells and beta-2-integrins on leukocytes) has been correlated with the degree of injury in various forms of glomerulonephritis [34-36].

4.3 Podocytes



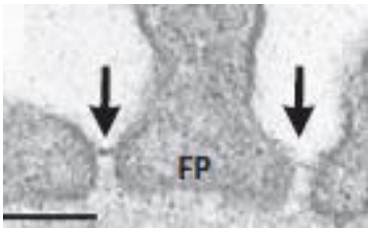


Figure 4.4 View of a rat glomerular capillary loop. Cell bodies of podocytes (P) extend into the urinary space, while their foot processes attach in a regular, interdigitating pattern. FP from different primary FPs (P1, P2) interdigitate with each other. Primary FPs contain abundant microtubules. Adjacent FPs are connected with th SD (arrows). US,urinary space; CL, capillary lumen. ×12,000.

The podocytes are terminally differentiated visceral epithelial cells with a complex cellular morphology that line the outer aspect of the GBM. They are important for the glomerular barrier, protein filtration and immune response of the glomerulus (**Figure**

4.4). They consist of a cell body, major processes and FPs that form a characteristic interdigitating pattern with FPs of neighboring podocytes, leaving in between 30 - 50nm wide filtration slits that are bridged by the glomerular SD (**Figure 4.5**). FPs are functionally defined by three membrane domains: the apical membrane domain, the SD and the basal membrane domain, also known as the sole plate, which is associated with GBM via several adhesion proteins such as α3β1 integrin and α-and β-dystroglycans [37]. Integrin associates on its cytoplasmic side with paxillin, talin, and vinculin [38], which mediate its connection to the actin cytoskeleton. In the SD area, attachment of the nephrin/podocin complex is mediated by CD2AP. P-cadherin may use ZO-1 for this purpose. Finally, in the apical membrane domain NHERV-2 and ezrin indirectly link podocalyxin to actin [39]. (**Figure 4.6)**

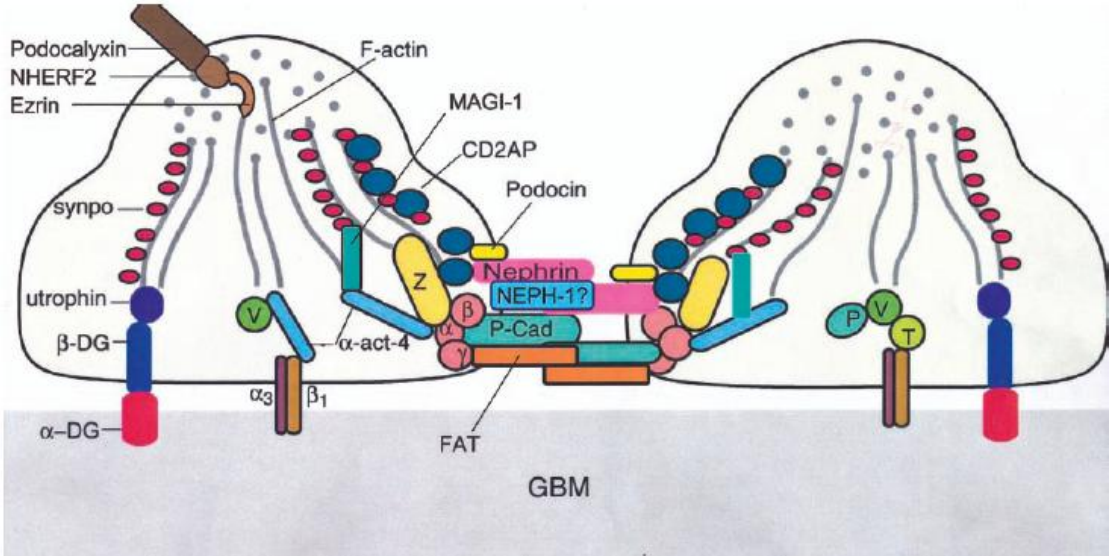


Figure 4.5 Molecular anatomy of the podocyte foot process (FP) actin cytoskeleton. This schematic shows two adjacent podocyte FP with the interposed slit diaphragm (SD) complex. The actin cytoskeleton is the common downstream pathway and receives input from the three podocyte domains. α-act4, α-actinin-4; α3β1, α3β1-integrin; α-DG, α-dystroglycan; β-DG, β-dystroglycan; NHERF2, Na/H exchanger regulatory factor 2; P, paxillin; P-cad, P-cadherin; Synpo, synaptopodin; T, talin; V, vinculin.

Podoplanin is responsible for the negative charge of the apical domain of the podocyte. Ephrin B1, a membrane bound protein that function as ligand-receptor pair with ephrs and regulate the paracellular permeability in epithelial cells has also been

found in rat podocyte. Ephrin B1 plays an important role in maintenance of the podocyte function. In experimental proteinuric disease it contributes to the redistribution of the CD2AP. Podocytes express receptors for all-trans-retinoic acid (ATRA). ATRA improved proteinuria in experimental anti-GBM disease, by preventing the decrease of nephrin and podocin [40]. Rho guanine dissociation inhibitor (GDI)-α, Nck1 and Nck2 adaptor proteins, FAT1, and synaptopodin regulate the plasticity of the actin cytoskeleton which is important for function of the filtration barrier. Moreover local podocyte production of vascular endothelial growth factor (VEGF) is required for glomerular endothelial cells integrity and protects from FP effacement and proteinuria [41, 42].

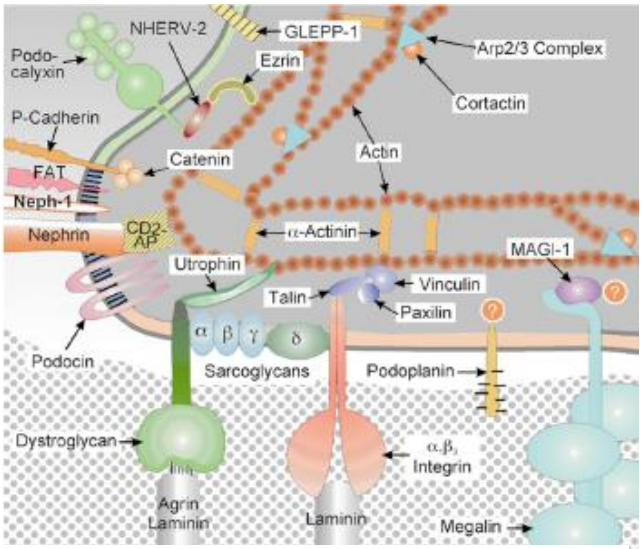


Figure 4.6 Schematic drawing of a lateral-basal portion of a podocyte. The three membrane domains are also depicted here.

4.3.1 The podocyte in kidney injury.

The role of the podocyte is crucial in a variety of diseases such as diabetes, FSGS, MN, MCD and LN. Podocytes are the target of many forms of injury (**Table 4.1**) including antibodies to podocyte membrane antigens (MN, MCD) [43], hemodynamic injury (diabetes) [44], gene mutations (nephrin, podocin), infections (HIV) and unknown causes (idiopathic FSGS) [45, 46]. Podocyte response to injury includes hypertrophy, epithelial-mesenchymal transition (EMT), and depletion (by apoptosis, detachment-migration, and lack of proliferation). Hypertrophy has been described in experimental model of ageing kidney [47]. It is initially an adaptive response to cover the denuded GBM by a cell incapable for proliferation. Increase in cyclin dependent

kinase (CDK) inhibitors (p21, p27) is found in experimental hypertrophy. Angiotensin II and mechanical stress induces podocyte hypertrophy mediated by CDK inhibitors [48].

EMT has been shown in human diabetic nephropathy [49]. In MCD podocytes undergo a phenotypic switch to a cell without FPs but with microvillus protrusions similar to renal tubular cells. This alteration is not associated with reduction in key podocyte proteins like nephrin and podocin, such as is seen in FSGS [50, 51].

Podocyte depletion contributes to the development of glomerulosclerosis. Apoptosis (programmed cell death) has been found to be mediated by angiotensin II in cultured rat podocytes [52], while TGF-β is a major contributor of apoptosis in models of toxic injury (PAN model) and is mediated through ROS. CD2AP and nephrin activate the phosphatidyl-inositol 3´-kinase kinase/AKT antiapoptotic signaling pathway thus they reduce podocyte apoptosis [53] (**Figure 4.7**).

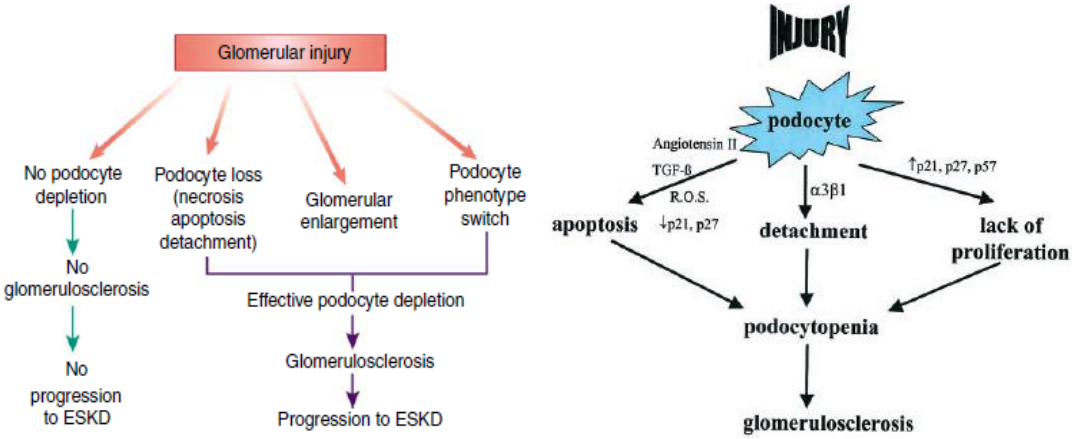


Figure 4.7 Schematic presentation of podocyte's various responses to injury and in particular podocyte loss theory that ultimately leads to glomerulosclerosis.

HIV-associated nephropathy and pamidronate toxicity are associated with a glomerular phenotype described as 'collapsing glomerulopathy' in which the glomerulus loses its normal structure including its capillary loops [54, 55, 56, 57]. In this condition, mature podocyte markers including WT1, nephrin, podocalyxin, GLEPP1, the cyclin-inhibitors p21, and p27 and foot processes are lost. People with active IgA nephropathy, SLE, and membranoproliferative glomerulonephritis have

increased numbers of podocytes in their urine [58-61], which can be eliminated by effective treatment [62]. Membranous nephropathy is well known to be associated with podocyte injury owing to insertion of complement C5b–9 complex into podocyte plasma membranes. In crescentic GN podocytes themselves can drive the crescentic process in part via expression of the hypoxia-inducible factor target gene Cxcr4 [63-68].

Table 4.1 Podocytopathies

Diseases of the podocyte

Podocyte disease	Cause of injury	Mechanism/mediator
Membranous nephropathy	Anti-podocyte antibodies	C5b-9
Minimal change disease	T cell mediated	Not well defined
Classic FSGS	Hereditary	α-Actinin-4 mutation
		Podocin mutation
		CD2AP haploinsufficiency
	Increased Pgc owing to:	Podocyte stress-tension
	• Obesity	
	• Diabetes	
	• Hypertension	
	• Reduced nephron number	
	↓Podocyte number	Apoptosis
		Detachment
		Lack of proliferation
		DNA damage
		Hypertrophy
	Circulating factors	Permeability factor(s)
	Sporadic disease	α-Actinin-4 mutation
		Podocin mutation
Cellular/collapsing FSGS	Infections	HIV
		Parvo B19?
	Drugs	Pamidronate
		Interferon
Diabetic nephropathy	Metabolic	Hyperglycemia
	Increased Pgc	Podocyte stress-tension
Amyloid	Amyloid protein deposition	Amyloid spicules directly injure podocyte
MPGN	Deposition of antigen–antibody complexes	Splitting of GBM
		Podocyte effacement

The clinical signature of podocyte damage is proteinuria. Apart from the previous mentioned causes a common finding in many proteinuric diseases is **FP effacement**. Effacement starts as a decrease in the degree of interdigitation by shortening and widening of foot processes. This is accompanied by degradation of some foot processes, followed by loss of the inter-digitating foot process pattern between individual cells (**Figure 4.8**).

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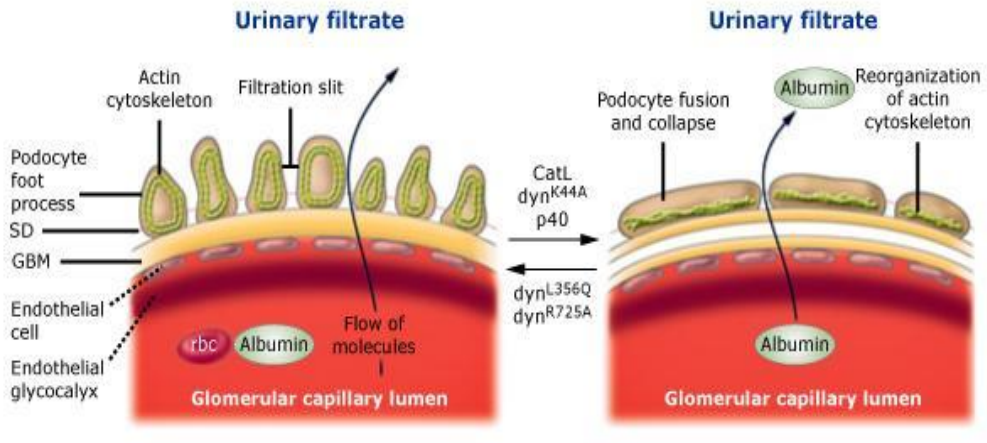

Figure 4.8 Glomerular filtration barrier. Podocyte FP effacement.

Foot process length decreases up to 70%, and the width increases up to 60% compared to normal. Four major causes can be identified that lead to FP effacement and proteinuria:

(*1*) interference with the SD complex and its lipid rafts;

(*2*) interference with the GBM or the podocyte-GBM interaction; [69-71] C5b-9 attack on podocytes induces ROS and protease production. ROS production is mediated by NADPH upregulation induced by arachidonic acid release. ROS contribute to collagen IV GBM degradation via lipid peroxidation. In podocytes, ROS also induce apoptosis, DNA damage and FP effacement [72]. Metalloproteinase-9 is markedly increased in podocytes following C5b-9 injury in experimental MN [73]. Cathepsin L, metalloproteinase-2 and metalloproteinase-9 are endogenous to podocytes, and their activities are increased by cytokines such as TGF-*β* [74].

(*3*) interference with the actin cytoskeleton and its associated protein α-actinin-4;

(*4*) interference with the negative apical membrane domain of podocytes, *e.g.*, neutralization of negative cell surface charges [75]. Podocytes express angiotensin II receptors type 1 (AT1) and type 2 (AT2) [76, 77]. Increased AT1 signaling in podocytes leads to protein leakage and structural podocyte damage progressing in FSGS [78]. In mouse podocytes, insulin activation of the insulin receptor results in the phosphorylation of Akt and Mapk42 or Mapk44, resulting in physiologic

remodeling of the actin cytoskeleton and preservation of cell function and survival. Thus in insulin resistance state, the absence of protection mechanism results in FP effacement, cell malfunction, death and proteinuria [79].

4.3.2 Slit diaphragm

As mentioned above interdigitating podocyte foot processes form a 40-nm-wide filtration slit and are connected by a continuous membrane-like structure called the slit diaphragm (SD).

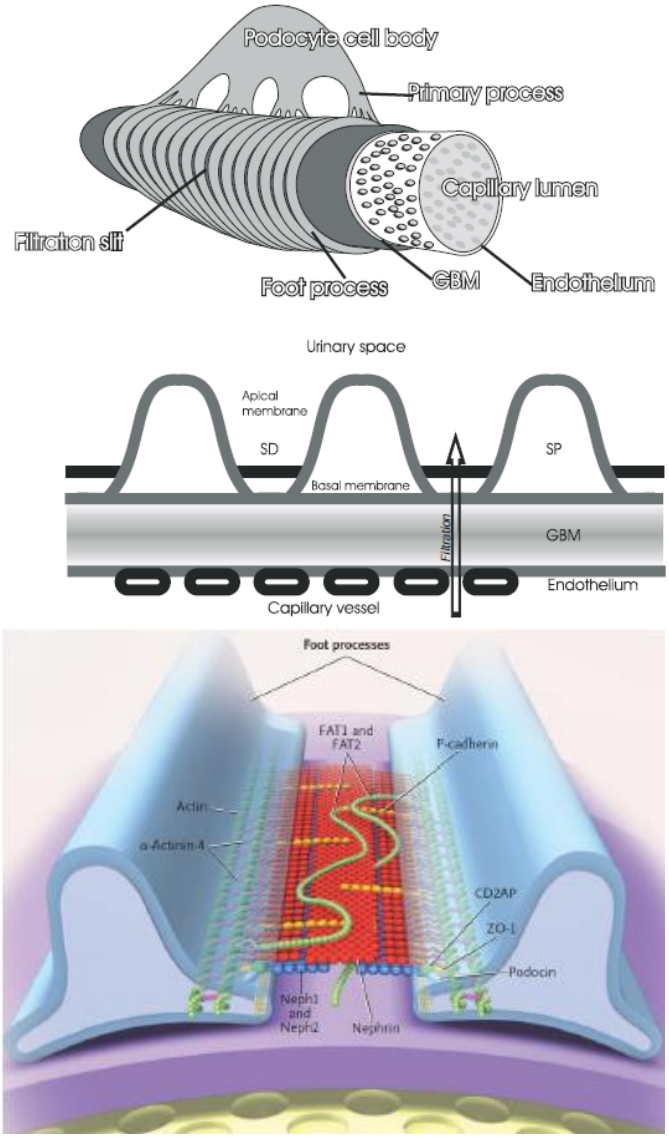

Figure 4.9 Slit diaphragm (SD) and its protein complex components.

The SD forms a zipper-like structure as reported by Rodewald and Karnovsky. The architecture of the SD is suggested to be mediated by protein-protein interactions across the foot process intercellular junction. The SD structure makes a selective sieve that prevents proteins > albumin from leaking into urine. It is estimated that this surface aintains approximately 40% of the hydraulic resistance of the whole filtration barrier. Unique membrane structural (e.g. nephrin, podocin, Neph1), adherens junctions proteins

(e.g. P-cadherin, FAT, catenins), tight junction proteins (e.g., JAM-A, cingulin) and Par3-Par6- sPKC polarity complex, receptors, signaling adaptors, ion channels, scaffolding proteins are the components of SD and are localized to lipid rafts [80-83] (**Figure 4.9**). The tight junction proteins are upregulated in PAN nephrosis and loosened their attachment to the actin cytoskeleton [84]. The polarity complex interacts with Neph-nephrin proteins and govern the appropriate distribution of nephrin and podocin [85-87]. Defects in α-actinin-4, nephrin, phospholipase C epsilon gene, podocin, transient receptor potential cation channel 6 result in rearrangement of the actin cytoskeleton. Densin, known due its involvement in the synaptic organization, maintenance of cell shape and polarity in nerve cells, has been identified in the SD area and interacts with nephrin [88]. NF-*κ*B activated by cell surface receptor signaling to meet stress and inflammatory responses, regulating innate and adaptive immunity and cell growth and survival is involved in GN. Animal data suggest that nephrin may normally limit NF-*κ*B activity thus discouraging the evolution of glomerular disease. CD2 associated protein (CD2AP) connects the nephrin complex with various modifying proteins. The protein ZO-1 directly associates with the cortical actin cytoskeleton, and densin binds to a-actinin-4. FAT-1 is also an organizer of actin polymerization. Dendrin, a cytosolic protein is found in FPs of mouse podocytes and associates with the SD complex in normal human kidney. In MCD patients, dendrin and ZO-1 are re-distributed within the podocytes [89, 90].

4.3.3 Nephrin

Twenty-five years after the hallmark finding by Rodewald and Karnovsky [79, 80], the discovery of the transmembrane protein nephrin as a major component of the SD complex by Tryggvason and others [91-93] provided a seminal progress in podocyte biology. Mutation analysis of the nephrin gene, NPHS1 (located on chromosome 19q13.1), elucidated the underlying genetic defect in congenital nephrotic syndrome

of the Finnish type as causative for FP effacement in this disease [94]. Nephrin is a large (1241–amino acid, 185-kD) transmembrane molecule with a large extracellular domain containing eight C2-type IgG like domains, a single membrane spanning domain, and a cytoplasmic portion that contains potential phosphorylation sites (**Figure 4.10**).

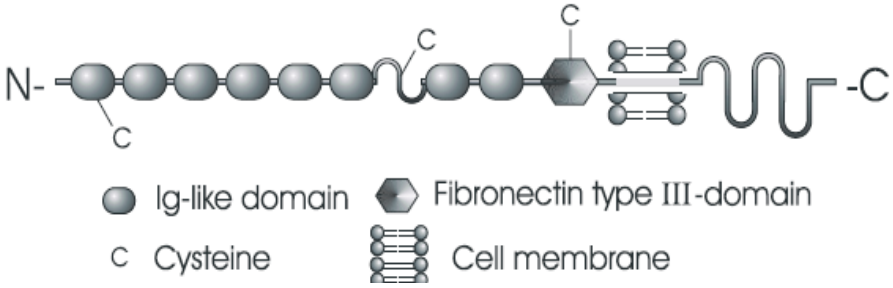

Figure 4.10 Schematic presentation of a nephrin molecule. One can see the Ig-like domain, which is extracellular, as well as the transmembrane and the cytosolic C-terminal end.

It is not only the main structural component of the podocyte SD, forming a scaffold for other SD molecules, but also a cell surface receptor participating in cell-cell signaling functions. Nephrin molecules interact with one another in a homophilic fashion in the middle of the slit to form a filtering structure. Nephrin has shown to interact with CD2AP and podocin linking the SD with the podocyte cytoskeleton. ZO-1 as well as α-actinin, IQ motif-containing GTPase activating protein 1(IQGAP1), membrane-associated guanylate cyclase inverted 2 (MAGI2) and calcium/calmodulin-dependent serine protein kinase (CASK) also couple the nephrin to the actin cytoskeleton [95-97]. NEPH1 interacts with nephrin in a heterophilic interaction (**Figure 4.9**) and along with podocin form a complex that preserves the FP architecture in the face of arterial pressure tranduced from glomerular capillaries [98]. Thus animal models lacking nephrin or anti-nephrin antibodies cause loss of SDs, FP effacement and eventually proteinuria. In addition nephrin serves as a signaling molecule. Src-family kinase Fyn phosphorylates the cytoplasmic domain of nephrin and modulates its interaction with podocin leading to enhanced association with podocin and downstream signaling of nephrin [99].

4.3.4 Podocin

The other SD protein that incorporates SD structure and signaling is podocin. It is a 42kD transmembrane protein of the stomatin family which is encoded by NPHS2 that is located on chromosome 1q25-31. It consists of a single, short transmembrane domain and cytosolic N- and C-terminal domains. NPHS2 gene mutations were originally detected in a type of autosomal recessive steroid resistant nephritic syndrome that affects children within the first years of life (**Figures 4.6** and **4.9**) [100]. Podocin almost exclusively localizes to the SD of podocytes and interacts with the cytoplasmic tail of nephrin. Podocin accumulates there in an oligomeric form in lipid rafts, which are microdomains rich in sphingolipids and cholesterol, in the outer leaflet of the plasma membrane and associates via its C-terminus with CD2AP and nephrin. Hence, podocin may act as a scaffolding protein, serving in the structural organization of the slit diaphragm and the regulation of its filtration function. Reduced podocin protein levels have been detected in the glomeruli of PAN nephrotic rats [101-105]. Recently [106, 107] nephrin and podocin have been found to have a crucial role in experimental GN, like anti-GBM and PHN.

4.4 Lupus nephritis

4.4.1 Overview

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease which can affect any organ of the body and thus have a broad array of manifestations such as rash, arthritis, anemia, serositis, nephritis, seizures and psychosis. The prevalence of lupus ranges from 40 to more than 200 cases per 100000 persons. The life expectancy of patients with SLE has improved from 4-year survival rate of 50% in the 1950s' to a 15-year survival rate of 80% today. LN is a common and serious manifestation of SLE associated with significant morbidity and mortality. The incidence of LN ranges from 25% to 75% among SLE patients and up to 20% of

these patients progress to renal failure. LN is regarded as a syndrome, including a variety of immunologic events [108, 109].

4.4.2 Clinical features – Diagnosis

As with SLE in general, the spectrum of LN is wide, encompassing the acute nephritic syndrome, nephrotic syndrome, acute or chronic renal failure, and isolated abnormalities of the urinary sediment. However, proteinuria is the most constant feature, being present in almost every patient with clinical lupus nephritis. Although microscopic hematuria is common, it rarely occurs in isolation. Hypertension is most prominent in patients with severe LN and about half will show a reduced glomerular filtration rate (GFR) and occasionally they will present with acute renal failure. Renal tubular function is occasionally disturbed (**Table 4.2**) [110]. Increased anti-dsDNA titers and reduction of serum complement is observed in the proliferative forms of disease. The diagnosis of LN is mainly established by kidney biopsy. A diverse histopathology characterizes LN. The latest ISN/RPS (2003) classification of LN is currently in use (**Table 4.3**) [111].

Table 4.2

Clinical features of patients with lupus nephritis[a]

Feature	% of Those with Nephritis
Proteinuria	100
Nephrotic syndrome	45 to 65
Granular casts	30
Red cell casts	10
Microscopic hematuria	80
Macroscopic hematuria	1 to 2
Reduced renal function	40 to 80
Rapidly declining renal function	30
Acute renal failure	1 to 2
Hypertension	15 to 50
Hyperkalemia	15
Tubular abnormalities[b]	60 to 80

In the following paragraphs we will focus on the current data regarding LN pathogenesis in particular. IC and complement deposition, interstitial and glomerular infiltration by inflammatory cells, mesangial cell proliferation, podocyte FP effacement are the initial findings and fibrosis follows.

Table 4.3 **Synopsis of International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN**

Class I Minimal mesangial LN	Minimal mesangial immune deposits
Class II Mesangial Proliferative LN	Mesangial hypercellularity - Normal glomeruli by light microscopy
Class III Focal LN	Segmental or global, endo- or extracapillary glomerulonephritis involving <50% of all glomeruli with or without messangial damage
Class III(A)	Active lesions
Class III (A/C)	Active + Chronic lesions
Class III (C)	Chronic lesions
Class IV Diffuse LN	Segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli with subendothelial deposites /with or without messangial damage
Class IV S(A)	Segmental Active lesions
Class IV G(A)	Global Active lesions
Class IVS(A/C)	Segmental Active+Chronic lesions
Class IV G(A/C)	Global Active+Chronic lesions
Class IV S(C)	Segmental Chronic lesions
Class IV G(C)	Global Chronic lesions
Class V Membranous LN	Segmental or global subepithelial deposits /with or without mesangial damage /with or without presence of class III or class V LN / with or without chronic lesions
Class VI Advanced sclerosing LN	≥90% global sclerosis

4.4.3 Pathogenesis of LN

4.4.3.1 Autoimmunity disorder in LN

Autoimmunity in SLE has been attributed to a loss of self-tolerance due to inadequate central or peripheral deletion or silencing of autoreactive lymphocytes, leading to multiple autoantibody specificities. Dysregulated apoptosis and inadequate removal of apoptotic cells and nuclear remnants may contribute to autoimmunity by causing prolonged exposure of the immune system to nuclear and cell membrane components. The characteristic development of autoantibodies to DNA and other nuclear antigens as well as to membrane phospholipids support the relevance of both mechanisms [112-114]. In addition to established genetic predisposition, altered immunoregulatory factors or environmental stimuli may trigger autoimmune phenomena. Autoantigens are released by both necrotic and apoptotic cells. Defects in the clearance of apoptotic cells have been described and these defects could lead to aberrant uptake by macrophages, which then present the previously intra cellular

antigens to T and B cells, thus driving the auto immune process [115]. Cytokine patterns might also be important in the pathogenesis of lupus. Investigations [116] have drawn attention to the overexpression of the type I interferon pathway in patients. Abnormal signal transduction could also be important in the pathogenesis of systemic lupus erythematosus. For example, decreased expression of T-cell receptor ζ chain and protein kinase C, decreased protein kinase C-dependent protein phosphorylation, impaired translocation of nuclear factor κB (NF-κB) p65, and decreased production of interleukin (IL) 2, have all been described in T cells from patients with this disorder [117].

Autoantibodies.

Renal injury in lupus nephritis may result from autoantibodies that bind to circulating antigens. Autoantibodies with antiphospholipid or cryoglobulin activity may also promote thrombotic and inflammatory vascular lesions in SLE. Antineutrophil cytoplasmic antigen autoantibodies (ANCA) have been described in a subgroup of patients with lupus nephritis and may initiate vasculitis and glomerulonephritis by “pauci-immune” neutrophil-dependent mechanisms similar to those described for microscopic polyangiitis or Wegener’s granulomatosis. Finally, it is also likely that other poorly characterized autoantibodies of unknown specificity (such as anti-endothelial antibodies) may be operant in the pathogenesis of some forms of lupus nephritis. Possible mechanisms by which autoantibodies cause LN are depicted in **Figure 4.11** (a) Some antibodies against dsDNA form complexes with DNA in the blood stream and are passively trapped in the glomeruli and (b) others attach directly to glomerular structures i.e. GBM. Not all autoantibodies cause renal damage (nephritogenic antibodies). The capacity of autoantibodies to form immune deposits and their location are dependent on properties unique to individual Ig in LN.

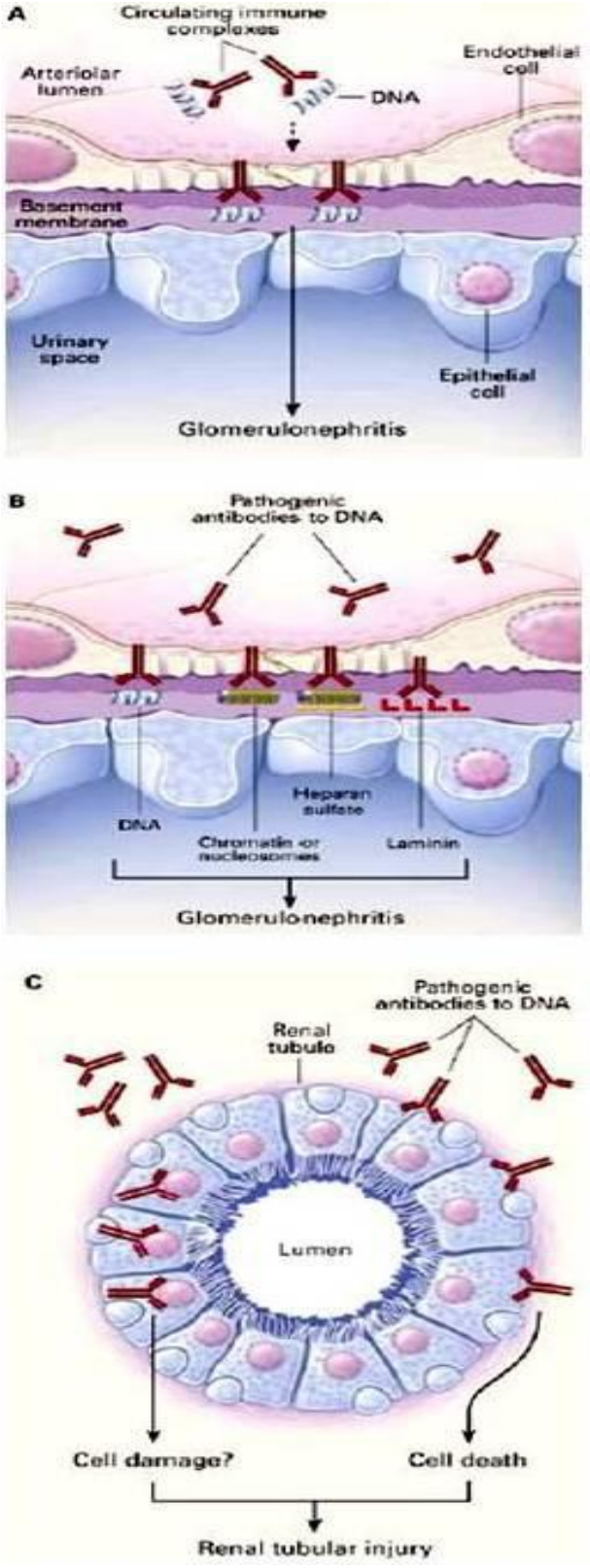

Figure 4.11 Pathogenesis of LN via autoantibody induced damage.

IgG1 and IgG3 anti nuclear antibodies are considered more nephritogenic. Extracellular double stranded DNA occurs in the form of nucleosomes, which are fragments of chromatin that cells release when they undergo apoptosis [118-120].

Positively charged chromatin fragments on GBM, as targets for autoantibodies [121] and high affinity antibodies to nucleosomal DNA mostly determines the pathogenic capacity of anti-DNA antibodies [122-124]. Their cross reactivity to alpha actinin, laminin, C1q and heparin sulphate attributes pathogenic properties [125-127].

Immune complexes-complement activation.

As mentioned before the complement system is an important part of innate immunity that defends the host against infectious microorganisms, clears ICs and dead cells, and connects innate to adaptive immunity. The ICs trapped in the GBM activate the complement pathway towards production of C3b and C4b. The next key protein is CR1, present on erythrocytes (or CFH on the rodent platelet), where it has binding affinity for C3b, and serves as a CFI cofactor for the cleavage of C3b to iC3b. ICs bound to human erythrocytes CR1 (or mouse platelet CFH) are transported and transferred to cells of the mononuclear phagocyte system such as liver or spleen. Glomerular podocytes also bear CR1, another cofactor which may play a role in IC processing locally and is decreased in LN [128, 129]. The Fc receptor is crucial for the LN development. NZB/W F1 mice with defective Fc receptors generated and deposited IC and activated complement but were protected from severe LN [130]. Also lupus MN is characterized by subepithelial IC deposition and FP effacement without inflammation. The subsequent development of proteinuria is complement dependent and involves the formation of MAC (C5b-9) in the same mechanism observed in idiopathic MN [131, 132]. Local expression of complement genes C3, Factor B, C2 and C4 is increased in murine LN [133]. In MRL/*lpr* lupus mouse kidneys, C3aR and C5aR expression was up-regulated significantly at both the mRNA and protein levels. Dysfunction or down-regulation of DAF may contribute to autoimmune disease pathogenesis and manifestations in an LN model of MRL/*lpr* mice [134]. Another rodent complement regulatory protein is p65 (Crry -*CR1-related gene/protein y*) because of its similarity to human CR1. Transgenic mice that

overexpressed a soluble form of Crry inhibited complement activation systemically and locally in the kidney. When crossed into the MRL/*lpr* strain, Crry inhibited complement of MRL/*lpr* mice [135, 136] and lessened LN.

B and T cells, macrophages, cytokines.

Lupus is a disease of failed immune tolerance to self that leads to the production of T and B autoreactive cells. The danger signal is delivered by costimulatory molecules expressed on antigen-presenting cells (APCs) and lymphocytes. Major costimulators involved in T and B cell collaboration are CD40/CD40L and members of the B7/CD28 family (B7/CD28, inducible costimulator ligand [ICOSL]/inducible costimulator [ICOS]). In addition, microbial products can trigger danger signals that permit bypass of tolerance by binding **Toll-like receptors (TLRs)** on B cells and APCs. TLR are microbe recognizing receptors expressed by B cells and by resident kidney cells and recognize self antigens thus contributing to autoimmune disease. TLR-9 is expressed in the tubulointerstitium and in the glomerulus in human LN. The ICs activate the renal cells through TLR to produce inflammatory mediators. TLR-9 protected against nephritis in the MRL/lpr mice as well as co-inhibition of the TLR-7 and TLR-9. Additionally TLR-9 ligand accelerated LN in MRL/lpr mice [137, 138].

Lupus **T cells** manifest defective TCR signaling; decreased activation threshold; upregulated expression of costimulatory molecules CD40L, ICOS, β2 integrins CD11a/ CD18 (LFA-1), and CD11c/CD18 (Mac-1); decreased expression of TCR-ζ chain; increased intracellular phosphorylation; upregulated Fc-γ receptor chain expression; deficient IL-2 production [139, 140].

Lupus **B cells** are hyperactive and show enhanced phosphorylation of mitogen-activated protein kinase pathways, altered Lyn expression, upregulated bcl2, upregulated CD40L, and defective B-cell receptor (BCR)-induced apoptosis. Circulating B-cell–activating factor (BAFF, or BLyS), a B-cell survival factor, is increased in a significant proportion of lupus patients, and mice overexpressing

BAFF develop a lupus-like disease. BAFF rescues autoreactive B cells from peripheral deletion and initiates CD40L-independent IgG isotype class switch [141-145].

Activated **macrophages** in the renal interstitium correspond with disease progression. Activation of renal dendritic cells occurs in murine and human LN, they induce T cell activation and are associated with expression of inflammatory cytokines [146].

Among the inflammatory **cytokines** TNF has been detected in macrophages infiltrating the glomerulus, as well as in glomerular podocytes, endothelial and mesangial cells and in the interstitium its concentration in the kidney correlates with disease activity [147, 148]. Increased IL-6 in LN is associated with mesangial cell proliferation. IL-1 has been detected in infiltrating monocytes and macrophages and in some podocytes and mesangial cells in human LN [146]. IL-18 is overexpressed in nephritic kidneys of MRL/lpr mice, both in infiltrating monocytes and in tubular epithelial cells. Upregulation of INFγ, the prototypical Th1 cytokine, produced by monocytes, has been shown in human and murine LN [149-151]. IL-4, the prototypical Th2 cytokine is found also in podocytes and in mesangial cells. IL-4 mRNA expression was associated with hypercellularity and extracellular matrix expansion in human LN [152].

4.4.3.2 Endothelial cells

Inflammatory cell recruitment requires endothelial cell activation. Endothelial hypoxia results in cell death and further activation of innate immunity. Active human LN is characterized by upregulation of Tie2 antagonist. Tie2 maintains endothelial integrity [153].

4.4.3.3 Mesangial cells

The IC deposition is followed by production of chemokines like MCP-1 and RANTES by the glomerular mesangial cells. The initial mesangial stimulation results in mesangial expansion and cellular infiltration. In a later stage a transition to fibroblast phenotype has been shown with an increase in expression of genes such as metalopeptidase, TGF-*β2*, insulin-like growth factor binding protein *2*. Activation of the TGF-*β2* signaling is the final common pathway leading to fibrosis, glomerulosclerosis and end stage renal failure [154].

4.4.3.4 Podocytes

Podocytes play an active role in immune response within the kidney. They express receptors for interleukins 4, 10, 13, functional CCR and CXCR chemokine receptors and respond to exogenous chemokines. They are also capable of producing inflammatory mediators such as interleukin 1, complement proteins including C3, TGF-*β* and fibroblast growth factor 2. They also express CR1 and Toll like receptor-4 (TLR-4). CR1 is localized exclusively in human podocytes on which is capable of binding C3b. Complement factor H (CFH) is uniquely expressed in rodent podocytes and is the functional surrogate for human CR1. DAF is also primarily a podocyte protein [155-159]**.** In conditions with proteinuria and FP effacement the podocytes upregulate B7-1, also called CD80, a transmembrane protein expressed on the surface of B cells and other APC. B7-1 induces proteinuria through the reorganization of the podocyte FP actin cytoskeleton and disruption of the SD. The induction of B7-1 in podocytes by LPS through TLR-4, signaling suggests that the podocyte is a component of the innate immune system, equipped with a danger signaling machinery [160, 161]. Podocyte expression of B7-1 correlated with the severity of human LN. In murine model of LN anti dsDNA antibodies that cross-react with α-actinin are pathogenic [162]. Blockage of type 1 angiotensin receptors in the

glomerulus reduced proteinuria, glomerular pathology and cytokine expression both in murine and in human LN [163, 164].

4.4.4 Treatment of LN

The optimal treatment of lupus nephritis (LN) varies with the type of disease that is present. Immunosuppressive therapy is indicated in the great majority of patients with diffuse and focal proliferative LN and in some selected patients with membranous LN including those with a severe nephrotic syndrome, an elevated serum creatinine, and/or associated proliferative disease. Immunosuppressive therapy is usually not indicated for minimal mesangial and mesangial proliferative LN. Induction immunosuppressive therapy includes glucocorticosteroids (GC) and cyclophosphamide (CY). The immunosuppressive drugs act by a variety of mechanisms, mainly by interfering with combinations of critical pathways in the inflammatory cascade.

4.4.4.1 Glucocorticosteroids (GC)

GC after binding to GC receptors alter gene regulation via direct transmission of signals to the nucleus. DNA binding of the glucocorticoid receptor mediates the antiinflammatory effects of glucocorticoids by: Recruiting transcription factors to promoter sequences of genes coding for antiinflammatory gene products including: I-kappa-B (IkBa), interleukin (IL)-1 receptor-II (IL-1RII), lipocortin-1 (annexin I), IL-10, alpha-2-macroglobulin, and secretory leukocyte protease inhibitor. GC inhibit the synthesis of almost all known cytokines, by blocking the function of transcription factors nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) that are required for transcription of proinflammatory mediators [165-170]. In addition to their effects on gene transcription, GC also inhibit secretion of inflammatory cytokines by affecting post-translational events. The stability of mRNA encoding IL-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF), and granulocyte-macrophage-colony stimulating

factor (GM-CSF) are diminished in the presence of GC. Effects on the cellular functions of phagocytic cells and endothelial cells result in reduced trafficking of leukocytes. Expression of adhesion molecules on the surfaces of both endothelial cells and leukocytes is markedly attenuated, resulting in a lesser degree of accumulation of phagocytic cells at sites of inflammation. The reduction in endothelial adhesion function may be due to direct effects of GC on expression of adhesion molecules as well as indirect effects due to the inhibitory effects of GC on transcription of cytokines such as IL-1 or TNF that upregulate endothelial adhesion molecule expression.

As far as the effects on acquired immunity, GC decrease the numbers of both circulating and organ resident dendritic cells in murine and rat models; a marked reduction in circulating dendritic cells is also seen in humans [171]. This effect appears to be mediated at least in part by GC-induced apoptosis of dendritic cells.

GC also produce a rapid depletion of circulating T cells due to a combination of effects that include a. enhanced circulatory emigration, b. inhibition of IL-2, a principal T cell growth factor, and IL-2 signaling, c. impaired release of cells from lymphoid tissues d. induction of apoptosis [172-175].

Numbers of circulating B cells and synthesis of antibodies by B cells are impacted by glucocorticoid administration to a much lesser extent [176, 177].

In addition to the effect on the immune system, GC may directly influence the unique structure and function of podocytes. Thus, GC can increase the stability of actin filaments, increase actin polymerization, and activate cytoskeleton-associated kinases in podocytes, suppress IL-6 and upregulate nephrin expression [178-180].

4.4.4.2 Cyclophosphamide (CY)

A number of clinical trials have demonstrated a benefit of intravenous CY in patients with proliferative lupus nephritis. CY is an alkylating agent that damages DNA repair mechanisms and is toxic to both resting and dividing cells, although proliferating cells

are generally more susceptible. CY has multiple effects on the immune system with both immunosuppressive and apparent immunostimulatory effects [181-183]. In murine models of NZB/W F1 and MRL/*lpr* mice it has been shown that CY given at intervals in high dose can stabilize established disease. This finding together with the demonstration that CY therapy decreases the frequency of autoreactive B cells for a period of only 3–4 wk even in prenephritic mice pointed the way to current protocols for treatment of human SLE nephritis with GC and monthly CY [184-186].

4.4.4.3 Other therapeutic approaches

Concerns about insufficient efficacy and poor tolerability have led to the use of other therapeutic approaches, e.g. methotrexate, azathioprine, mycophenolate mofetil (MMF), or rituximab. MMF, a selective inhibitor of inosine-monophosphatase-dehydrogenase induces renal remission [187]. Anti-tumor necrosis factor (TNFa) agents' use in lupus is tempered since they exacerbate disease in the NZB/W mouse and induce antibodies to dsDNA and lupus syndromes in humans [188]. Thus TNFa appears to protect against initiation of lupus-like diseases. Another therapy is B cells depletion with anti-CD20 monocloanal antibody (rituximab) [189]. Novel targets identified the last years using mouse studies that emerge the possibility of new therapeutic for LN. Restoration of some aspects of self-tolerance was achieved both in TLR9 knockout mice [190] and in NZB/W mice treated with a combination of CTLA4Ig and CD40L co-stimulatory blockade [191]. Several studies in mice address the therapeutic potential of T-regulatory cells for LN with various results [192].

4.4.5 NZB/W F1 lupus-prone mice.

Several features of NZB/W F1 mice model are consistent with lupus in humans. Females develop disease at a frequency 10 times that of males, and IC and complement deposition in glomeruli are observed. Significant proteinuria and severe GN is seen concomitant with the serological appearance of antibodies to DNA as well

as ICs of the immunoglobulin G1 (IgG1), IgG2a, and IgG2b subclasses beginning at months. Median survival is 6 months, with mortality resulting from renal failure. Several studies have demonstrated the essential role of B cells and autoantibodies in disease development [193-195].

5. RESEARCH QUESTIONS AND AIM OF STUDY

Recent data suggest a critical role for the podocyte SD and its components in the pathogenesis of GN and in LN. In view of these findings, therapeutic strategies to protect podocytes have been pursued by several groups [196]. Although *in vitro* studies to suggest direct effect of glucocorticoids (GC) and other immunosuppressive drugs on podocytes through regulation of slit diaphragm components and intracellular signaling pathways relevant for cytoskeletal stability, cell maturation, and survival [197], yet, there is paucity of *in vivo* data to demonstrate the effect of the aforementioned therapies on glomerular podocytes.

We have recently shown reduced expression of the slit diaphragm proteins nephrin and podocin early during the course of LN in NZB/W mice, when podocytes structure is still unaffected and immune activity is low [198]. In these mice, GC and CY are well-established therapies that can delay the clinical and histologic progression of renal disease primarily by exerting anti-inflammatory or immunosuppressive properties. We asked whether the favorable effects of these treatments are accompanied by changes in glomerular podocyte structure and slit diaphragm components. To this end, NZB/W mice were treated with GC or CY starting at early stages of renal disease.

6. MATERIALS AND METHODS

6.1 Animal studies

Female NZB/W mice were obtained from Harlan, UK. Animal care and treatment were conducted in accordance with the guidelines for the care and use of laboratory animals, approved by the University Of Crete School Of Medicine. Mice were housed in constant temperature with a 12-hr dark/12-hr light cycle. They had free access to tap water and standard mouse chow throughout the study and were euthanatized by CO2. We have previously demonstrated that the expression of slit diaphragm proteins is reduced in NZB/W mice starting at the age of 3 months, and this reduction precedes the appearance of microscopic podocyte abnormalities and severe proteinuria [198]. We therefore used 3-month-old NZB/W mice, which were randomly assigned into one of the following groups: a) untreated (control) mice (*n* = 24),
b) GC–treated mice, which received dexamethasone (Soldesanil or.dr.sol., Diapit; 0.4 mg/kg/day p.o. in drinking water) for 3 (*n* = 4) or 6 (*n* = 5) consecutive months, and c) CY–treated mice, which received CY (50mg/kg intraperitoneally every 10 days) (Endoxan pd.inj.sol., Baxter) for 3 (*n* = 4) or 6 (*n* = 4) consecutive months [199, 200]. C57BL6 were chosen as controls for normal protein excretion as spontaneous glomerulonephritis and impaired autoimmunity was not observed in such animals. Also 3 NZB and 3 NZW mice as parent strains of the NZB/W hybrid were studied. Little is known about renal characteristics of NZB and NZW. We sought to study females (n=3) NZB and males NZW (n=3) as the NZB/W are hybrids from a mating of a female NZB and a male NZW mouse.

6.2 Proteinuria

Individual mice were placed in metabolic cages (Tecniplast) for 24h urine collections the day before they were sacrificed. Urine samples were aliquoted and stored at -80 ˚C without any protease inhibitors until processed further. Urine samples were assayed for total protein using Bio-Rad Protein Assay reagent (Bio-Rad

Laboratories), using bovine serum albumin (BSA) as standard. Samples were read on an Ultrospec 2100 Pro (Biochrom Ltd) spectrophotometer at 590nm. Baseline values for urinary protein excretion, as measured in 1-month old NZB/W mice and in C57BL6 controls were equal or less than 4mg/24h. Thus, abnormal proteinuria was defined as protein excretion above 4mg/24h, as previously described. NZB/W mice were categorized: a) non proteinuric: <2mg/24h; b) mild proteinuric: 2-10mg /24h and, c) severe proteinuric >10mg/24h [201].

6.3 Determination of anti-dsDNA.

Serum anti-dsDNA autoantibodies were detected by an enzyme-immunoassay (Mouse anti-ds DNA IgG (Total) ELISA kit; ADI V3-5100) according to the manufacturer‟s instruction. Briefly, blood serum samples, diluted 1:100 in assay diluent buffer (100κl/well), was plated in antigen coated, 96-well strip plate, then incubated for 30 minutes and subsequently washed three times with 1 x washing buffer. HRP labelled goat anti-mouse IgG conjugated antibody (1:100 in assay diluent buffer – 100κl/well) was added and incubated for 30min and then washed. Positive and negative control wells were also prepared. Finally 100 κl/well of TMB solution was used (15 minutes incubation in the dark) and the reaction was terminated by the addition of 100κl/well of stop solution. The plates were read at 450 nm in a Model 680 Microplate Reader, Bio-Rad Laboratories Ltd, UK. All incubations were performed at room temperature (RT).

6.4 Renal histology

The left mouse kidney was processed for histological studies. Briefly, one part was fixed in 10% buffered formalin stored at room temperature and later dehydrated in alcohol, and embedded in paraffin and stored at RT for standard light microscopy staining techniques (hematoxyline - eosin, PAS, Jones, Masson‟s Trichrom). Another part of the kidney was snap frozen by liquid nitrogen in 22-oxycalcitriol

(OCT) compound and stored at -80°C for immunofluorescence (IgG, nephrin). A third part was fixed in 2.5% glutaraldehyde in phosphate buffer ($NaH_2PO_4H_2O$: 0.1 M + Na_2HPO_4: 0.1M pH: 7.2) stored at 4°C for electron microscopy study.

6.4.1 Light microscopy (LM)

Four κm sections of formalin-fixed paraffin-embedded tissue were examined in a Nikon THP Japan microscope. Kidney biopsies were evaluated for active lesions (mesangial expansion, endocapillary proliferation, glomerular deposits, extracapillary proliferation and interstitial infiltrates), as well as chronic lesions (tubular atrophy and interstitial fibrosis) of LN. At least 30 randomly selected glomeruli, visualized and photographed, from each animal were examined. All slides were scored by an experienced renal pathologist (L. N.) masked to the group. The 2003 International Society of Nephrology / Renal Pathology Society (ISN/RPS) classification of LN was adopted [202].

6.4.2 Immunofluorescence

For immunofluorescence (IgG and nephrin) studies, kidney cryosections (5 κm) were fixed in acetone at -80°C and were blocked with 20% normal goat serum, before incubation with antibodies against IgG (1:50 for 1 hour at room temperature) and nephrin (1:100 overnight at 4°C). Secondary species-appropriate fluroscein-isothyocyanate (FITC) conjugated antibody (1:20 for 1 hour at room temperature) was used and slides were examined in a Nikon eclipse 80i microscope. Pictures were captured with a Nikon Digital Sightt using the W/S – Element F Software. Glomerular IgG staining was assessed as follows: 0, no signal; 1+, mild; 2+, moderate; 3+, strong signal intensity. At least 15 glomeruli per animal were studied. For distribution and quantification purposes, a semiquantitive score was assigned for glomerular nephrin expression according to the following patterns: 0, negative; 1+, very weak; 2+, mild; 3+, moderate; 4+, strongly positive.

6.4.3 Electron microscopy

For electron microscopy (EM) studies, specimens were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, post-fixed for 1 hour in 1% osmium tetroxide, dehydrated in a series of graded ethanols, cleared in propylene oxide and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with uranyl acetate/lead citrate and examined in a JEM-1010 transmission electron microscope by the same pathologist (KK), in a blind manner.

Analysis was based on micrographs representing ≥20 glomeruli for each group of mice. The presence of mesangial, subendothelial and subepithelial electron dense deposits (EDD) was evaluated in a semiquantitive way according to the following pattern: 0, negative; 1+, frequent; 2+, extensive. The podocyte fusion was characterized as 0, negative; 1+, rare; 2+, extensive.

6.5 Glomerular isolation

The right kidney was processed for biochemical and molecular studies immediately after sacrifice. Glomeruli were enriched by differential sieving on ice as been described elsewhere [203]. One half of the kidney was minced in pasta like consistency, then passed through a 90 micrometer nylon sieve (Nitex 03-50/31B B B B, Sefar inc) and subsequently trapped the glomeruli on a 50 micrometer nylon sieve (Nitex 03-90/49B B B B, Sefar). Glomeruli from one half of the kidney were lysed in 1ml RIPA buffer ((0.1 M HEPES, 0.15M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol, 0.1 M sodium fluoride, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 1% Nonidet P-40, and protease inhibitors) [204]. The lysed glomeruli were centrifuged at 4°C at 14,000 x *g* for 15 minutes and the supernatant was aliquoted stored at -80°C until used for Western blot studies.

6.5.1 Western blot (WB) analysis

For Western blot analysis, 40κg of total protein from glomerular extract were diluted in Laemmli sample buffer with 5% 2-mercaptoethanol, boiled for 5 minutes and separated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN 3 Mini Vertical Electrophoresis System (BIO-RAD). Proteins were transferred to a nitrocellulose membrane (Protan BA85 - Schleicher & Schuell Bioscience), blocked for 1 h in 5% skim milk in TBS-T (0.05% Tween 20) at room temperature, and then incubated with the indicated primary antibody (1:500) diluted in 5% skim milk in TBS-T (0.05% Tween 20) at 4°C overnight. After three wash with TBS-T (0.05% Tween 20), membranes were incubated with the appropriate horseradish peroxidase (HRP) conjugated IgG diluted 1:3000 – 1:10000 according the manufacturer's instruction at room temperature for 1 hour. Blots were developed using enhanced chemiluminescence (ECL) by Amersham (RNP2209) and exposed (SuperRX 12x18 – FUJIFILM). Nephrin and podocin were normalized to actin detected by a pan actin monoclonal antibody diluted 1:1000 in TBS-T (0.05% Tween 20) at room temperature for 1.5 hours. The density of the positive bands was quantitated by Tinascan.

6.5.2 RNA isolation, cDNA preparation and real time PCR.

Total **RNA was isolated** from kidneys by a single-step guanidinium isothiocyanate-phenol-chloroform extraction (Trizol Reagent Invitrogen) according to the manufucturer's instructions. After tissue homogenation in Trizol reagent (50-100mg of tissue/1ml Trizol Reagent) at 4°C, the nucleoprotein complex was completely dissociated by sample incubation at room temperature. The phase separation was performed by the incubation with 0.2ml of chloroform at room temperature for 3 minutes and centrifugation at 12000 x *g* at 4°C for 15 minutes. The RNA precipitation from the resultant aqueous phase was done by mixing with 0.5ml isopropyl alcohol, incubation at room temperature for 10 minutes and centrifugation at 12000 x *g at* 4°C

for 10 minutes. One wash of the RNA pellet with 1ml of 75% ethanol followed and subsequently the RNA pellet was air-dried and finally redissolved in 30κl of RNAase-free water and stored at -80°C.

cDNA preparation

One microgram of total RNA was used to prepare first strand cDNA in a total reaction volume of 20κl by AMV Reverse Transcriptase (Promega). The appropriate volume of RNAase free water and 0.5μg primer/μgRNA was added to the 1μg total RNA, heated at 65°C for 5 minutes, chilled on ice for 5 minutes and centrifuged briefly. Then 4μl of 25mM MgCl2, 2μl of Reverse Transcriptase Reaction 10x buffer, 2 μl of 10mM dNTPs, 0.5μl of 40u/ml RNasin and 0,75μl of 20U/ml RT enzyme (AMV) were added to the RNA solution up to the volume of 20μl. The mix then was sequentially incubated at 25°C for 15 minutes, at 42°C for 60 minutes, at 95°C for 5 minutes and finally at 4°C for 5 minutes. The cDNA was stored at -20°C until used.

Real Time PCR reaction

A typical 20μl real time PCR reaction was performed in the ABI Prism 7700 Sequence Detection System using BioRad SYBR green Super mix 1x. Each reaction contained 10 μl of 1× SYBR green Super Mix, 0.6μM (1.2μl) of mouse nephrin or podocin or GAPDH primers, 5 μl of 1:5 dilution of the cDNA prepared as above, and water to 20 μl. The reactions were then followed by 40 cycles of 30 seconds at 95°C, of 30 seconds at 55°C, and of 72 seconds at 72°C. All measurements were performed in duplicate. The primers used in this study were as follows: mouse GAPDH, forward 5′– AATGTGTCCGTCGTGGATCTGA-3′ and reverse 5′– GATGCCTGCTTCACCACCTTCT-3′, mouse nephrin forward 5′– ACACAAGAAGCTCCACGGTTAG-3′ and reverse 5′– TGGCGATATGACACCTCTTCC-3, mouse podocin forward 5' GTGTCCAAAGCCATCCAGTT 3' and reverse 5' GGCAACCTTTACATCTTGGG 3'. Nephrin and podocin mRNA quantity was normalized to GAPDH mRNA.

6.6 Antibodies.

Anti-mouse IgG (H+L) (BA 2000, Vector Lab.) and FITC-conjugated streptavidin (F72, Biomeda) were used for mouse IF. A polyclonal rabbit anti-nephrin antibody directed against the cytoplasmic domain of mouse nephrin was kindly provided from Dr. L. B. Holzman [93] for the WB and IF studies. FITC-conjugated polyclonal swine anti-rabbit IgG (F0205, DAKO) was used as secondary antibody for nephrin IF. Podocin was detected in western blot by a polyclonal rabbit anti-podocin antibody (ab50339, Abcam) and actin by pan-actin monoclonal antibody (MAB 1501, Chemicon) (1:3000). HRP-conjugated mouse anti-rabbit and HRP-conjugated mouse anti-mouse were obtained from Cell Signalling (7074 and 7076, respectively) (both used at 1:3000).

6.7 Statistical analysis.

Data are expressed as the mean ± standard error of the mean (SEM) value. Comparisons were performed with the Mann-Whitney *U* test for numerical data and with the Fisher‟s exact test for categorical. Correlation analysis was done by Spearman‟s rho (ρ) test. All analysis was done with SPSS 16.0 (SPSS, Inc.) and *p*-values (two-tailed) <0.05 were considered as statistically significant.

7. RESULTS

7.1 Clinical features and histology of treated and untreated mice.

We first determined the clinical picture and histology of renal disease in our experimental animals.

NZB/W F1 treated and untreated mice

Twenty four hour urine protein excretion and serum anti-dsDNA titers were determined in 17 NZB/W females that were used as controls and in 17 NZB/W female mice that were treated with either GC or CY for 3-6 months. All were positive for serum anti-dsDNA titers from the age of three months.

Five out of 6 NZB/W aged 3 months had mild and one no proteinuria. Three out of nine aged 6 months had significant and the remaining six severe proteinuria as well as all (n=2) aged 9 months. By the age of 6 months proteinuria was approximately 33,4 ± 28.1 mg/24-hr, which was further increased at 9 months of age (46.3 ± 13.3 mg/24-hr). Mice treated with GC had reduced proteinuria at both 6 and 9 months of age (17.3 ± 13.9 mg/24-hr and 7.4 ± 4.6 mg/24-hr, respectively). CY–treated mice also had significantly reduced proteinuria levels (13.9 ± 5.2 mg/24-hr at 6 months, 2.2 ± 0.5 mg/24-hr at 9 months) compared to their untreated littermates, and comparable to the levels of 3-month-old mice (5.8 ±1.2 mg/24-hr) (**Figure 7.1**).

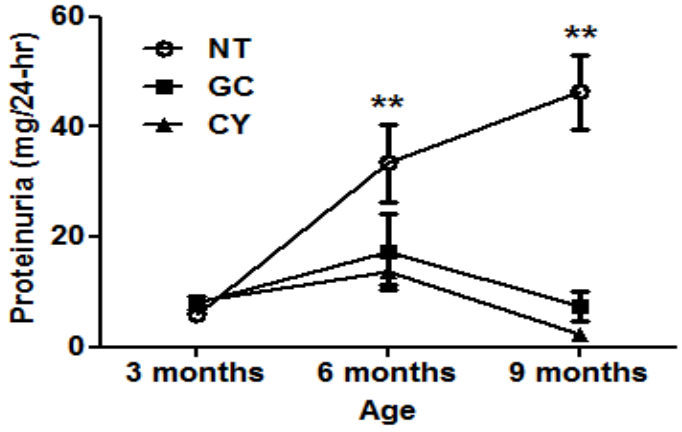

Figure 7.1 Untreated (NT) NZB/W mice developed overt proteinuria by the age of 6 months, which was further increased at 9 months of age. Mice treated with either GC or CY had reduced proteinuria at both 6 and 9 months of age. Bars represent standard error of the mean value. * p <0.05; ** p <0.005

We next examined the effect of therapy with GC or CY on renal histology in NZB/W lupus mice. Four out of six (4/6) of three-month-old NZB/W mice demonstrated

normal histology findings and the remaining 2 (2/6) showed mild segmental mesangial hyperplasia (MMLN). Six out of 9 aged 6 months and 1 out of 2 aged 9 months exhibited segmental or global, endo- or extracapillary glomerulonephritis involving <50% of all glomeruli (focal proliferative nephritis) and were encoded as FPLN. The remaining, 3 (n=2 aged 6 months, n=1 aged nine months), showed global extracapillary glomerulonephritis involving ≥50% of all glomeruli with active lesions, cellular crescents, pseudothrombi and subendothelial deposits and two aged 6 months with mainly chronic lesions – fibrocellular crescents. Those 4 were categorized as diffuse proliferative nephritis (DPLN). In GC– and CY–treated mice, glomerulonephritis was milder with markedly reduced interstitial inflammation and mesangial hyperplasia; all of mice treated with GC for 3 months (6 months old) showed MMLN and 50% (n=2) of mice treated with CY for the same period had normal LM and the remaining 2 (50%) showed again MMLN. seven out of eight (7/8) NZB/W mice treated for 6 months (9 months old) had normal LM histology with only one of the GC-group having MMLN (**Figure 7.2**).

IgG immunofluorescence was performed in 13 control NZB/W females and 17 treated mice. Five out of six (5/6) of aged 3 months control NZB/W mice showed weak signal of IgG IF, whereas all the remaining aged 6-9 months exhibited intense signal. On the contrary, IgG kidney deposition was significantly reduced in treated mice being undetectable in mice treated with CY for 6 months (**Table 7.1** and **Figure 7.3**). The findings on LM and IF of the NZB/W females are presented in **Table 7.1**.

Thirteen NZB/W mice were examined in electron microscope (EM). At the age of 3 months a segmental increase of mesangial matrix was observed. Electron dense deposits were present in mesangial areas and in GBM, in both subendothelial (rare)

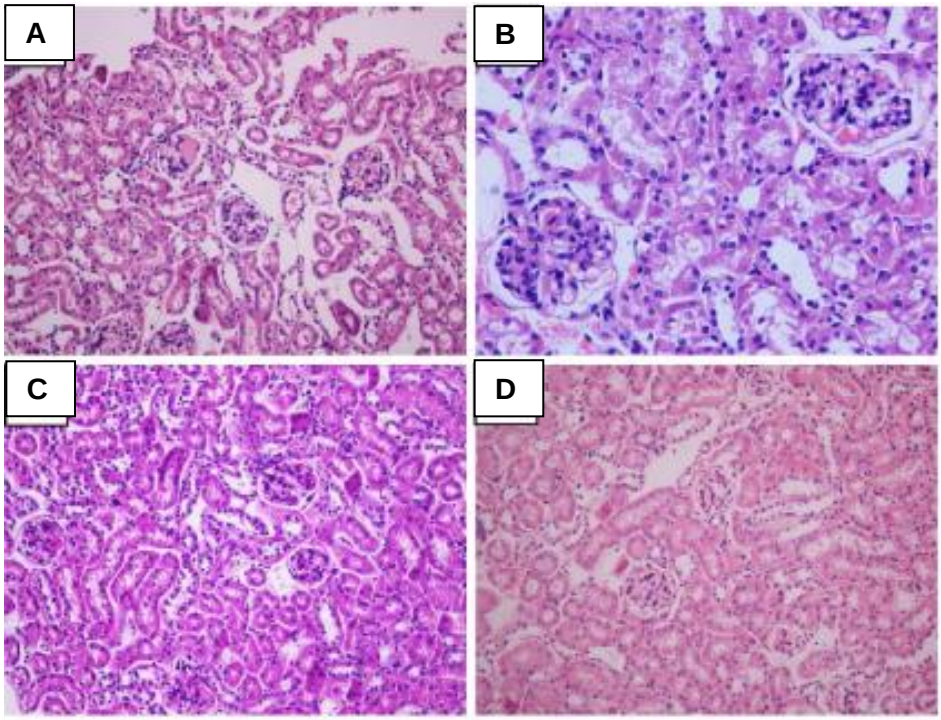

Figure 7.2 (**A**) Glomerular infiltration in a 3-month-old NZB/W (control) mouse. (**B**) Focal proliferative nephritis in a 6-month-old untreated NZB/W mouse. (**C**) Minimal mesangial nephritis in a 6-month-old NZB/W mouse treated with GC for 3 consecutive months. (**D**) Normal light microscopy findings in a 6-month-old NZB/W mouse treated with CY for 3 consecutive months. Original magnification ×100 in panels A, C, D, ×250 in panel B.

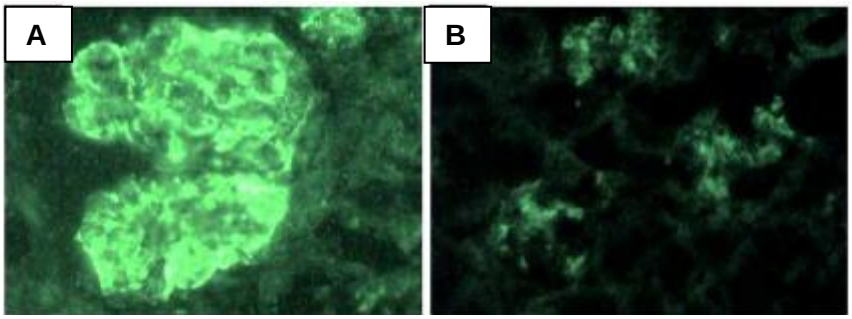

Figure 7.3 (**A**) Immunofluorescence IgG staining pattern in a 6-month-old untreated NZB/W mouse with moderate (++) mesangial deposits and intense (+++) glomerular basement membrane deposits. (**B**) Weak (+) mesangial IgG deposits in 6-month-old NZB/W mouse that was treated with CY for 3 consecutive months. Original magnification ×250 in panel B and ×400 in panel A.

and subepithelial deposits (more frequent). The sub-epithelial deposits were larger and more prominent. Food processes were relatively well preserved, but there was

Table 7.1. Renal histology and IgG immunofluorescence findings in NZB/W mice that were left untreated or were treated with glucocorticoids or cyclophosphamide for 3–6 months.

	Renal histology (LM)				IgG deposits (IF)	
NZB/W mice	**Normal**	**MMLN**	**FPLN**	**DPLN**	**<2+**	**≥2+**
3 months-old						
NT group	4 / 6	2 / 6	0 / 6	0 / 6	5 / 6	1 / 6
6 months-old						
NT group	0 / 9	0 / 9	6 / 9	3 / 9	0 / 5	5 / 5
GC group	0 / 5	5 / 5	0 / 5	0 / 5	4 / 5	1 / 5
CY group	2 / 4	2 / 4	0 / 4	0 / 4	4 / 4	0 / 4
9 months-old						
NT group	0 / 2	0 / 2	1 / 2	1 / 2	0 / 2	2 / 2
GC group	3 / 4	1 / 4	0 / 4	0 / 4	4 / 4	0 / 4
CY group	4 / 4	0 / 4	0 / 4	0 / 4	4 / 4	0 / 4

Abbreviations: LM, light microscopy; IF, immunofluorescence; Normal, normal LM findings; MMLN, mild mesangial hypercellularity; FPLN, focal proliferative lupus nephritis; DPLN, diffuse proliferative lupus nephritis; NT, no therapy; GC, corticosteroids therapy; CY, pulse cyclophosphamide therapy.

evidence of podocyte fusion first seen at this age in 2 out of 6. In all mice, at the age of 6 and 9 months there was a variable but at times extensive presence of electron dense deposits (EDD). Deposits were present in mesangial areas as well as within GBMs, also in both subendothelial and subepithelial locations. The expansion of the mesangium had caused occlusion of the capillary loops and fusion of podocytes that was very extensive. As we have previously demonstrated [198] there is evidence for podocytopathy, including effacement of podocyte FPs and destruction of slit diaphragm, in NZB/W mice with FPLN or DPLN. In view of the favorable effects of GC and CY on proteinuria and renal histology, we examined whether either therapy could preserve podocyte and slit diaphragm structure. In mice treated with GC or CY, the mesangium was segmentally increased with only few sub-endothelial EDD. Both

slit diaphragms and podocytes FPs were well preserved with rare effacements (**Figure 7.4** and **Table 7.2**).

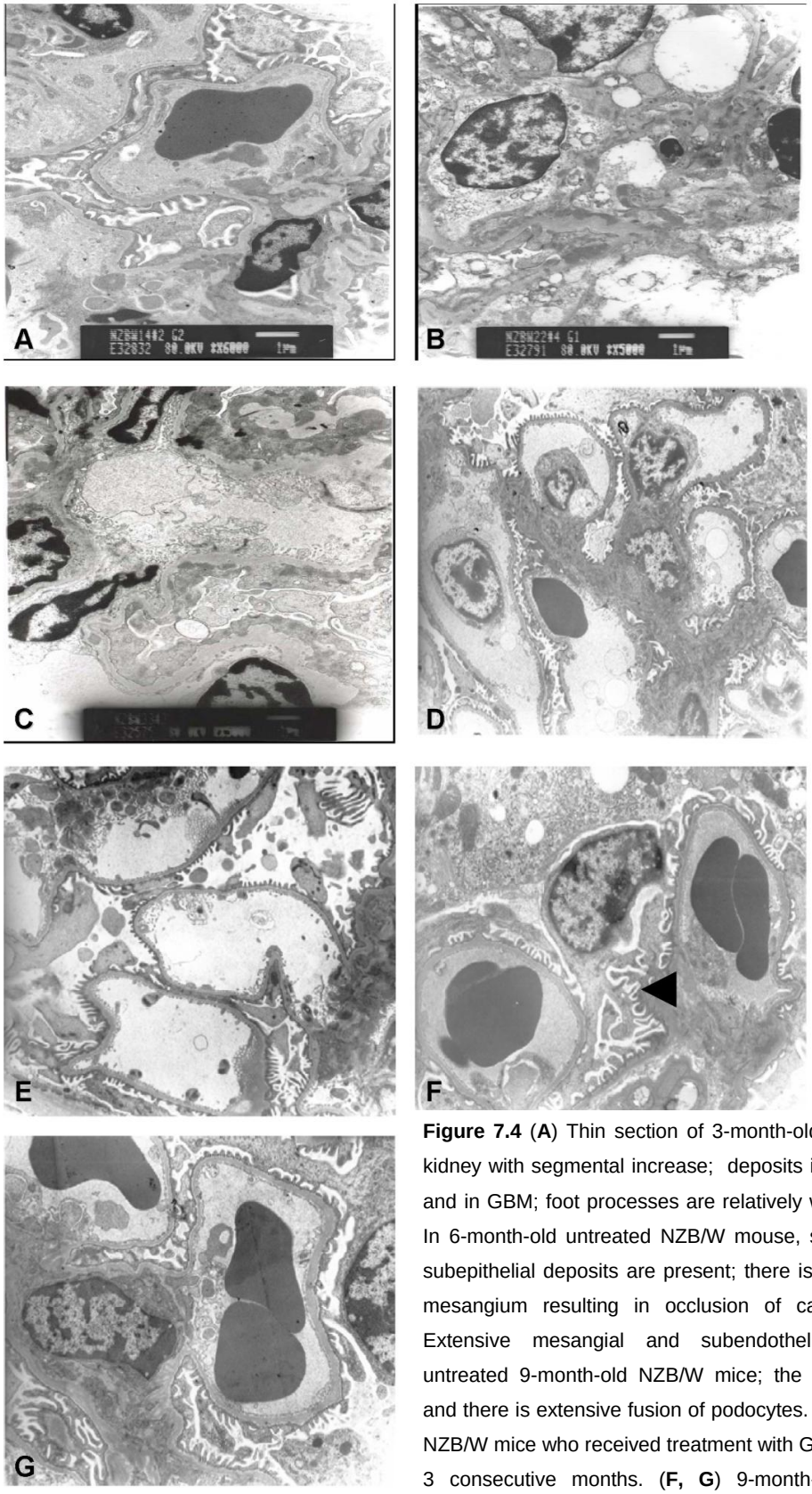

Figure 7.4 (**A**) Thin section of 3-month-old untreated mouse kidney with segmental increase; deposits in mesangial areas and in GBM; foot processes are relatively well preserved. (**B**) In 6-month-old untreated NZB/W mouse, subendothelial and subepithelial deposits are present; there is also expansion of mesangium resulting in occlusion of capillary loops. (**C**) Extensive mesangial and subendothelial deposition in untreated 9-month-old NZB/W mice; the GBM is thickened and there is extensive fusion of podocytes. (**D, E**) 6-month-old NZB/W mice who received treatment with GC (D) or CY (E) for 3 consecutive months. (**F, G**) 9-month-old NZB/W mice treated with GC (F) or CY (G) for 6 months. In panels D, E, F there is mesangial expansion with subendothelial deposits, whereas in panel G there are occasional mesangial deposits. Podocytes and slit diaphragms in all panels are well preserved (arrowhead). Magnification × 5000.

Table 7.2. Electron microscopy of kidney biopsies in NZB/W mice that were left untreated or were treated with glucocorticoids or cyclophosphamide for 3–6 months

	Mesangial EDD		Subendothelial EDD		Subepithelial EDD		Podocyte FP Effacement		
NZB/W mice	–/±	+/++	–/±	+/++	–/±	+/++	–	+	++
3 months-old									
NT group	0/2	2/2	2/2	0/2	2/2	0/2	0/2	2/2	0/2
6 months-old									
NT group	0/5	5/5	0/5	5/5	0/5	5/5	0/5	0/5	5/5
GC group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1
CY group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1
9 months-old									
NT group	0/2	2/2	0/2	2/2	0/2	2/2	0/2	0/2	2/2
GC group	1/1	0/1	0/1	1/1	1/1	0/1	1/1	0/1	0/1
CY group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1

Abbreviations: EDD, electron-dense deposits, FP, foot process, NT, no treatment, GC, glucocorticoids, CY, cyclophosphamide. Electron microscopy findings were annotated as follows: – = normal or absent, ± = rare, + = present, ++ = severe/extensive

C57BL/6 characteristics

C57BL/6 females a widely used control which exhibit neither autoimmune disorder nor glomerulonephritis was also studied. Results from preliminary experiments confirmed that all C57BL/6 of different ages (3 to 9 months) had the same phenotype and did not differ significantly in between for all tested variables except in proteinuria which was generally within the normal range reaching the levels of mild proteinuria sporadically. All C57BL/6 were anti ds DNA negative. Neither focal nor diffuse proliferative glomerulonephritis was observed in any of the C57BL/6. Large quantities

of IgG deposits were absent in all tested C57BL/6 mice. Eight out of 11, 2 out of 4 and 4 out of 5 aged 3, 6, 9 months respectively had normal LM findings. The remaining showed very rare segmental mesangial hyperplasia. Electron microscopy confirmed a normal renal tissue without mesangial expansion and deposits.

NZB and NZW characteristics

We studied 3 female NZB ≥ 6 months old and 3 male NZW 3 months old with mean proteinuria 5,2 ± 8,4 mg/24-h and 21 ± 5,7 mg/24-h respectively. LM showed normal histology except from one NZB mice with FPLN. IF was negative. EM showed subendothelial, mesangial deposits and effaced FPs, more extensive in NZB mice.

7.2 Increased nephrin protein levels in NZB/W treated animals.

We next assessed the effect of the treatment with GC or CY on glomerular expression of nephrin and podocin, which are major slit diaphragm components. Western blot (WB) analysis of kidney glomerular extracts showed increased nephrin levels in mice treated with either agent for 6 months compared to untreated (NT) littermates ($p<0.05$ for the GC *versus* NT and the CY *versus* NT pairwise comparisons) **(Figure 7.5)**. Glomerular nephrin expression showed negative association with the histological class of nephritis (ρ=-0.69, $p<0.001$), and positive association with podocin protein levels (ρ=0.53, $p=0.007$). Nephrin expression and localization was further assayed by IF in kidney sections from NZB/W mice. In 3-month-old mice with normal or MMLN LM histology there was intense nephrin staining, which was reduced in older untreated mice with FPLN or DPLN, and became diminished in 9-month-old diseased mice (**Figure 7.6A–C**).

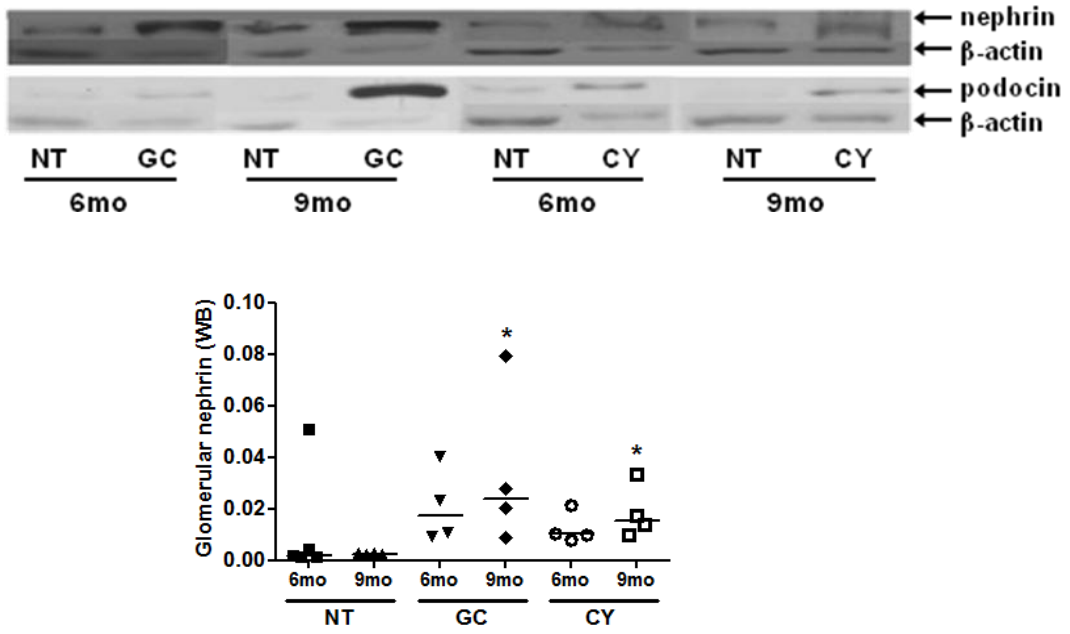

Figure 7.5 Glomerular expression of nephrin in NZB/W untreated and treated mice.
(**Top**) Western blot analysis of nephrin, podocin and *β*-actin in glomerular extracts of NZB/W control and treated mice. Nephrin and podocin band density was quantified following normalization with *β*-actin density. (**Bottom**) Nephrin is increased after 3 and 6 months therapy with GC and CY. Dots represent individual values, and bars represent medians. * p <0.05 for pairwise comparisons between age-matched treated and untreated mice. *Abbreviations*: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

In accordance with the WB results, nephrin IF staining was stronger in GC– or CY–treated mice than their untreated littermates **(Figure 7.6D)**. Nephrin IF score correlated with the nephritis histological class (ρ=0.55, p=0.008) and nephrin expression assayed by WB (ρ=0.71, p=0.004).

7.3 Podocin protein levels are increased in NZB/W treated mice.

Similar to nephrin treatment of NZB/W mice for 3–6 months resulted also in higher glomerular podocin protein levels compared to untreated aged-matched mice with FPLN or DPLN **(Figure 7.5(top), Figure 7.7)**. Again, podocin levels correlated inversely with the histological class of nephritis (ρ=-0.62, p=0.001).

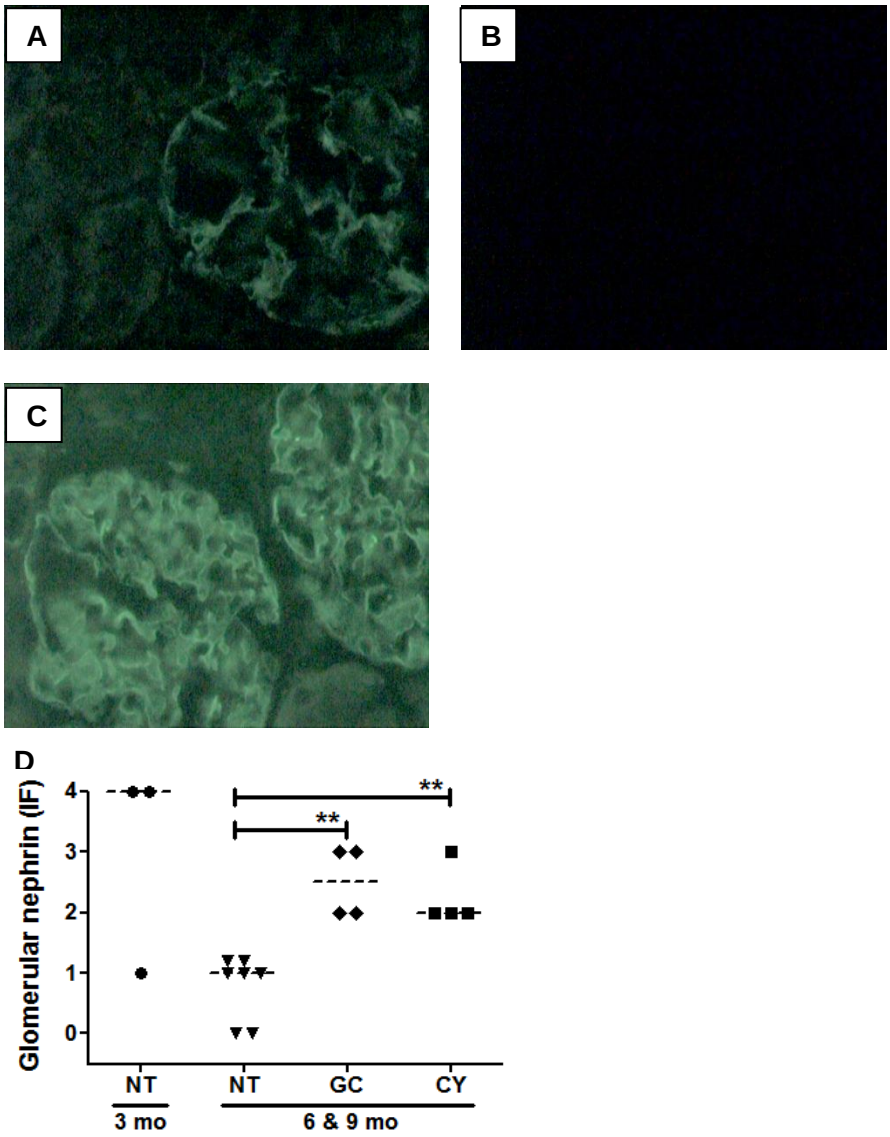

Figure 7.6 Immunofluorescence for nephrin expression in kidney biopsies of untreated and treated NZB/W mice.(**A**) Nephrin immunofluorescence indicating decreased deposition of nephrin in podocytes (+) in untreated 6-month-old NZB/W mice. (**B**) Absent nephrin immunofluorescence in 9-month-old untreated NZB/W mice. (**C**) Increased nephrin intensity (+++) in 6-month-old NZB/W mice treated with CY for 3 months. Original magnification ×400 in panels A-C. **(D)** Glomerular nephrin staining in immunofluorescence was normal in two out of three untreated 3-month-old NZB/W mice with minimal mesangial nephritis, and severely reduced or absent in all untreated 6- or 9-month-old mice with focal or proliferative nephritis. Nephrin immunofluorescence was enhanced in NZB/W mice that received GC and CY therapy and had normal or minimal mesangial nephritis histology. ** p <0.01 for pairwise comparisons between age-matched treated and untreated mice. *Abbreviations*: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

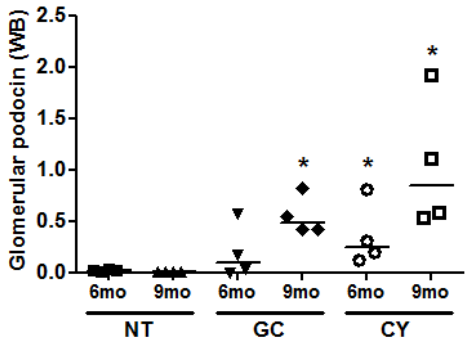

Figure 7.7 Glomerular expression of podocin in NZB/W untreated and treated mice.
Podocin protein expression is significantly increased after 6 months therapy with GC, and after 3-6 months therapy with CY. Dots represent individual values, and bars represent medians. * p <0.05 for pairwise comparisons between age-matched treated and untreated mice. *Abbreviations*: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

7.4 Nephrin and podocin mRNA levels are enhanced in treated animals.

To determine whether changes in nephrin and podocin expression in NZB/W treated mice correlate with altered transcription in corresponding genes, we performed quantitative real-time PCR in total RNA extracted from kidney glomeruli of treated and untreated mice. Glomerular nephrin mRNA was significantly increased after 3–6 of GC and CY therapy compared to agematched untreated littermates (**Figure 7.8A**).

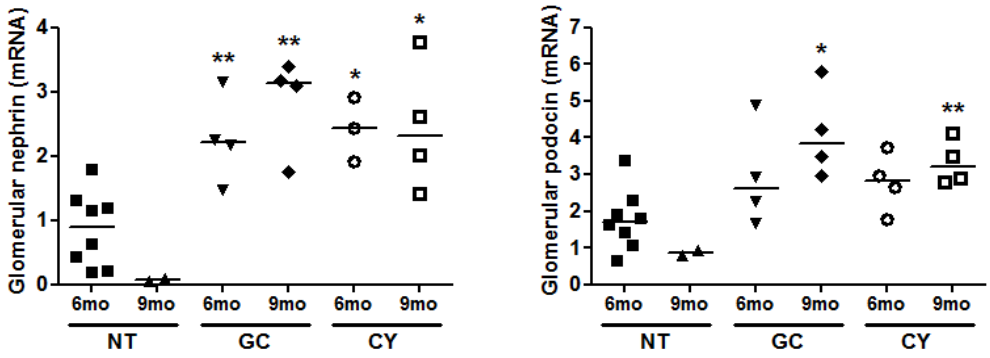

Figure 7.8 Glomerular nephrin and podocin mRNA in NZB/W untreated and treated mice. Nephrin mRNA (**A**) was significantly increased after 3 to 6 months therapy with either GC or CY. Podocin mRNA (**B**) levels were significantly enhanced only in treated with GC or CY mice for 6 consecutive months. Dots represent individual values, and bars represent medians. * p <0.05; ** p <0.01 for pairwise comparisons between age-matched treated and untreated mice. *Abbreviations*: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

NZB/W mice treated with either GC or CY had also higher podocin mRNA levels than untreated mice but differences were statistically significant only in mice treated for 6 months (ie, 9-months-old) (**Figure 7.8B**). We observed a significant correlation between nephrin and podocin mRNA both in untreated (ρ=0.67, p=0.005, *n* = 16) and in GC/CY-treated (ρ=0.72, p=0.002, *n* = 16) mice.

7.5 Slit diaphragm alterations in NZB/W treated mice correlate with histology.

Next, we addressed whether electron microscopy findings in kidney biopsies correlated with differential expression of nephrin and podocin of untreated and treated NZB/W mice aged 3–9 months. Indeed, mesangial, subendothelial, and

Table 7.3. Correlation between kidney electron microscopy findings and glomerular nephrin and podocin expression in NZB/W lupus mice

	Mesangial EDD [1]	Subendothelial EDD	Subepithelial EDD	Podocyte FP effacement [2]
Glomerular nephrin (protein)	ρ = -0.75 [3] (p = 0.013)	ρ = -0.85 (p = 0.002)	ρ = -0.79 (p = 0.007)	ρ = -0.77 (p = 0.009)
Glomerular podocin (protein)	ρ = -0.91 (p < 0.001)	ρ = -0.88 (p < 0.001)	ρ = -0.93 (p < 0.001)	ρ = -0.93 (p < 0.001)
Glomerular nephrin (mRNA)	ρ = -0.62 (p = 0.023)	ρ = -0.49 (p = 0.090)	ρ = -0.60 (p = 0.030)	ρ = -0.66 (p = 0.015)
Glomerular podocin (mRNA)	ρ = -0.37 (p = 0.210)	ρ = -0.30 (p = 0.318)	ρ = -0.35 (p = 0.240)	ρ = -0.44 (p = 0.134)

[1] EDD were scored as follows: 0 = absent, 1 = rare, 2 = present, 3 = extensive

[2] Podocyte FP effacement was scored as follows: 0 = no effacement, 1 = minimal effacement, 2 = effacement

[3] Spearman's rho coefficient

subepithelial EDD showed significant inverse correlation with glomerular nephrin (ρ correlation coefficient ranging from -0.75 to -0.85) and podocin (ρ ranging from -0.88 to -0.93) protein expression (**Table 7.3**). Similarly, podocyte FP effacement showed

negative association with both nephrin (ρ = -0.77, p = 0.009) and podocin (ρ= -0.93, p < 0.001). With respect to mRNA levels, EM findings of EDD (any type) and podocyte FP effacement showed also inverse association with nephrin – but not podocin – expression. Together, these results suggest a putative pathogenic link between expression of the slit diaphragm components nephrin and podocin and severity of kidney lesions and podocyte distortion in NZB/W LN.

7.6 Slit diaphragm proteins levels in NZB/W treated mice compared to their parental strains and normal controls.

We next sought to examine whether nephrin and podocin are increased up to normal levels. Indeed, nephrin and podocin protein expression of treated mice with either GC or CY for 3-6 months was not significantly different from that of C57Bl/6 mice that are normal. Also nephrin expression in treated animals was not different from that of their ancestors NZB and NZW which are not lupus-prone and exhibit minimal autoimmunity. Accordingly podocin expression in treated mice was slightly enhanced compared to NZB or NZW, but the difference was not statistically significant. Nephrin and podocin mRNA levels were found well preserved between GC- or CY-treated mice, the parental strains and the normal control mice (**Figure 7.9**).

Moreover, nephrin and podocin glomerular expression of NZB/W treated mice and their ancestors was increased compared to untreated mice with FPLN or DPLN, but not statistically signicantly. With respect to mRNA levels of nephrin and podocin, NZB/W treated animals exhibited a trend of enhanced transcription of the corresponding genes compared to NZB, NZW and untreated animals with FPLN or DPLN (**Figure 7.10**).

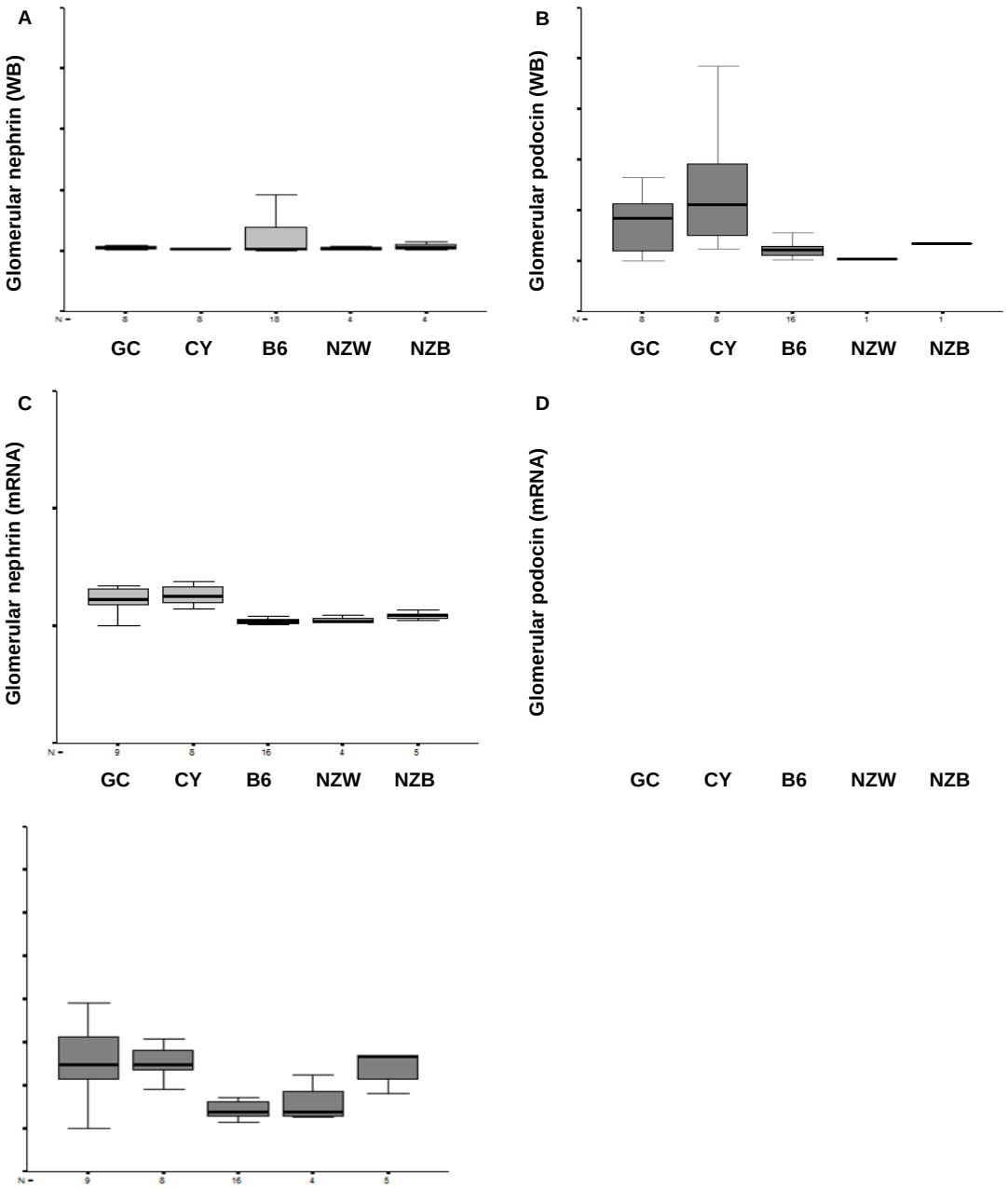

Figure 7.9 Glomerular expression and mRNA levels of nephrin and podocin in NZB/W treated mice, C57Bl/6, NZW and NZB. (**A**) Nephrin and (**B**) podocin expression as measured by western blot was not statistically different between treated with GC or CY mice and untreated normal control B6 and their parental strains NZW and NZB. Similarly (**C**) nephrin and (**D**) podocin mRNA levels were also the same for treated mice, normal controls and their parental strains. Data are expressed as means ± SEM. *Abbreviations*: GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice, B6, C57Bl/6 normal control mice, NZW, NZW mice, NZB, NZB mice.

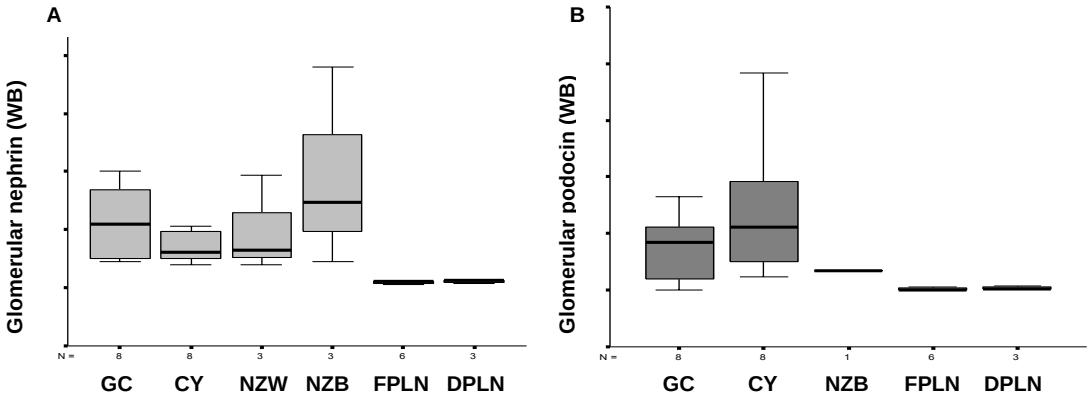

Figure 7.10 Glomerular expression and mRNA levels of nephrin and podocin in NZB/W untreated mice, NZB/W treated mice, NZW, NZB. (*Continued to next page*)

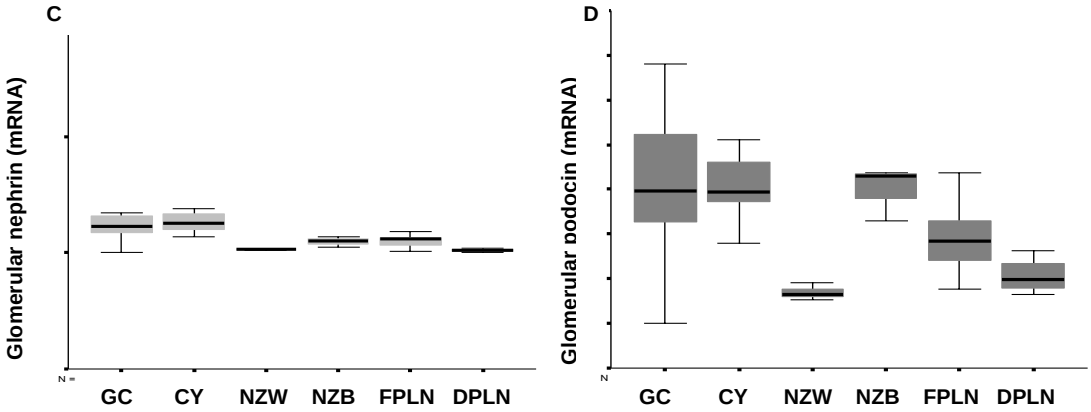

Figure 7.10 Glomerular expression and mRNA levels of nephrin and podocin in NZB/W untreated mice, NZB/W treated mice, NZW, NZB. (*Continued*) (**A**) Nephrin and (**B**) podocin expression as measured by western blot was not statistically different between treated with GC or CY mice and NZW and NZB, but increased compared to FPLN and DPLN. Similarly (**C**) nephrin and (**D**) podocin mRNA levels were also not different between treated mice and their parental strains, while FPLN and DPLN had not signicantly lessened mRNA levels. Data are expressed as means ± SEM. *Abbreviations*: GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice, NZW, NZW mice, NZB, NZB mice, FPLN, NZB/W untreated mice with focal proliferative lupus nephritis, DPLN, NZB/W untreated mice with diffuse proliferative lupus nephritis.

8. DISCUSSION

Podocytopathy is increasingly recognized as an important pathogenic aspect in acquired kidney diseases associated with significant proteinuria [205, 206]. We [198] and others [207-211] have previously demonstrated effacement of podocyte FPs, distorted slit diaphragm structure, and reduced nephrin and podocin expression correlating with advancing stages of lupus glomerulonephritis. Herein, we report that early treatment of murine NZB/W lupus with GC or CY prevents proteinuria, and this effect correlates with preserved podocyte structure and restored expression of the major slit diaphragm components, nephrin and podocin.

Loss of podocyte and slit diaphragm integrity may occur early in the course of glomerular diseases such as diabetic nephropathy and LN [198, 212, 213]. In murine lupus, podocyte abnormalities and reduction in glomerular nephrin expression are detected in young NZB/W mice with MMLN, before the appearance of significant proteinuria [198]. More specific, glomerular nephrin protein levels are reduced in mild LN and become diminished at more advanced stage, thus change in nephrin expression could precede ultra-structural changes in SD. Podocin protein levels are reduced only at proliferative disease stages, probably due to the fact that following podocyte injury and before proteinuria onset, podocin dissociates from nephrin, resulting in the urinary excretion of the latter [214].

To directly access the effects of treatment on podocytopathy -irrespective of the immunologic effects which ensue later in the course of the disease- we treated 3-month-old NZB/W mice with therapeutic doses of GC or CY, and found that administration of either agent for 3–6 months retained the levels of nephrin and podocin and preserved podocyte FPs. From a clinical point of view, persistent podocyte injury harbors great risk to severe and progressive glomerular damage that can ultimately lead to glomerulosclerosis and end-stage renal failure [215-217]. Accordingly, early identification of podocytopathy could allow for institution of pre-

emptive targeted therapies to improve long-term kidney outcomes. To this end, substantive efforts have been made [218-220].

We have also previously shown [221] that the parental strains of NZB/W mice (i.e female NZB and male NZW) exhibit immune mediated glomerular alterations early in their life with proteinuria being the prevalent clinical manifestation. Proteinuria may be the result of FP effacement which is documented on EM, despite the fact that LM findings are normal. We gave evidence that the ultrastructural alterations regarding reduced SD proteins in the NZB/W ancestors might be a co-factor contributing to NZB/W predisposition to renal disease. NZB nephritis and the probability that NZW might be more susceptible to immune mediated renal damage have also been reported by others [193, 222, 223]. Our findings regarding NZW nephritis demonstrate immune mediated nephritis, that is indistinguishable to NZB/W and NZB nephritis, in the previously regarded healthy NZW as has others have shown as well [224]. The trend of slightly increased nephrin and podocin expression in treated mice compared to NZB and NZW supports the previous findings. The favorable effects of treatment on podocytopathy are also supported by the fact that SD protein expression and their genes' transcription in treated animals are almost the same with the ones of normal control mice.

In our study serum anti-dsDNA levels were not different between treated and untreated lupus-prone mice. This could be due to the fact that anti-dsDNA antibodies by themselves cannot predict the severity of the disease. The ability of an anti-dsDNA antibody to induce nephritic change depends on the affinity to renal antigens, site of localization of the immune-complex deposits, complement-fixing ability and amount of antibody [225]. Other researchers used several antibodies to monitor disease activity (e.g. anti-C1q, especially in proliferative LN, or anti-nucleosome) [226, 227].

The exact mechanisms through which podocytopathy develops in LN remain elusive. There is evidence for immune-mediated podocyte damage that involves kidney-

infiltrating cytotoxic CD8+ T cells [228], suppression of nephrin gene transcription by macrophages associated cytokines [229, 230], and cross-reaction of anti-dsDNA antibodies with podocyte proteins like α-actinin-4 [231]. Notably, podocytes can produce inflammatory mediators and upregulate the costimulatory molecule B7-1 (CD80) via toll-like receptor (TLR)–3 and –4 signaling [208, 232]. Other researchers have shown induction of TLR-9 expression in renal podocytes in childhood-onset LN, associated with reduced nephrin and podocin expression [233]. TLR-9 may respond to DNA containing immune complexes, which promote podocyte injury. Accordingly, broad immunosuppressive agents such as GC and CY, routinely used in the treatment of LN, may exert antiproteinuric effects -at least in part- through downregulation of the immune activity directed against podocytes.

Intriguingly, recent evidence shows that immunosuppressive agents, in addition to the effect on the immune system, may directly influence the unique structure and function of podocytes. Thus, glucocorticoids can increase the stability of actin filaments, increase actin polymerization, and activate cytoskeleton-associated kinases in podocytes, suppress IL-6 and upregulate nephrin expression [178-180]. Podocytes are also a direct target of the calcineurin inhibitor cyclosporine A (CsA), independent of NFAT inhibition in T cells [234]. Other immunosuppressive agents, such as mizoribine, an immunosuppressant that has been used in renal transplantation, vasculitis, and LN, with known antiproteinuric effects, has been shown to restore post-translational processing of nephrin by improving intracellular energy balance, thereby normalizing nephrin trafficking [235]. More recently, rituximab (anti-CD20 mAb) was shown to prevent recurrent focal segmental glomerulosclerosis by modulating podocyte function in a sphingomyelin phosphodiesterase acid-like 3b-dependent manner [236]. Our data corroborate earlier studies in patients with proliferative LN whereby CY more than azathioprine reduced the number of urinary podocytes implying an amelioration of podocyte injury [237].

Together, these data suggest that the podocyte-preserving outcome of GC and CY therapy may be mediated – at least in part – by a direct effect on podocytes independently of the immunosuppressive effects since podocytopathy was present prior to immune reactivity and immunosuppressive treatment suppressed immune reactivity.

However, alternative experimental approaches employing transgenic mouse models would be required to directly dissect immune from non-immune effects of GC and CY on podocytes in lupus.

In conclusion, our data demonstrate that early treatment of murine LN with GC or CY prevents the development of proteinuria and proliferative nephritis, in association with preserved podocyte structure and increased glomerular nephrin and podocin expression. These findings further emphasize the role of podocytes and the slit diaphragm in the pathogenesis of immune-mediated glomerulonephritis, and point toward the potential benefits of development of selective, antiproteinuric, and podocyte-protective drugs [238].

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Early treatment with glucocorticoids or cyclophosphamide retains the slit diaphragm proteins nephrin and podocin in experimental lupus nephritis

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Abstract:	Renal podocytes and their slit diaphragms ensure the integrity of renal basement membrane and prevent urinary protein loss. We have previously reported that decreases of the podocyte slit diaphragm proteins nephrin and podocin represent early events in the podocytopathy of lupus nephritis. We asked whether immunosuppressive agents such as glucocorticoids and cyclophosphamide, may have direct effects on podocytes. We assessed in New Zealand Black / New Zealand White F1 lupus nephritis mice glomerular nephrin and podocin expression and localization by the use of western blot and immunofluorescence; mRNA levels were measured by real-time PCR and renal histology by light and electron microscopy. Early treatment with glucocorticoids and cyclophosphamide halted the histologic alterations associated with lupus nephritis, preserving podocyte foot processes. Nephrin and podocin protein expression significantly increased in both glucocorticoid and cyclophosphamide groups as early as after 3 months of therapy. Real-time PCR revealed similar enhancement in nephrin and podocin mRNA levels after 3-6 months of treatment. This study documents that early treatment in experimental lupus nephritis with glucocorticoids or cyclophosphamide preserves slit diaphragm proteins in podocytes and halts histological changes of the glomeruli, thus raising the possibility of a direct protective effect of these drugs on podocytes.

Early treatment with glucocorticoids or cyclophosphamide retains the slit diaphragm proteins nephrin and podocin in experimental lupus nephritis: Evidence for a direct effect on podocytes

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ABSTRACT

Renal podocytes and their slit diaphragms ensure the integrity of the renal basement membrane and prevents urinary protein loss. Recently, we have reported that decreases of the main podocyte slit diaphragm (SD) proteins, nephrin and podocin, are an early event in podocytopathy in lupus nephritis preceding overt immunological activity. We asked whether immunosuppressive agents such as corticosteroids and cyclophosphamide, may have direct effects on podocytes independent of their immunosuppressive effects. To this end, we accessed in NZB/W LN mice glomerular nephrin and podocin expression and localization by the use of western blot and immunofluorescence; mRNA levels were measured by real-time PCR and renal histology by light and electron microscopy. Early treatment with corticosteroids and cyclophoshamide halted the histologic alterations associated with nephritis preserving podocyte foot processes (FP). Nephrin expression was significantly increased in both corticosteroids and cyclophosphamide groups as early as after 3 months of therapy. Podocin expression was increased as early as 3 months reaching maximum expression after 6 months of therapy. Real-time PCR revealed similar substantial enhancement in nephrin and podocin mRNA levels after 6 months of treatment. This study documents that early treatment in experimental LN preserves SD proteins in podocytes and halts histological changes of the glomeruli and raises the possibility of a direct protective effect of immunosuppressive drugs on podocytes.

Key words. Lupus nephritis treatment, podocytes, nephrin, podocin.

INTRODUCTION

One or more forms of glomerulonephritis develop in 40–60% of patients with systemic lupus erythematosus (SLE), and contribute to significant morbidity and mortality. Despite advances in the treatment of SLE, still a considerable proportion (10–30%) of patients with lupus nephritis (LN) will progress to end-stage renal disease.[1,2]

Female New Zealand Black (NZB)/New Zealand White (NZW) F1 (NZB/W) hybrid mice spontaneously develop lupus-like autoimmune disease and are an invaluable tool in exploring SLE etiopathogenesis.[3,4] Similar to humans, the hallmark of the disease in NZB/W is formation of autoantibodies against multiple epitopes of chromatin and double-stranded (ds)-DNA molecules.[5,6] These autoantibodies are deposited in the kidneys starting at 5 months of age, and cause proliferative glomerulonephritis which results to progressive renal failure by the age of 9 months.[4] In addition to autoantibody deposition, signals from the innate and the adaptive immune system are important for the initiation and maintenance of renal damage.[3,7]

It is increasingly recognized that renal podocyte damage (podocytopathy) may contribute to pathogenesis and progression of proteinuric renal diseases.[8-10] Podocytes, the visceral glomerular epithelial cells, are terminally differentiated cells with primary and secondary interdigitating branches, named foot processes (FPs). Ultrafiltration in the glomerulus is accomplished by a fenestrated endothelial layer, the glomerular basement membrane, and the overlying podocytes. The latter form the final barrier to protein loss. FPs of adjacent podocytes are connected by a continuous membrane-like structure called the slit diaphragm.[11-13] When podocytes are injured, the slit diaphragm and cytoskeletal structure of the FPs are altered resulting in effacement of the podocyte, thus disrupting the filtration barrier and leading to proteinuria.[12,14]

Major components of the slit diaphragm are nephrin, a trans-membrane protein with immunoglobulin-like domains, and podocin, which interacts with the cytoplasmic tail of nephrin. Mutations in the genes encoding for these proteins, *NPHS1* and *NPHS2* respectively, are implicated in familial cases of nephrotic syndromes.[15-18] Accordingly, disruption of the slit

diaphragm structure in nephrin-deficient mice results in loss of glomerular permselectivity and gross proteinuria.[19] Further studies have shown that nephrin and podocin mRNA and protein expression are reduced in acquired human kidney diseases, including LN.[20-25]

In view of these findings, therapeutic strategies to protect podocytes have been pursued by several groups.[26] Although *in vitro* studies to suggest direct effect of glucocorticoids and other immunosuppressive drugs on podocytes through regulation of slit diaphragm components and intracellular signaling pathways relevant for cytoskeletal stability, cell maturation, and survival[27], yet, there is paucity of *in vivo* data to demonstrate the effect of the aforementioned therapies on glomerular podocytes.

We have recently shown reduced expression of the slit diaphragm proteins nephrin and podocin early during the course of LN in NZB/W mice, when podocytes structure is still unaffected and immune activity is low.[10] In these mice, glucocorticoids (GC) and cyclophosphamide (CY) are well-established therapies that can delay the clinical and histologic progression of renal disease primarily by exerting potent anti-inflammatory properties. To this end, we asked whether the favorable effects of these treatments are accompanied by changes in glomerular podocyte structure and slit diaphragm components. To this end, NZB/W mice were treated with GC or CY starting at early stages of renal disease. Our results suggest that treatment of murine lupus nephritis with GC and CY may exert significant antiproteinuric effects associated with improved podocyte structure and preserved expression of the main slit diaphragm components, nephrin and podocin.

MATERIALS AND METHODS

Animal studies

Female NZB/W mice were obtained from Harlan, UK. Animal care and treatment were conducted in accordance with the guidelines for the care and use of laboratory animals, approved by the University Of Crete School Of Medicine. Mice were housed in constant

temperature with a 12-hr dark/12-hr light cycle. They had free access to tap water and standard mouse chow throughout the study and were euthanatized by CO_2. We have previously demonstrated that the expression of slit diaphragm proteins is reduced in NZB/W mice starting at the age of 3 months, and this reduction precedes the appearance of microscopic podocyte abnormalities and severe proteinuria.[10] We therefore used 3-month-old NZB/W mice, which were randomly assigned into one of the following groups: a) untreated (control) mice (n = 24), b) GC–treated mice, which received dexamethasone (Soldesanil or.dr.sol., Diapit; 0.4 mg/kg/day p.o. in drinking water) for 3 (n = 4) or 6 (n = 5) consecutive months, and c) CY–treated mice, which received CY (50mg/kg intraperitoneally every 10 days) (Endoxan pd.inj.sol., Baxter) for 3 (n = 4) or 6 (n = 4) consecutive months.[28,29]

Assessment of proteinuria

Mice were placed in metabolic cages (Tecniplast) for 24-hr urine collections the day before they were euthanized. Urine samples were assayed for total protein using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). Clinically significant proteinuria was defined as urine protein excretion >4 mg/24-hr.[30]

Determination of serum anti-dsDNA

Serum anti-dsDNA autoantibodies were detected by enzyme immunoassay (mouse anti-dsDNA IgG Total ELISA kit; ADI V3-5100) according to manufacturer's instructions. Briefly serum samples, diluted 1:100, were plated for 30 minutes in antigen coated 96-well strip plates. HRP–labeled goat anti-mouse IgG conjugated antibody was added to the wells and incubated for 30 minutes. TMB solution was used and plates were read at 450nm in a Model 680 Microplate Reader.

Renal histology

Light microscopy (LM)

Mouse kidney tissue was fixed in 10% buffered formalin, dehydrated in alcohol, and embedded in paraffin for standard LM. Active LN lesions (mesangial expansion, endocapillary proliferation, glomerular deposits, extra-capillary proliferation, interstitial infiltrates) and chronic lesions (tubular atrophy, interstitial fibrosis) were evaluated. At least 30 randomly selected glomeruli from each animal were examined. Slides were scored by an experienced renal pathologist (L.N.) masked to the group. The 2003 International Society of Nephrology / Renal Pathology Society (ISN/RPS) classification was used.[31]

Immunofluorescence (IF)

Kidney tissue was snap frozen by liquid nitrogen in OCT compound and stored at -80°C. Cryosections (5μm) were fixed in acetone at -80°C and were blocked with 20% normal goat serum before incubation with antibodies against mouse IgG (1:50) and nephrin (1:100). Secondary FITC-conjugated anti-mouse antibody (1:20) was used and slides were examined in Nikon eclipse 80i microscope. At least 15 glomeruli per animal were and glomerular IgG staining was semiquantified as follows: 0, no signal; 1+, mild; 2+, moderate; 3+, strong signal intensity. Nephrin expression was similarly scored: 0, negative; 1+, very weak; 2+, mild; 3+, moderate; 4+, strongly positive.

Electron microscopy (EM)

Specimens were fixed in 2.5% glutaraldehyde, post-fixed 1% osmium tetroxide, dehydrated in ethanol, cleared in propylene oxide and embedded in Epon/Araldite mixture. Ultrathin sections were stained with uranyl acetate/lead citrate and examined in JEM-1010 transmission electron microscope by a blinded cell biologist (K.K.). Assessment was based on the evaluation of electron micrographs representing ≥20 glomeruli from each group of mice. The presence of mesangial, subendothelial and supepithelial electron dense deposits (EDD) was

evaluated in a semi-quantitative way: 0, negative; 1+, frequent; 2+, extensive. Podocyte FP effacement was characterized as 0, negative; 1+, rare; 2+, extensive.

Glomerular isolation

Glomeruli were enriched by differential sieving on ice as described elsewhere.[32] Kidney tissue was minced and passed through 90μm and 50μm nylon sieves (Nitex, SefarInc.). Glomeruli were lysed in RIPA buffer with protease inhibitors[33], centrifuged at 4°C at 14,000 g for 15 minutes, and the supernatant was stored at -80°C.

Western blot (WB) analysis

Glomerular protein extracts were separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with the indicated primary antibody (1:500). After washes, membranes were incubated with HRP-conjugated IgG (1:10000) and blots were developed using enhanced chemiluminescence (Amersham RNP 2209). Nephrin and podocin band density were normalized to actin expression (Tinascan).

RNA Isolation, cDNA preparation, and real-time PCR

Total RNA was isolated after kidney tissue homogenization by Trizol Reagent (Invitrogen) according to the manufacturer's instructions. One μg RNA was used to prepare first strand cDNA by AMV Reverse Transcriptase (Promega) in 20μl total reaction volume. Real-time PCR was performed in the ABI Prism 7700 Sequence Detection System using BioRad SYBR-Green Super mix. All measurements were performed in duplicate. The primers used were: *GAPDH*, forward 5′- AATGTGTCCGTCGTGGATCTGA -3′ and reverse 5′-GATGCCTGCTTCACCACCTTCT -3′, *nephrin*, forward 5′-ACACAAGAAGCTCCACGGTTAG-3′ and reverse 5′-TGGCGATATGACACCTCTTCC-3′, *podocin*, forward 5'-GTGTCCAAAGCCATCCAGTT-3' and reverse 5'-GGCAACCTTTACATCTTGGG-3'.

Antibodies

Anti-mouse IgG (H+L) (BA 2000, Vector Lab.) and FITC-conjugated streptavidin (F72, Biomeda) were used for mouse IF. A polyclonal rabbit anti-nephrin antibody directed against the cytoplasmic domain of mouse nephrin was kindly provided from Dr. L. B. Holzman[17] for the WB and IF studies. FITC-conjugated polyclonal swine anti-rabbit IgG (F0205, DAKO) was used as secondary antibody for nephrin IF. Podocin was detected in western blot by a polyclonal rabbit anti-podocin antibody (ab50339, Abcam) and actin by pan-actin monoclonal antibody (MAB 1501, Chemicon) (1:3000). HRP-conjugated mouse anti-rabbit and HRP-conjugated mouse anti-mouse were obtained from Cell Signalling (7074 and 7076, respectively) (both used at 1:3000).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) values. Comparisons were performed with the Mann-Whitney *U* test for numerical data. The Spearman's rho (ρ) test was used for correlation analysis. All analyses were done with SPSS 16.0 (SPSS, Inc.) and *p*-values (two-tailed) <0.05 were considered as statistically significant.

RESULTS

Treatment of young NZB/W lupus-prone mice with glucocorticoids or cyclophosphamide prevents the development of proteinuria, kidney inflammation, and glomerular IgG deposits

In accordance with previous studies[4], untreated NZB/W mice developed overt proteinuria by the age of 6 months (33.4 ± 28.1 mg/24-hr), which was further increased at 9 months of age (46.3 ± 13.3 mg/24-hr) (**Figure 1A**). Increased anti-dsDNA titers were detected in sera of all

mice aged 6–9 months (*data not shown*). Mice treated with GC had reduced proteinuria at both 6 and 9 months of age (17.3 ± 13.9 mg/24-hr and 7.4 ± 4.6 mg/24-hr, respectively). CY–treated mice also had significantly reduced proteinuria levels (13.9 ± 5.2 mg/24-hr at 6 months, 2.2 ± 0.5 mg/24-hr at 9 months) compared to their untreated littermates, and comparable to the levels of 3-month-old mice (5.8 ± 1.2 mg/24-hr).

We next examined the effect of therapy with GC or CY on renal histology in NZB/W lupus mice. **Table 1** shows the results of LM and IF for IgG deposits on kidney biopsies of untreated and treated mice. Three-month-old NZB/W mice demonstrated normal histology or findings of mild mesangial LN (MMLN). In contrast, all untreated 6- and 9-month-old mice developed focal or diffuse proliferative glomerulonephritis (FPLN and DPLN, respectively) associated with numerous IgG deposits. In GC– and CY–treated mice, glomerulonephritis was milder with markedly reduced interstitial inflammation and mesangial hyperplasia; seven out of eight (7/8) NZB/W mice treated for 6 months had normal LM histology (**Figure 1B–E**). Accordingly, IgG kidney deposition was significantly reduced in treated mice being undetectable in mice treated with CY for 6 months (**Table 1** and **Figure 1F–G**).

Treatment of young NZB/W lupus-prone mice with glucocorticoids or cyclophosphamide preserves podocytes and slit diaphragm structure

We have previously demonstrated evidence for podocytopathy, including effacement of podocyte FPs and destruction of slit diaphragm, in NZB/W mice with FPLN or DPLN.[10] In view of the favorable effects of GC and CY on proteinuria and renal histology, we examined whether either therapy could preserve podocyte and slit diaphragm structure. EM examination of kidney sections from untreated 6- and 9-month-old mice with FPLN or DPLN revealed extensive mesangial, subendothelial, and subepithelial EDD that resulted in occlusion of capillary loops, fusion of podocytes and effacement of FPs, and destruction of slit diaphragms (**Figure 2A–C**). Conversely, in mice treated with GC or CY, the mesangium was segmentally

increased with only few sub-endothelial EDD. Both slit diaphragms and podocytes FPs were well preserved with rare effacements (**Figure 2D–G** and **Table 2**).

Preserved glomerular nephrin and podocin protein expression in NZB/W mice treated with glucocorticoids or cyclophosphamide

We next assessed the effect of these treatments on glomerular expression of nephrin and podocin, which are major slit diaphragm components. Western blot (WB) analysis of kidney glomerular extracts showed increased nephrin levels in mice treated with either agent for 6 months compared to untreated (NT) littermates ($p<0.05$ for the GC *versus* NT and the CY *versus* NT pairwise comparisons) **(Figure 3A–B)**. Glomerular nephrin expression showed negative association with the histological class of nephritis (ρ=-0.69, $p<0.001$), and positive association with podocin protein levels (ρ=0.53, p=0.007) (*data not shown*). Treatment of NZB/W mice for 3–6 months resulted also in higher glomerular podocin protein levels compared to untreated aged-matched mice with FPLN or DPLN **(Figure 3A, C)**. Similar to nephrin, podocin levels correlated inversely with the histological class of nephritis (ρ=-0.62, p=0.001) (*data not shown*).

Nephrin expression and localization was further assayed by IF in kidney sections from NZB/W mice. In 3-month-old mice with normal or MMLN LM histology there was intense nephrin staining, which was reduced in older untreated mice with FPLN or DPLN, and became diminished in 9-month-old diseased mice (Figure 4A–C). In accordance with the WB results, nephrin IF staining was stronger in GC– or CY–treated mice than their untreated littermates **(Figure 4D)**. Nephrin IF score correlated with the nephritis histological class (ρ=0.55, p=0.008) and nephrin expression assayed by WB (ρ=0.71, p=0.004) (*data not shown*).

Nephrin and podocin mRNA levels are increased in NZB/W mice treated with glucocorticoids or cyclophosphamide

To determine whether changes in nephrin and podocin expression in NZB/W treated mice correlate with altered transcription in corresponding genes, we performed quantitative real-time PCR in total RNA extracted from kidney glomeruli of treated and untreated mice. Glomerular nephrin mRNA was significantly increased after 3–6 of GC and CY therapy compared to age-matched untreated littermates (**Figure 3D**). NZB/W mice treated with either GC or CY had also higher podocin mRNA levels than untreated mice but differences were statistically significant only in mice treated for 6 months (ie, 9-months-old) (**Figure 3E**). We observed a significant correlation between nephrin and podocin mRNA both in untreated (ρ=0.67, p=0.005, n = 16) and in GC/CY-treated (ρ=0.72, p=0.002, n = 16) mice (*data not shown*).

Reduced electron dense kidney deposits and preserved podocyte structure correlate with higher glomerular nephrin and podocin levels in NZB/W mice

Next, we addressed whether electron microscopy findings in kidney biopsies correlated with differential expression of nephrin and podocin of untreated and treated NZB/W mice aged 3–9 months. Indeed, mesangial, subendothelial, and subepithelial EDD showed significant inverse correlation with glomerular nephrin (ρ correlation coefficient ranging from -0.75 to -0.85) and podocin (ρ ranging from -0.88 to -0.93) protein expression (**Table 3**). Similarly, podocyte FP effacement showed negative association with both nephrin (ρ = -0.77, p = 0.009) and podocin (ρ = -0.93, p < 0.001). With respect to mRNA levels, EM findings of EDD (any type) and podocyte FP effacement showed also inverse association with nephrin – but not podocin – expression. Together, these results suggest a putative pathogenic link between expression of the slit diaphragm components nephrin and podocin and severity of kidney lesions and podocyte distortion in NZB/W lupus nephritis.

DISCUSSION

Podocytopathy is increasingly recognized as an important pathogenic aspect in acquired kidney diseases associated with significant proteinuria.[11, 12] We [10] and others [8, 9, 21-23] have previously demonstrated effacement of podocyte FPs, distorted slit diaphragm structure, and reduced nephrin and podocin expression correlating with advancing stages of lupus glomerulonephritis. Herein, we report that early treatment of murine NZB/W lupus with GC or CY prevents proteinuria, and this effect correlates with preserved podocyte structure and restored expression of the major slit diaphragm components, nephrin and podocin.

Loss of podocyte and slit diaphragm integrity may occur early in the course of glomerular diseases such as diabetic nephropathy and LN.[10, 34, 35] In murine lupus, podocyte abnormalities and reduction in glomerular nephrin expression are detected in young NZB/W mice with MMLN, before the appearance of significant proteinuria.[10] To directly access the effects of treatment on podocytopathy-irrespective of the immunologic effects which ensue later in the course of the disease- we treated 3-month-old NZB/W mice with therapeutic doses of GC or CY, and found that administration of either agent for 3–6 months retained the levels of nephrin and podocin and preserved podocyte FPs. From a clinical point of view, persistent podocyte injury harbors great risk to severe and progressive glomerular damage that can ultimately lead to glomerulosclerosis and end-stage renal failure.[36-38] Accordingly, early identification of podocytopathy could allow for institution of pre-emptive targeted therapies to improve long-term kidney outcomes. To this end, substantive efforts have been made.[39-41]

The exact mechanisms through which podocytopathy develops in LN remain elusive. There is evidence for immune-mediated podocyte damage that involves kidney-infiltrating cytotoxic $CD8^+$ T cells[42], suppression of nephrin gene transcription by macrophages and their associated cytokines[43, 44], and cross-reaction of anti-dsDNA antibodies with podocyte proteins like α-actinin-4.[45] Notably, podocytes can produce inflammatory mediators and upregulate the co-stimulatory molecule B7-1 (CD80) via toll-like receptor (TLR)–3 and –4 signaling.[9, 46] Other researchers have shown induction of TLR-9 expression in renal podocytes in

childhood-onset LN, associated with reduced nephrin and podocin expression.[47] TLR-9 may respond to DNA-containing immune complexes, which promote podocyte injury. Accordingly, broad immunosuppressive agents such as GC and CY, routinely used in the treatment of LN, may exert antiproteinuric effects -at least in part- through downregulation of the immune activity directed against podocytes.

Intriguingly, recent evidence shows that immunosuppressive agents, in addition to the effect on the immune system, may directly influence the unique structure and function of podocytes. Thus, glucocorticoid receptors are present on podocytes and translocate to the nucleus upon dexamethasone treatment.[48] Glucocorticoids can increase the stability of actin filaments, increase actin polymerization, and activate cytoskeleton-associated kinases in podocytes, suppress IL-6 and upregulate nephrin expression.[48-50] Podocytes are also a direct target of the calcineurin inhibitor cyclosporine A (CsA). The antiproteinuric effect of CsA may result from the stabilization of synaptopodin in podocytes, independent of NFAT inhibition in T cells.[51] More recently, rituximab (anti-CD20 mAb) was shown to prevent recurrent focal segmental glomerulosclerosis (FSGS) by modulating podocyte function in a sphingomyelin phosphodiesterase acid-like 3b-dependent manner.[53] Our data corroborate earlier studies in patients with proliferative lupus nephritis whereby cyclophoshamide more than azathioprine reduced the number of urinary podocytes implying a amelioration of podocyte injury.[54] However, alternative experimental approaches employing transgenic mouse models would be required to directly dissect immune from non-immune effects of GC and CY on podocytes in lupus.

In conclusion, our data demonstrate that early treatment of murine LN with GC or CY prevents the development of proteinuria and proliferative nephritis, in association with preserved podocyte structure and increased glomerular nephrin and podocin expression. These findings further emphasize the role of podocytes and the slit diaphragm in the pathogenesis of immune-mediated glomerulonephritis, and point toward the potential benefits of development of selective, antiproteinuric, and podocyte-protective drugs.[55]

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TABLES

Table 1. Renal histology and IgG immunofluorescence findings in NZB/W mice that were left untreated or were treated with glucocorticoids or cyclophosphamide for 3–6 months

	Renal histology (LM)				IgG deposits (IF)	
NZB/W mice	**Normal**	**MMLN**	**FPLN**	**DPLN**	**<2+**	**≥2+**
3 months-old						
NT group	4 / 6	2 / 6	0 / 6	0 / 6	5 / 6	1 / 6
6 months-old						
NT group	0 / 9	0 / 9	6 / 9	3 / 9	0 / 5	5 / 5
GC group	0 / 5	5 / 5	0 / 5	0 / 5	4 / 5	1 / 5
CY group	2 / 4	2 / 4	0 / 4	0 / 4	4 / 4	0 / 4
9 months-old						
NT group	0 / 2	0 / 2	1 / 2	1 / 2	0 / 2	2 / 2
GC group	3 / 4	1 / 4	0 / 4	0 / 4	4 / 4	0 / 4
CY group	4 / 4	0 / 4	0 / 4	0 / 4	4 / 4	0 / 4

Abbreviations: LM, light microscopy; IF, immunofluorescence; Normal, normal LM findings; MMLN, mild mesangial hypercellularity; FPLN, focal proliferative lupus nephritis; DPLN, diffuse proliferative lupus nephritis; NT, no therapy; GC, corticosteroids therapy; CY, pulse cyclophosphamide therapy.

Table 2. Electron microscopy of kidney biopsies in NZB/W mice that were left untreated or were treated with glucocorticoids or cyclophosphamide for 3–6 months

	Mesangial EDD		Subendothelial EDD		Subepithelial EDD		Podocyte FP Effacement		
NZB/W mice	–/±	+/++	–/±	+/++	–/±	+/++	–	+	++
3 months-old									
NT group	0/2	2/2	2/2	0/2	2/2	0/2	0/2	2/2	0/2
6 months-old									
NT group	0/5	5/5	0/5	5/5	0/5	5/5	0/5	0/5	5/5
GC group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1
CY group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1
9 months-old									
NT group	0/2	2/2	0/2	2/2	0/2	2/2	0/2	0/2	2/2
GC group	1/1	0/1	0/1	1/1	1/1	0/1	1/1	0/1	0/1
CY group	1/1	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1

Abbreviations: EDD, electron-dense deposits, FP, foot process, NT, no treatment, GC, glucocorticoids, CY, cyclophosphamide. Electron microscopy findings were annotated as follows: – = normal or absent, ± = rare, + = present, ++ = severe/extensive

Table 3. Correlation between kidney electron microscopy findings and glomerular nephrin and podocin expression in NZB/W lupus mice

	Mesangial EDD [1]	Subendothelial EDD	Subepithelial EDD	Podocyte FP effacement [2]
Glomerular nephrin (protein)	ρ = -0.75 [3] (p = 0.013)	ρ = -0.85 (p = 0.002)	ρ = -0.79 (p = 0.007)	ρ = -0.77 (p = 0.009)
Glomerular podocin (protein)	ρ = -0.91 (p < 0.001)	ρ = -0.88 (p < 0.001)	ρ = -0.93 (p < 0.001)	ρ = -0.93 (p < 0.001)
Glomerular nephrin (mRNA)	ρ = -0.62 (p = 0.023)	ρ = -0.49 (p = 0.090)	ρ = -0.60 (p = 0.030)	ρ = -0.66 (p = 0.015)
Glomerular podocin (mRNA)	ρ = -0.37 (p = 0.210)	ρ = -0.30 (p = 0.318)	ρ = -0.35 (p = 0.240)	ρ = -0.44 (p = 0.134)

[1] EDD were scored as follows: 0 = absent, 1 = rare, 2 = present, 3 = extensive

[2] Podocyte FP effacement was scored as follows: 0 = no effacement, 1 = minimal effacement, 2 = effacement

[3] Spearman's rho coefficient

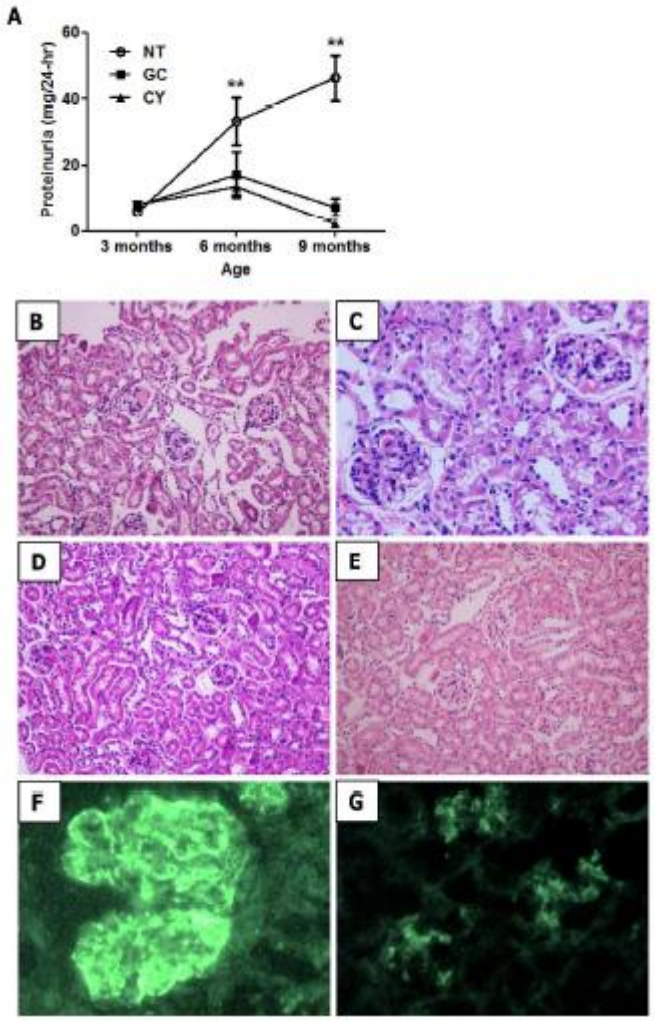

FIGURE 1. Proteinuria, light microscopy and immunofluorescence findings in NZB/W untreated and treated mice

(A) Untreated (NT) NZB/W mice developed overt proteinuria by the age of 6 months, which was further increased at 9 months of age. Mice treated with either GC or CY had reduced proteinuria at both 6 and 9 months of age. Bars represent standard error of the mean value. * $p < 0.05$; ** $p < 0.005$ (B) Glomerular infiltration in in 3-month-old NZB/W (control) mouse. (C) Focal proliferative nephritis in a 6-month-old untreated NZB/W mouse. (D) Minimal mesangial nephritis in a 6-month-old NZB/W mouse treated with GC for 3 consecutive months. (E) Normal light microscopy findings in a 6-month-old NZB/W mouse treated with CY for 3 consecutive months. (F) Immunofluorescence IgG staining pattern in a 6-month-old untreated NZB/W mouse with moderate (++) mesangial deposits and intense (+++) glomerular basement membrane deposits. (G) Weak (+) mesangial IgG deposits in 6-month-old NZB/W mouse that was treated with CY for 3 consecutive months. Original magnification ×100 in panels B, D, E, ×250 in panels C, G, and ×400 in panel F.

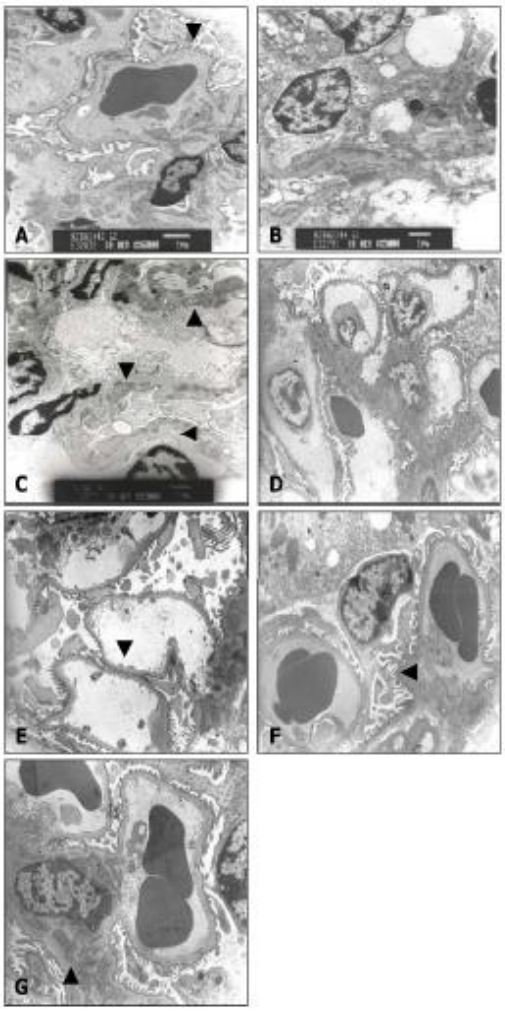

FIGURE 2. Electron microscopy findings in NZB/W untreated and treated mice
(A) Thin section of 3-month-old untreated mouse kidney with segmental increase in mesangial deposits; electron deposits in mesangial areas and in GBM; foot processes are relatively well preserved (arrow). (B) In 6-month-old untreated NZB/W mouse, subendothelial and subepithelial deposits are present; there is also expansion of mesangium resulting in occlusion of capillary loops. (C) Extensive mesangial (top arrow) and subendothelial (bottom arrow) deposition in untreated 9-month-old NZB/W mice; the GBM is thickened and there is extensive fusion of podocytes (left arrow). (D, E) 6-month-old NZB/W mice who received treatment with GC (D) or CY (E) for 3 consecutive months. (F, G) 9-month-old NZB/W mice treated with GC (F) or CY (G) for 6 months. In panels D, E, F there is mesangial expansion with subendothelial deposits (arrows in E, F), whereas in panel G there are occasional mesangial deposits (arrow). Podocytes and slit diaphragms in all panels are well preserved (arrowhead). Magnification ×5000.
179x350mm (300 x 300 DPI)

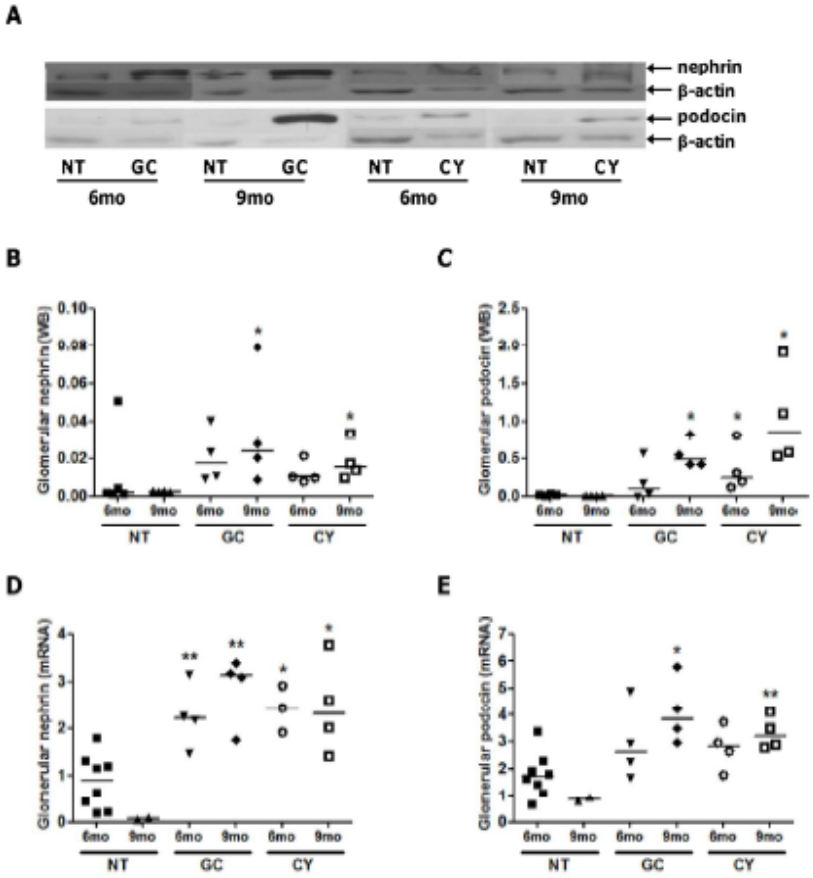

FIGURE 3. Glomerular expression of the slit diaphragm proteins, nephrin and podocin, in NZB/W untreated and treated mice

(A) Western blot analysis of nephrin, podocin and β-actin in glomerular extracts of NZB/W control and treated mice. Nephrin and podocin band density was quantified following normalization with β-actin density. (B) Nephrin is increased after 3 and 6 months therapy with GC and CY. (C) Podocin protein expression is significantly increased after 3 months therapy with GC, and after 3-6 months therapy with CY. (D) Nephrin and (E) podocin glomerular mRNA levels were measured by real-time RT-PCR with GAPDH serving as housekeeping gene. Nephrin mRNA (D) was significantly increased after 3 to 6 months therapy with either GC or CY. Podocin mRNA (E) levels were significantly enhanced only in mice which were treated with either GC or CY for 6 consecutive months. Dots represent individual values, and bars represent medians. * $p < 0.05$; ** $p < 0.01$ for pairwise comparisons between age-matched treated and untreated mice. Abbreviations: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

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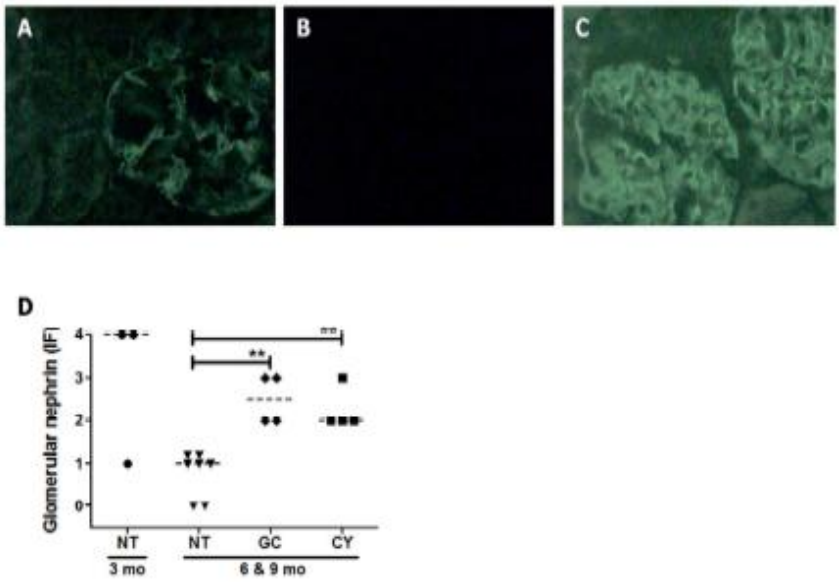

FIGURE 4. Immunofluorescence for nephrin expression in kidney biopsies of untreated and treated NZB/W mice

(A) Nephrin immunofluorescence indicating decreased deposition of nephrin in podocytes (+) in untreated 6-month-old NZB/W mice. (B) Absent nephrin immunofluorescence in 9-month-old untreated NZB/W mice. (C) Increased nephrin intensity (+++) in 6-month-old NZB/W mice treated with CY for 3 months. Original magnification ×400 in panels A-C. (D) Glomerular nephrin staining in immunofluorescence was normal in two out of three untreated 3-month-old NZB/W mice with minimal mesangial nephritis, and severely reduced or absent in all untreated 6- or 9-month-old mice with focal or proliferative nephritis. Nephrin immunofluorescence was enhanced in NZB/W mice that received GC and CY therapy and had normal or minimal mesangial nephritis histology. ** p <0.01 for pairwise comparisons between age-matched treated and untreated mice. Abbreviations: NT, NZB/W control mice that received no therapy, GC, corticosteroids therapy of NZB/W mice, CY, cyclophosphamide therapy of NZB/W mice.

104x74mm (300 x 300 DPI)