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Article

Pulmonary surfactants and the respiratory-renal connection in steroid-sensitive nephrotic syndrome of childhood

Minimal Change Disease (MCD)

Upper Respiratory Tract Infections (virus, bacteria)

Exacerbated Pulmonary Anti-inflammatory Response (inc. **Surfactants**)

Endothelial Dysfunction Glycocalyx Degradation

Virus and **high** level of Surfactants In bloodstream

Robust SIRP-α dependent **podocyte activation and inflammation**

Robust, uncontrolled proteinuria

Gabriel Cara-Fuentes, Ana Andres-Hernando, Colin Bauer, ..., Michiko Shimada, Richard J. Johnson, Miguel A. Lanaspa

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Highlights

A key pulmonary-renal interplay is evidenced in minimal change disease

Urinary SIRPα is elevated in subjects with steroid-sensitive nephrotic syndrome

Circulating surfactants, soluble SIRPα ligands, are elevated in minimal change disease

Surfactants interact with SIRPα in podocytes causing foot effacement and proteinuria

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Pulmonary surfactants and the respiratory-renal connection in steroid-sensitive nephrotic syndrome of childhood

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SUMMARY

Steroid-sensitive nephrotic syndrome (SSNS) in childhood is usually due to minimal change disease (MCD). Unlike many glomerular conditions, SSNS/MCD is commonly precipitated by respiratory infections. Of interest, pulmonary inflammation releases surfactants in circulation which are soluble agonists of SIRPα, a podocyte receptor that regulates integrin signaling. Here, we characterized this pulmonary-renal connection in MCD and performed studies to determine its importance. Children with SSNS/MCD in relapse but not remission had elevated plasma surfactants and urinary SIRPα. Sera from relapsing subjects triggered podocyte SIRPα signaling via tyrosine phosphatase SHP-2 and nephrin dephosphorylation, a marker of podocyte activation. Further, addition of surfactants to MCD sera from patients in remission replicated these findings. Similarly, nasal instillation of toll-like receptor 3 and 4 agonists in mice resulted in elevated serum surfactants and their binding to glomeruli triggering proteinuria. Together, our data document a critical pulmonary-podocyte signaling pathway involving surfactants and SIRPα signaling in SSNS/MCD.

INTRODUCTION

Steroid-sensitive nephrotic syndrome (SSNS) is the most common cause of nephrotic syndrome in childhood and is usually due to minimal change disease (MCD) (Vivarelli et al., 2017). The etiology is unknown but it is thought to be mediated by a circulating factor that either directly or indirectly causes podocyte activation leading to an increase in permeability of the glomerular filtration barrier to albumin (Davin, 2016; Noone et al., 2018). While less is known about the circulating factor, alterations in the podocyte have been reported, including expression of angiopoietin-like 4 (Angptl-4) (Clement et al., 2011, 2014), phosphorylated focal adhesion kinase (FAK) (George et al., 2012), Src homology-2 domain-containing protein tyrosine phosphatase-2 (SHP2) (Aoudjit et al., 2011; Verma et al., 2015; Zha et al., 2013), and decreased expression of phosphorylated nephrin (P-Nephrin) (Kurihara et al., 2010; Uchida et al., 2008). These studies suggest a possible integrin mediated activation of the podocyte that alters nephrin function.

We also reported that CD80 (also known as B7.1) is induced in glomeruli of subjects with MCD and that membrane-associated, not soluble, CD80 is excreted in the urine, likely shed from glomerular cells (Garin et al., 2009, 2010). The role of CD80 has been controversial, as some groups have not been able to identify CD80 in the glomeruli of subjects with MCD. However, using multiple methodologies, we could demonstrate *de novo* expression of CD80 in both glomerular endothelial cells and podocytes in a subset of patients with minimal change disease (Cara-Fuentes et al., 2020). Furthermore, several other groups have also confirmed that urinary CD80 excretion increases markedly in subjects with MCD compared to other proteinuric glomerular diseases, although the clinical significance is yet to be determined (Ahmed et al., 2018; Gonzalez Guerrico et al., 2020; Ling et al., 2015, 2018; Mishra et al., 2017). As CD80 is a coregulatory molecule in T cell response, this is consistent with evidence for immune modulation in the glomeruli in MCD (Colucci et al., 2018).

MCD is commonly precipitated by respiratory events. Culture-positive infections of the respiratory tract have been identified in 70 percent of children relapsing with childhood nephrotic syndrome (MacDonald et al., 1986). Forty percent of children with SSNS also have asthma, eczema, or hay fever which may

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A

Tubules | SIRP-α | Glomeruli

SIRP-α | Synaptopodin

Merge | Merge

B

SIRP-α, 1 mg creatinine

Pt1 | Pt2 | Pt3 | Pt4 | Pt5 | Pt6

C1 C2 | Rm Rl Rm | Rm Rl Rm | Rl Rm | Rl Rm | Rl Rm | Rl

90 kDa

80 kDa

C

SIRPα (Fold change)

0.2330

<0.001 <0.001 <0.001

Control
Remission
Relapse

D

Pt10

E

Urine Protein/Creatinine ratio (mg/g)

r=0.836, <0.001

SIRPα (fold-change)

F

Urine Protein/Creatinine ratio (mg/g)

r=0.575, 0.008

SIRPα (fold-change)

Figure 1. Characterization of renal SIRPα in MCD

(A) Immuno-colocalization of SIRPα (green) with synaptopodin (red) in mouse glomeruli. Merged signal is shown in yellow. Size bar: 200 μm Tubules were negative for SIRPα expression.

(B) SIRPα in urine of two age-matched control patients (C1 and C2, black symbols) and six patients with MCD (Pt1 to Pt6) in relapse (Rl, blue symbols) or steroid-dependent remission (Rm, red symbols).

(C) Densitometry analysis of expression of SIRPα in the urine of controls and ten patients with SSNS/MCD in consecutive relapse/remission cycles (from 2 to 4 cycles).

(D) Densitometry analysis of patient 10 (Pt10) with 2 remission and 2 relapse cycles.

(E) Correlation analysis between urinary protein excretion and urinary SIRPα levels in samples from two controls (black symbols) and ten subjects with SSNS/MCD in relapse (blue symbols) and remission (red symbols).

(F) Correlation analysis between urinary protein excretion and urinary SIRPα levels in samples from ten subjects with SSNS/MCD in relapse.

Statistical Analysis: One-Way ANOVA. For (E and F) Pearson r correlation analysis.

precipitate relapse (Abdel-Hafez et al., 2009; Thomson et al., 1976). Nevertheless, investigation of a respiratory-renal connection with MCD has, to our knowledge, not been investigated.

We hypothesized that respiratory infection or inflammation may result in the release of factors that enter the circulation and potentially activate the glomerular podocyte. In this regard, the lung produces surfactant proteins A and D (SP-A and SP-D) that normally circulate at low levels in the blood but can increase or decrease with respiratory inflammation or infection (Takahashi et al., 2006). In turn, surfactant proteins bind and activate signal regulatory protein alpha (SIRPα) (Janssen et al., 2008). Of interest, SIRPα is expressed on glomerular podocytes where it localizes to the slit diaphragm (Kurihara et al., 2010).

SIRPα is a cell membrane receptor involved in integrin signaling (Tsuda et al., 1998) that has intracellular tyrosine motifs that are normally phosphorylated and bind Src homology region 2 domain-containing phosphatase-1 (SHP1, also known as PTPN6) and SHP-2 (also known as PTPN11). Some ligands, such as CD47, maintain SIRPα in a phosphorylated state, which helps keep the cell in a differentiated state (Johansen and Brown, 2007). Other ligands, such as SP-A and SP-D, result in a loss of the phosphorylation of SIRPα, resulting in release of SHP1 and SHP2, thereby causing cell activation. Similarly, a loss of total SIRPα from the cell surface also be associated with activation (Johansen and Brown, 2007; Kong et al., 2007). For example, incubation of macrophages with toll-like receptor (TLR) ligands results in downregulation of SIRPα with the intracytosolic release of SHP-1 and SHP-2, with the former localizing to the IκK complex where it activates NF-κB signaling(Kong et al., 2007). Both SHP-1 and SHP-2 can also bind nephrin, and affect the phosphorylation state of nephrin, with SHP-1 being associated with a reduction in phosphorylated nephrin (Denhez et al., 2015) while SHP-2, according to some reports may increase phosphorylated nephrin (Hsu et al., 2017; Verma et al., 2015). In turn, since nephrin has a key role in slit diaphragm function, an alteration in nephrin status might be expected to affect glomerular permeability to protein, resulting in proteinuria (Uchida et al., 2008). Indeed, phosphorylated nephrin has been reported to be low in glomeruli of subjects with MCD (Uchida et al., 2008) in association with a redistribution of nephrin from the slit diaphragm to the cytoplasm (Wernerson et al., 2003).

Given these observations, we conducted studies to evaluate the hypothesis that surfactant proteins might be able to reach the glomerulus in children with SSNS/MCD and induce podocyte activation via SIRPα.

RESULTS

SIRPα is expressed in the podocyte and elevated in the urine of patients with steroid-sensitive nephrotic syndrome

Consistent with previous reports, we identified the presence of SIRPα in glomeruli but not tubules of naive mice (Kurihara et al., 2010; Li et al., 2019). Within the glomeruli, SIRPα colocalized with the podocyte-specific marker synaptopodin (Figure 1A). In view of its recently proposed role in maintaining podocyte survival (Li et al., 2019), we analyzed the urinary expression of this molecule in subjects with a podocyte-specific condition such as SSNS/MCD. A full characterization of the patient cohort is provided in the methods section. Different subjects in continuous relapse-remission cycles were analyzed. As shown in Figure 1B, little or no SIRPα expression was found in the urine of age-matched control subjects. In contrast, a robust SIRPα signal was observed in the urine of six different subjects (Pt1 to Pt6) with SSNS/MCD in relapse. In these same patients, SIRPα levels were then consistently lower when the condition was in remission suggestive of a direct correlation between urinary shedding of SIRPα and the relapse of SSNS/MCD (Figures 1B and 1C). A more detailed correlation between urinary SIRPα and the MCD state (relapse or remission) is
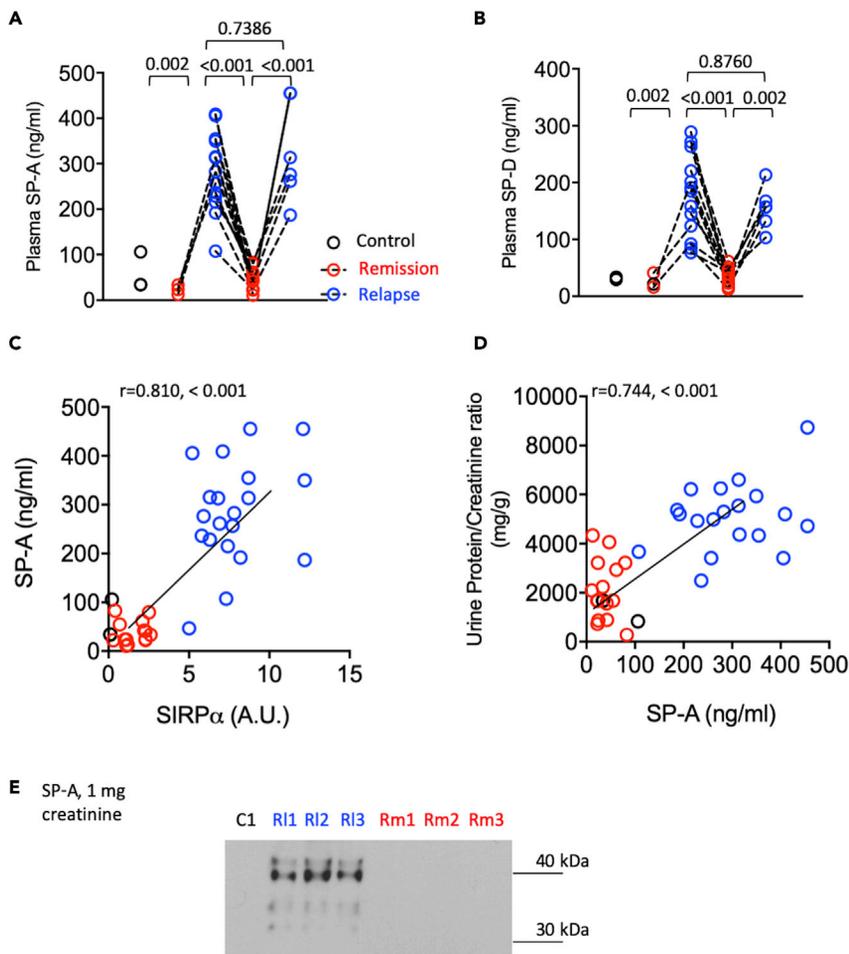
Figure 2. Surfactant levels and urinary protein in SSNS/MCD

(A) Plasma SP-A in control and ten subjects with SSNS/MCD in consecutive relapse/remission cycles (from 2 to 4 cycles).

(B) Plasma SP-D in control and ten subjects with SSNS/MCD in consecutive relapse/remission cycles (from 2 to 4 cycles).

(C) Correlation analysis between plasma SP-A and urinary SIRPα levels in samples from two controls (black symbols) and ten subjects with SSNS/MCD in relapse (blue symbols) and remission (red symbols).

(D) Correlation analysis between plasma SP-A and urinary protein in samples from two controls (black symbols) and ten subjects with SSNS/MCD in relapse (blue symbols) and remission (red symbols).

(E) Representative western blot for SP-A in the urine of a control subject (C1) and three patients with MCD in relapse (Rl1 to Rl3) and remission (Rm1 to Rm3).

Statistical Analysis: One-Way ANOVA for A and B. For (C and D) Pearson r correlation analysis.

showcased in Figure 1D for a patient undergoing two relapse/remission cycles. Of interest, urine SIRPα correlated closely with urinary protein excretion (Figure 1E), and particularly when subjects were in relapse (Figure 1F). Overall, these findings suggest that urinary SIRPα may be a marker for SSNS/MCD in children, and that its levels would correlate with the severity of the condition.

Serum and urine surfactant proteins are elevated in MCD

Surfactant protein A and D (SP-A and SP-D) are recognized ligands for SIRPα (Janssen et al., 2008). As soluble factors, they are present in the circulation of healthy children and adults, typically in the range of 40–60 ng/mL (Bratcher and Gaggar, 2014; Takahashi et al., 2006). SP-A and SP-D are considered anti-inflammatory proteins and circulating levels can be normal, low, or high with upper respiratory infections (URI) and pneumonia (Leth-Larsen et al., 2003; Nishida et al., 2011; Shu et al., 2015). However, and as shown in Figures 2A and 2B, subjects with relapsing MCD demonstrated a marked elevation in serum levels of both SP-A and SP-D compared to either the same subjects in remission or age-matched healthy controls.

In general, SP-A levels averaged almost 10-fold higher in patients with SSNS/MCD in relapse (303.3 ± 53.4 ng/mL in relapse vs 32.3 ± 16.5 ng/mL in remission).

We also evaluated the relationship of SP-A levels with the urinary excretion of SIRPα to ascertain whether both events are connected. As shown in Figures 2C and 2D, plasma SP-A strongly correlated with both urinary SIRPα and urinary protein excretion. This would suggest that the release of surfactants in the circulation could lead to the activation of the podocyte, possibly via its interaction with SIRPα. Furthermore, and consistent with greater excretion of SIRPα, we also detected elevated SP-A in the urine of children with MCD in relapse but not in remission (Figure 2E).

Surfactants interact with SIRPα and activate the podocyte *in vivo*

Surfactants are part of the anti-inflammatory response in the lung. Therefore, we next explored whether experimental induction of inflammation of the respiratory tract might cause proteinuria *in vivo*. To this end, we instilled a combination of polyinosinic:polycytidylic acid (Poly:IC) and lipopolysaccharide (LPS) (TLR ligands for TLR3 and 4, respectively) and compared this to the classical intraperitoneal models of TLR ligand-induced proteinuria (Ma et al., 2010). An optimization of the dose of both ligands is presented in the supplemental material (Figures S1 and S2). Different parameters including a 24-h elevation of pulmonary surfactant and anti-inflammatory cytokines were evaluated in order to calculate the dose for each TLR ligand in our model (Figure S1A). After multiple titrations, a dose of 80 μg LPS (Figures S1B–S1E) and 240 μg Poly I:C was selected as it induced the most robust inflammatory response in the lung with low mortality (Figures S1F–S1I). Of interest, when compared with a classical model of proteinuria, intraperitoneal (IP) injection of 200 μg LPS, our model resulted in similar elevations in albuminuria (Figure S2A). However, unlike IP LPS-treated mice, animals exposed to LPS and Poly I:C combination via instillation demonstrated a generally healthier phenotype with greater 24-h urinary output (Figure S2B) and paralleled lower urinary osmolality (Figure S2C). Together, the total amount of albumin excreted in the urine in a 24-h period was markedly higher in instilled LPS + Poly I:C mice compared to LPS-treated animals (Figure S2D). Similarly, water intake was significantly higher with markedly lower mortality rates in LPS + Poly I:C-exposed mice (Figures S2E and S2F).

To test the effect of repeated pulmonary inflammation in surfactant release and proteinuria, we studied mice on vehicle or LPS + Poly I:C combinations for 3 consecutive days as depicted in Figure 3A. Every morning, an instillation of LPS + Poly I:C was administered and urine was collected in 6-h intervals. After 3 days, an extra instillation was administered and plasma and tissues were collected 3 h later for analysis. As shown in Figure 3B, the instillation model resulted in a significant rise in serum SP-A compared to unmanipulated mice or saline-instilled controls (5.54 ± 2.81 ng/mL in saline-administered mice vs 90.20 ± 36.57 ng/mL in LPS + Poly I:C-treated animals, p < 0.01). Furthermore, SP-A was localized to podocytes, as noted by its colocalization with synaptopodin (Figure 3C). Approximately, 70%–80% of glomeruli of mice analyzed were positive for SP-A in LPS/Poly I:C-instilled mice. In contrast, SP-A was absent in glomeruli from saline-exposed mice. More importantly, we identified the interaction between SP-A and SIRPα in the glomeruli both by colocalization (Figure 3D) and biochemically (pull-down, Figure 3E). Functionally, LPS + PolyI:C-treated mice demonstrated elevated albumin excretion compared to unmanipulated or saline-treated animals. This elevation was more noticeable during the first 12 h post administration peaking at around 12–18 h with values of 20–30 mg albumin excretion in a 6-h period (Figure 3F). Overall, on average, the accumulated 24-h albumin excretion was significantly higher in mice receiving a combination of LPS and Poly I:C (18.88 ± 4.25 mg albumin/24 h in saline-administered mice vs 53.46 ± 4.01 mg albumin/24 h in LPS + Poly I:C-treated animals, p < 0.01, Figure 3G). Of note, saline-treated mice also demonstrated a substantial elevation in urinary albumin excretion at 6–12 h post instillation compared to unmanipulated animals suggesting that perhaps any potential pulmonary insult could in turn activate the podocyte via surfactant release. Finally, nasal instillation of TLR3 and TLR4 ligands resulted in podocyte foot process effacement as demonstrated by electron microscopy (Figure 3H).

Surfactants promote nephrin dephosphorylation in human podocytes via SIRPα and SHP-2

SIRPα has been reported to colocalize and interact with phosphatases including both SHP-1 and SHP-2 in macrophages (Kong et al., 2007) and with SHP-2 in podocytes (Kurihara et al., 2010). Nephrin phosphorylation is key for the podocyte to maintain its polarity and cell-cell interaction (Uchida et al., 2008). Therefore, we hypothesized that the activation of SIRPα by surfactants would lead to the translocation of SHP-2 to nephrin for its dephosphorylation. In turn, reduced nephrin phosphorylation would result in the podocyte

A
Intranasal instillation

Urine Collection: 6 hr, 12 hr, 18 hr, 24 hr

Urine Collection: 6 hr, 12 hr, 18 hr, 24 hr

Urine Collection: 6 hr, 12 hr, 18 hr, 24 hr

Plasma, Kidney Collection: 3 hr

Time (hours)

80 μg LPS/ 240 poly I:C
Saline

B
SP-A (ng/ml)
<0.001
Unmanipulated
Saline
80 μg LPS/ 240 poly I:C

C
LPS/ poly I:C
SP-A   Synaptopodin

LPS/ poly I:C
SP-A | Synaptopodin | Merge

Saline
SP-A | Synaptopodin | Merge

D
LPS/ poly I:C
SIRP-α | SP-A | Merge

E SIRP-α
Input,PCI Unman Saline LPS/ poly I:C
50 kDa
30 kDa

F
Albumin excretion (mg albumin/6 hr)
Time (hours)
LPS/ poly I:C
Saline
Unmanipulated

G 0 → 24 hours
Albumin excretion (mg albumin/24 hr)
<0.001
<0.001

H
Saline

LPS/ poly I:C

Figure 3. Intranasal instillation of LPS and PolyI:C and proteinuria in mice

(A) Schematic depicting the protocol to induce proteinuria. Saline (red) or a combination of LPS (80 µg) and Poly I:C (240 µg) (blue) is administered intranasally once daily for three consecutive days. Urine is collected every 6 h and 3 h after the fourth injection, plasma, lung, and kidney tissue are collected for analysis.

(B) Plasma SP-A levels in unmanipulated (black) mice or receiving either saline (red) or LPS/Poly I:C combination (n = 5 mice per group).

(C) Representative confocal images for SP-A (green) and synaptopodin (red) colocalization in kidney glomeruli of mice receiving saline or LPS/Poly I:C. Merged colocalization is shown in yellow. Size bar: 200 µm. Images are representative from n = 5 mice per group

(D) Confocal analysis of SIRPα (green) and SP-A (red) colocalization in kidney glomeruli of mice receiving saline or LPS/ Poly I:C. Merged colocalization is shown in yellow. Size bar: 200 µm. Images are representative from five mice.

(E) Co-immunoprecipitation of SIRPα and detection of SP-A in kidney cortex extracts of unmanipulated, saline-exposed, and LPS/Poly I:C-exposed mice. Total input (500 ug) and amount retained in preclearance is shown in lanes one and two; n = 3 mice per group.

(F) Six hour albumin excretion over the 72-h period in unmanipulated (black) mice receiving either saline or LPS/Poly I:C.

(G) 72-h accumulated albumin excretion in the urine of the same mice in F). n = 5 mice per group.

(H) Representative electron microscopy podocyte images from saline- or LPS/PolyI:C-treated mice for 24 h. Blue arrows represent areas of foot effacement.

Statistical Analysis: One-Way ANOVA, Tukey post hoc analysis for (B) and (G), data are represented as mean ± SEM.

activation, inflammation, and the loss of its polarity (Figure 4A). As expected, under normal conditions, in confluent human podocyte cultures, SIRPα colocalized with SHP-2 (Figure 4A). However, when cultured podocytes were incubated with serum from a child with MCD (Pt1) in relapse (who had serum SP-A levels of 380 ng/mL and SP-D of 286 ng/mL), the SHP-2- SIRPα interaction faded and SHP-2 coimmunoprecipitated with nephrin (Figures 4B and 4C). Of interest, the interaction between nephrin and SHP-2 was not evident in podocytes exposed to serum from the same patient in remission (with serum SP-A levels of 68 ng/mL and SP-D levels of 89 ng/mL) (Figures 4B and 4C). A similar pattern was observed when serum from six different subjects with SSNS/MCD in relapse or remission was employed with serum SP-A levels ranging from 234 to 412 ng/mL and SP-D of 113–286 ng/mL in relapse and serum SP-A levels of 16–42 ng/mL and SP-D of 12– 35 ng/mL in remission (Figure 4D). Consistently, phosphorylated nephrin levels were significantly reduced over time after exposure of podocytes to serum of patients with MCD in relapse but not in remission (Figures 4E–4G). Furthermore, decreased nephrin phosphorylation was paralleled with a marked reduction in its expression in cell-cell junctions as denoted by confocal microscopy (Figure 4H). Consistent with activation of the podocyte (Shimada et al., 2012), the serum of patients with MCD induced the reorganization of the actin cytoskeleton (Figure 4I) and local inflammation with elevation of proinflammatory cytokines IL6 and TNFa (Figure 4J) and the costimulatory molecule CD80 (Figure 4K). To better assess whether the surfactants in the serum were responsible for nephrin dephosphorylation, we next performed studies in which we evaluated the effect of supplementing serum from MCD in remission with specific surfactants including the two SP-A isoforms, SPA-1 and SPA-1b. As shown in Figures 4L and 4M, adding SPA-1, SPA-1b, or their combination at a concentration of 500 ng/mL to sera from patients in remission resulted in a marked decrease in phosphorylated nephrin similar to that observed when podocytes were incubated with sera form the same patients in relapse. Taken together, this would suggest that surfactants are important soluble factors present in the plasma of subjects with active MCD that activate the podocyte via SIRPα and nephrin dephosphorylation.

Steroids do not modify SIRPα activation in the podocyte but ameliorate downstream podocyte activation and inflammation

Steroids are commonly used to achieve remission in subjects with MCD but the mechanism whereby this occurs is not fully understood. In cultured podocytes, dexamethasone did not affect the translocation of SHP2 from SIRPα to nephrin in podocytes incubated with sera from patients with active relapses (Figures 5A–5C) and consistently, nephrin dephosphorylation was not prevented in dexamethasone-treated podocytes (Figures 5D and 5E). However, while the direct effects of SIRPα activation were not modified, steroids effectively suppressed several downstream mechanisms associated with nephrin loss including actin reorganization (Figure 5F) (Zhu et al., 2008) and inflammation (Figure 5G) (Hussain et al., 2009). These results would suggest that steroids are downstream modulators of SIRPα activation in the podocyte. Possibly, steroids could also act *in vivo* by reducing the surfactant release to the bloodstream after a pulmonary tract infection thereby reducing the availability of SIRPα ligands in the circulation (Figure 5H).
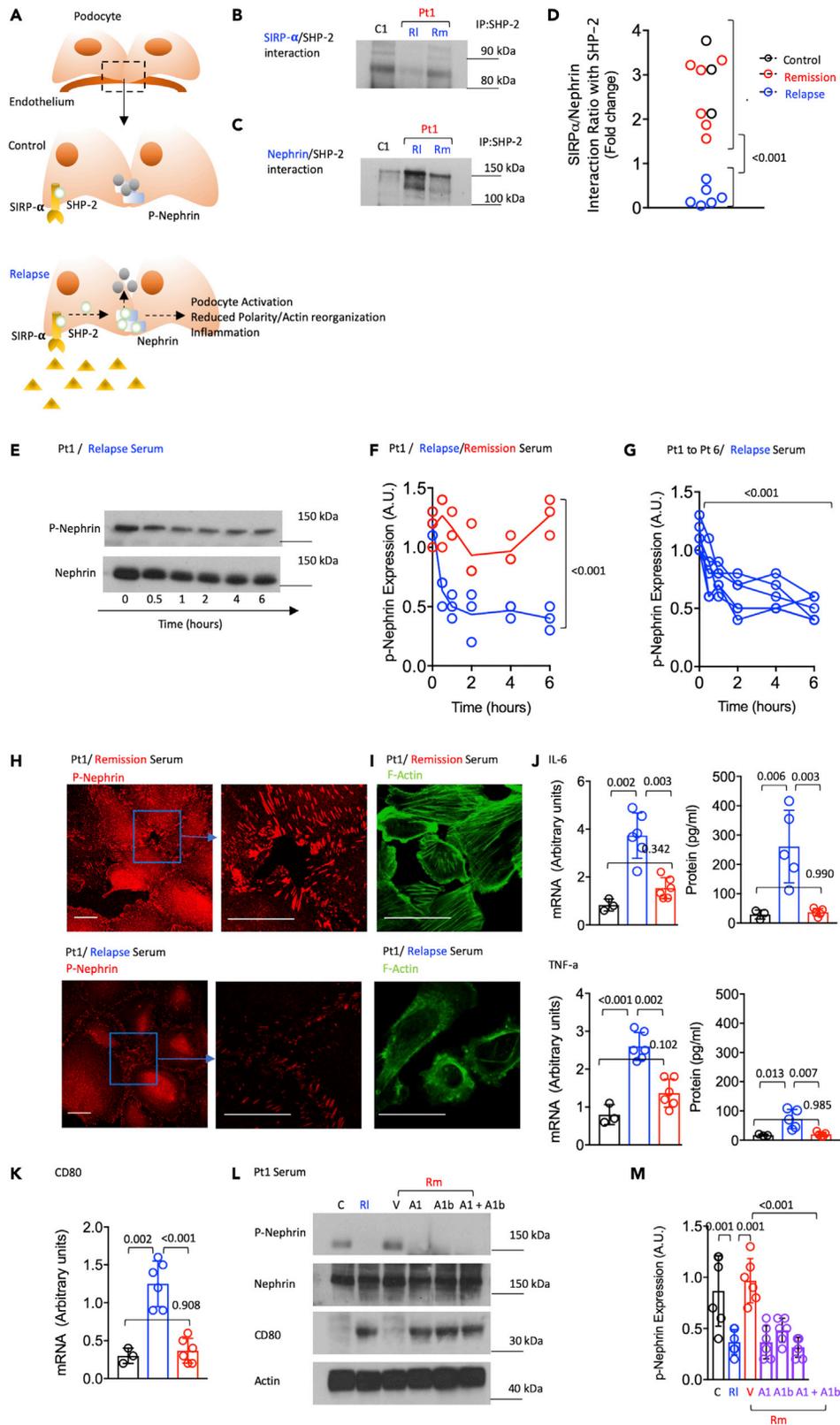
Figure 4. *In vitro* analysis of the effects of surfactants on podocyte activation

(A) Schematic depicting the proposed changes in the podocyte following stimulation with surfactants. Surfactants bind and activate podocyte SIRPα releasing SHP-2 to the cytosol where it binds and interacts with nephrin among other proteins. In turn, SHP-2 dephosphorylates nephrin leading to the loss of cell-cell interaction and polarity, actin reorganization, and inflammation.

(B) Representative co-immunoprecipitation image between SIRPα and SHP-2 in cultured human podocytes incubated with serum from an age-matched control subject (C1) or from a patient with MCD (Pt1) in relapse (Rl) or remission (Rm).

(C) Representative co-immunoprecipitation between SIRPα and nephrin in cultured human podocytes incubated with serum from an age-matched control subject (C1) or from a patient with MCD (Pt1) in relapse (Rl) or remission (Rm).

(D) Ratio of interaction between SHP-2 and SIRPα or nephrin in cultured human podocytes incubated with serum from three controls (black symbols) or six subjects with SSNS/MCD in relapse (blue symbols) or remission (red symbols).

(E) Representative western blot for total and phosphorylated nephrin (P-Nephrin) expression in cultured human podocytes incubated with serum from a patient with MCD (Pt1) in relapse for 6 h.

(F) Densitometry analysis of P-Nephrin expression corrected for total nephrin following incubation with serum from a patient with MCD (Pt1) in relapse or remission for 6 h. Data presented are for three independent replicates for each time point.

(G) Densitometry analysis of P-Nephrin expression corrected for total nephrin following incubation with serum from six patients with SSNS/MCD (Pt1 to Pt6) in relapse for 6 h. Data presented are an average from three independent replicates for each subject and time point.

(H) Confocal imaging of P-Nephrin expression (red) in cell-cell junction of cultured human podocytes incubated with serum from a patient with MCD (Pt1) in remission or relapse for 6 h. Size bars: 20 μm.

(I) Confocal imaging of polymerized actin (F-actin, green) in cultured human podocytes incubated with serum from a patient with MCD (Pt1) in remission or relapse for 6 h. Size bars: 20 μm.

(J) IL6 -top- and TNFa -bottom- mRNA and protein (supernatant) levels in human cultured podocytes incubated for 24 h with serum from three age-matched control subjects (black symbols) or six patients with SSNS/MCD in relapse (blue symbols) or remission (red symbols). Data presented are an average from three independent replicates for each subject.

(K) CD80 mRNA expression in human cultured podocytes incubated for 24 h with serum from three age-matched control subjects (black symbols) or six patients with MCD in relapse (blue symbols) or remission (red symbols). Data presented are an average from three independent replicates for each subject.

(L) Representative western blot for total and phosphorylated nephrin (P-Nephrin), CD80, and actin loading control in human cultured podocytes incubated for 24 h with serum from a patient with MCD (Pt1) in relapse, or remission (red symbol) in the presence of vehicle (v), human SP-A1, SP-A1b, or a combination of both surfactants (500 ng/mL each). Data presented are an average from three independent replicates for each subject.

(M) Densitometry analysis for P-Nephrin in human cultured podocytes incubated for 24 h with serum from six patients with SSNS/MCD (Pt1 to Pt6) in relapse, remission or remission in the presence of vehicle (v), human SP-A1, SP-A1b, or a combination of both surfactants (500 ng/mL each).

Data presented are an average from three independent replicates for each subject and condition. Statistical Analysis: One-Way ANOVA. Data in panel (I, K, and M) are represented as mean ± SEM.

DISCUSSION

Our data support a respiratory-renal signaling pathway that could potentially have a role in the pathogenesis of MCD (Figure 6).

First, we found that both SP-A and SP-D are elevated in the sera of subjects with SSNS/MCD in relapse, and the levels were highest in those with high urinary SIRPα levels. The levels of SP-A and SP-D in our patients appear to be some of the highest levels observed for the various pulmonary diseases studied based on the assay we used (Bratcher and Gaggar, 2014; Takahashi et al., 2006). The levels of SP-A and SP-D in our patients varied from 150 to 400 ng/mL and are higher than that observed with the same Biovendor ELISA in most other respiratory conditions (Bratcher and Gaggar, 2014; Takahashi et al., 2006). SP-A and SP-D are anti-inflammatory proteins and circulating levels usually remain the same or fall with most pulmonary infections (URI, pneumonia) (Leth-Larsen et al., 2003; Nishida et al., 2011; Shu et al., 2015) and levels are minimally elevated (100–150 ng/mL) in the majority of subjects with chronic obstructive pulmonary disease (COPD) or acute respiratory distress syndrome (ARDS) (Lomas et al., 2009; Tzouvelekis et al., 2005). This suggests that the children with SSNS/MCD may have an exaggerated response to respiratory infections. The exacerbated surfactant response in subjects with MCD could be attributed to several factors. First, high surfactant levels in plasma could be attributed to both a greater surfactant response by the alveolar epithelium spilling over the circulation, and the destruction of the endothelium glycocalyx. Second, there could be increased susceptibility of podocytes to the effects of surfactants, particularly in the settings of low podocyte density in MCD as demonstrated by Kikuchi et al. (2017).
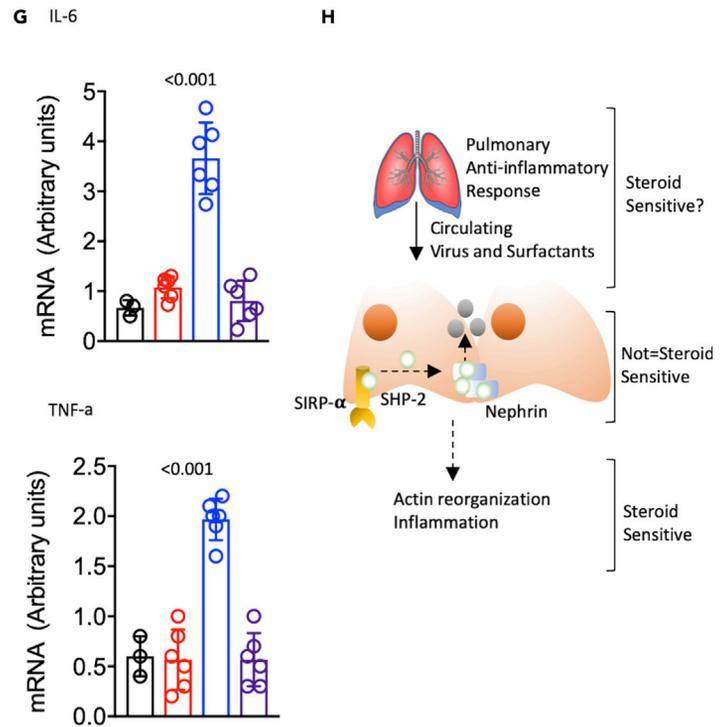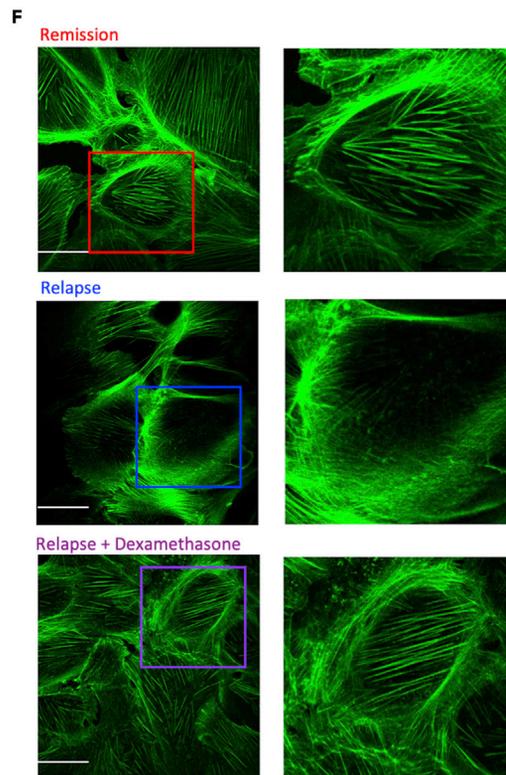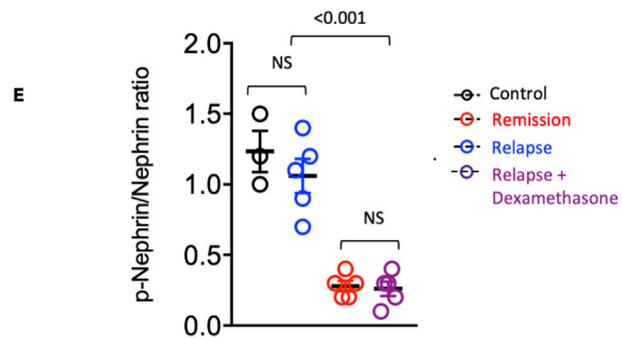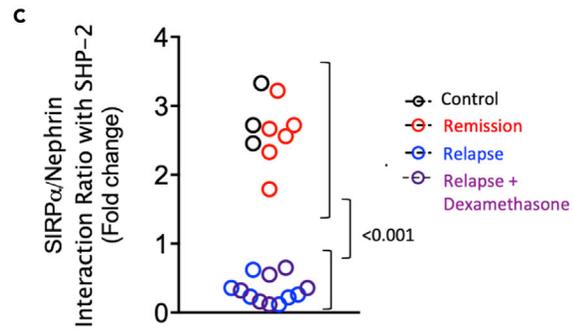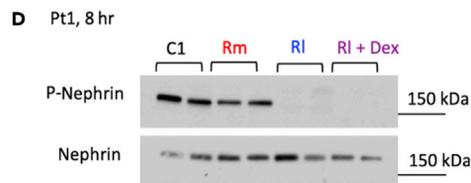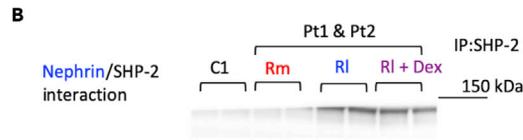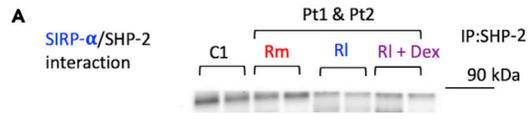
Figure 5. Effect of steroids on SIRPα and podocyte activation

(A) Co-immunoprecipitation between SIRPα and SHP-2 in cultured human podocytes incubated with serum from an age-matched control subject (C1) or from two different subjects with MCD (Pt1 and Pt2) in remission, relapse (Rl), or relapse in the presence of dexamethasone (100 nM) (Rl + Dex).

(C) Co-immunoprecipitation between SIRPα and nephrin in cultured human podocytes incubated with serum from an age-matched control subject (C1) or or from two different subjects with MCD (Pt1 and Pt2) in remission, relapse (Rl), or relapse in the presence of dexamethasone (100 nM) (Rl + Dex).

(D) Ratio of interaction between SHP-2 and SIRPα or nephrin in cultured human podocytes incubated with serum from three controls (black symbols) or six subjects with SSNS/MCD in remission (red symbols), relapse (blue symbols), or relapse and dexamethasone (purple symbols). D) Representative western blot for total and phosphorylated nephrin (P-Nephrin) expression in cultured human podocytes incubated with serum from a control (C1) subject or from a patient with MCD (Pt1) in remission (Rm), relapse (Rl), or relapse in the presence of dexamethasone (Rl + dex) for 8 h.

(E) Densitometry analysis of P-Nephrin expression corrected for total nephrin following incubation with serum from three control subjects (black) or six patients with SSNS/MCD in remission, relapse, or relapse in the presence of dexamethasone for 8 h. Data presented are an average from three independent replicates for each subject and time point.

(F) Confocal imaging of polymerized actin (F-actin, green) in cultured human podocytes incubated with serum from a patient with MCD (Pt1) in remission, relapse, or relapse in the presence of dexamethasone for 8 h. Size bars: 20 μm.

(G) IL6 -top- and TNFa -bottom- mRNA expression in human cultured podocytes incubated for 24 h with serum from three age-matched control subjects (black symbols) or six patients with SSNS/MCD in remission (red symbols), relapse (blue symbols), or relapse in the presence of dexamethasone (Purple symbols). Data presented are an average from three independent replicates for each subject.

(H) Proposed steroid-sensitive areas in podocytes. Steroids will have no effect on direct podocyte SIRPα activation by surfactants, the cytosolic release of SHP2 and the dephosphorylation of nephrin. However, steroids ameliorate the cytoskeletal depolymerization of actin and podocyte inflammation. Possibly, steroids may also act *in vivo* by reducing the surfactant release to the circulation after a pulmonary tract infection.

Statistical Analysis: One-Way ANOVA, Tukey post hoc analysis. Data in panles (C) and (G) are represented as mean ± SEM.

SP-A and SP-D bind SIRPα on lung macrophages and dendritic cells (Janssen et al., 2008) where they have a local role in blocking Th1-dependent and Th2-dependent airway inflammation (Janssen et al., 2008; Raymond et al., 2010). However, the surfactant proteins can also bind and activate SIRPα. Activation of SIRPα may be involved in integrin signaling and leads to the release of SHP-2 (Tsuda et al., 1998) that may dephosphorylate proteins such as nephrin. Our studies suggest SP-A and SP-D reach the glomerulus where they bind SIRPα, which has already been found on podocytes (Kurihara et al., 2010). Our finding that SIRPα is present in the urine of children with MCD in relapse but not remission suggests that some SIRPα may be shed into the urine, similar to what we and others postulated for CD80. In this regard, Li et al. recently found that the expression of SIRPα is decreased in subjects with another podocyte-derived condition that results in proteinuria, focal segmental glomerular sclerosis (FSGS) (Li et al., 2019). Based on our data, we propose that this reduction in podocyte SIRPα is the result of its shedding into the urine thus making this protein a potential marker for glomerular conditions. Interestingly, the role of SIRPα in FSGS and MCD seems to be different and dependent on a specific factor or ligand. In this regard, our data suggest that when pulmonary surfactants interact with SIRPα, the end result is the activation of the podocyte and proteinuria. Unlike SSNS/MCD, FSGS is a more chronic condition that is not necessarily preceded or triggered by upper respiratory tract infections; this would suggest that SIRPα may act differently regarding the ligand with which it is interacting. In this regard, multiple factors have been shown to interact with surfactants, including CD47 (Kurihara et al., 2010) which has antiphagocytic effects, and in these settings, the interaction of SIRPa with CD47 would prevent podocyte loss by reducing autophagy as proposed by Li et al., while its interaction with surfactants would in turn activate the podocyte.

The second major finding was based on the studies of podocytes in culture. Specifically, we provide evidence that sera from children with MCD in relapse also activates SIRPα with the release of SHP-2 phosphatase that colocalizes with nephrin, leading to reduced levels of phosphorylated nephrin with podocyte actin rearrangement. The inability of sera in remission to activate the podocyte was reversed by adding back recombinant SP-A to the sera.

The majority of MCD patients achieve remission with steroids. Therefore, we analyzed the effect of steroids on SIRPα activation in the podocyte. Surprisingly, we found that dexamethasone did not affect the SIRPα signaling although it effectively reduced the associated inflammation and overall podocyte activation. This would suggest that steroids while they are effective in accelerating podocyte recovery and achieve remission, oral steroids may not block the initial triggering mechanism. This is consistent with a recent double blind clinical trial which concluded that oral steroids do not reduce the risk of new relapses in patients with MCD with upper respiratory tract infections (Christian et al., 2022).

Finally, we present an animal model generated by the intranasal instillation of TLR ligands, and that is associated with a rise in serum SP-A with its localization in glomeruli and the development of albuminuria. The
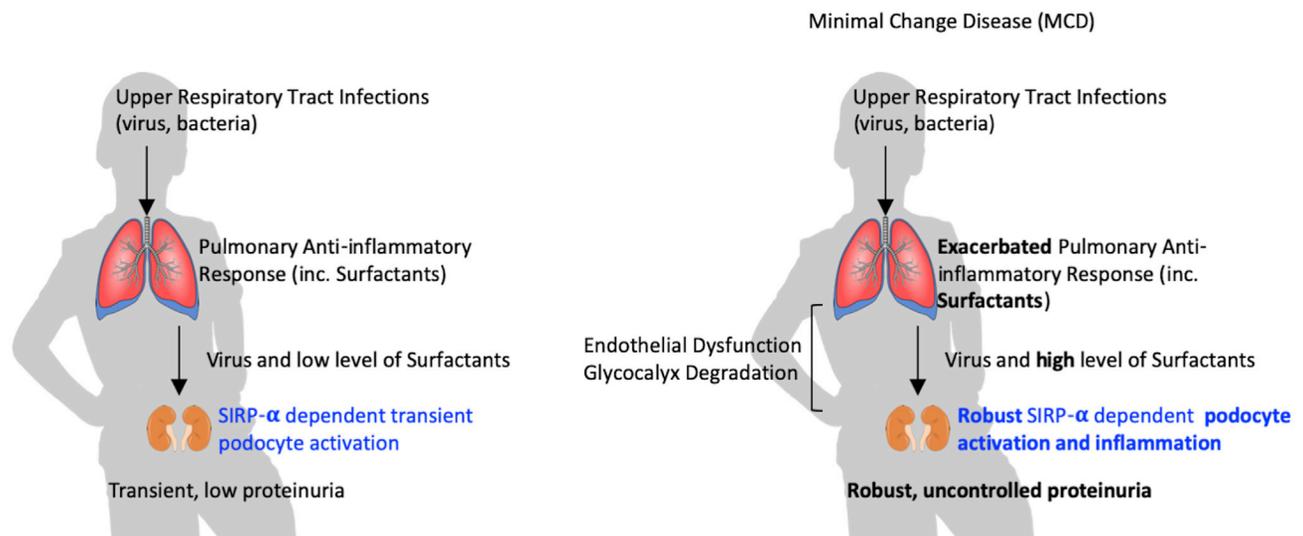
Figure 6. Pulmonary determinants in MCD-associated proteinuria

Proposed mechanism: upper respiratory tract infections commonly precede the onset of MCD in children. In healthy subjects -left-, these infections lead to a regulated pulmonary anti-inflammatory response. Part of this response include the release to the circulation of low levels of signaling factors including surfactant which transiently activate the podocyte in the kidney to help the clearance of organisms in infections. In subjects with MCD, -right- there is an exacerbated and dysregulated pulmonary response leading to marked levels of surfactants in circulation and thus a robust activation of the podocyte via SIRPα. This is favored by endothelial dysfunction and glycocalyx degradation present in both lung and renal capillaries. In this context, anti-inflammatory steroids commonly used to treat MCD would help to ameliorate both the inflammation and the activation of the podocyte as well as the reducing the anti-inflammatory response in the lungs.

observation that proteinuria was double than that observed with the classical intraperitoneal TLR models is of interest and warrants further studies. However, the interpretation of our mouse model does not allow to ascertain what infection (viral or bacteria) is more relevant in triggering the surfactant response as it requires using both TLR3 and TLR4 ligands at the same time. Of interest, nose instillations with saline resulted in some yet detectable albumin spill in the urine compared to unmanipulated mice. This would suggest the presence of a defensive pulmonary-renal connection to rapidly eliminate from the lungs and the circulation with any potentially injurious antigens/viruses. However, in cases like MCD in which the pulmonary and/or the renal (podocyte) responses are exacerbated or desynchronized, this mechanism would result in uncontrolled excessive proteinuria.

Limitations of the study

A limitation of our study is the absence of confirmatory biopsies in most patients. However, the use of steroid-sensitive patients allowed us to identify a different pattern in plasma of these subjects in that patients in remission following corticoid treatment resulted in substantially lower surfactant levels in plasma. This would suggest that besides the already characterized local inflammation in the podocyte, one of the potential mechanisms whereby corticoids work in SSNS/MCD may involve ameliorating the pulmonary inflammatory response and the release of surfactants thus preventing the onset of further relapses. We also recognize that other mechanisms are likely involved in SSNS/MCD and the persistence of the proteinuria after the bacterial or viral infection has subsided.

While MCD is commonly preceded by upper respiratory tract infections, just a very small percentage of children with respiratory infections develop nephrotic syndrome. This would suggest the presence of an underlying predisposition to proteinuria in these subjects. While numerous genetic mutations have been identified that affect the podocyte integrity, they do not account for most cases of nephrotic syndrome. In this regard, our group recently demonstrated that endothelial dysfunction is commonly observed in children with MCD which could potentially account for the predisposition of some subjects to nephrotic syndrome (Bauer et al., 2022). More specifically, we found that plasma levels of markers of endothelial glycocalyx degradation (syndecan and heparan sulfate fragments), heparan sulfate proteoglycan cleaving enzymes (heparanase and MMP2), and endothelial activation (vWF and thrombomodulin) are elevated in MCD. Rizzo et al. also demonstrated that glycocalyx degradation in the lung is associated with

surfactant release to the circulation (Rizzo et al., 2022). Based on these observations, endothelial dysfunction in MCD could act as a mechanism favoring the release to the circulation of surfactants from active respiratory tract infections as well as facilitate the interaction of high molecular weight surfactants with podocyte SIRPα.

In summary, the pathogenesis of SSNS/MCD remains unknown. Here, we provide evidence for a respiratory-renal connection, mediated by the release of surfactant proteins from the lung that can induce podocyte activation *in vivo*. Further studies are warranted to investigate this mechanism for proteinuria and nephrotic syndrome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104694.

AUTHOR CONTRIBUTIONS

G.C.-F., R.J.J., and M.A.L. designed the research; G.C.-F., A.A.-H., M.S., M.K., M.B., R.J.J., and M.A.L. analyzed the data; G.C.-F., M.B., R.J.J., and M.A.L. provided key resources; G.C.-F., A.A.-H., C.C., C.B., G.E.G., and M.A.L. performed the research; G.C.-F., M.A.L., and R.J.J. wrote the paper.

DECLARATION OF INTERESTS

Authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. We worked to ensure ethnic or other types of diversity in the recruitment of human subjects. We worked to ensure that the study questionnaires were prepared in an inclusive way. We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti human/mouse SIRPα	Cell Signaling	13,379 RRID:AB_2798196
Anti mouse synaptopodin	Santa Cruz Biotechnologies	SC-21537 RRID:AB_2201166
Anti mouse SP-A	Santa Cruz Biotechnologies	SC-13977 RRID:AB_66194
Anti human SPA (SFTPA)	ThermoFisher	PA5-79986 RRID:AB_2747101
Anti human Nephrin	ThermoFisher	PA5-25932 RRIB:A_2,543,432
Anti human P-Nephrin	Abcam	ab80299
Anti human β-actin	Cell Signaling	4970 RRIB:AB_2,223,172
Anti human SHP2 (PTPN11)	Cell Signaling	3752 RRID:AB_2300607
Goat Anti-Rabbit IgG HRP conjugated	Cell Signaling	7074; RRID:AB_2099233
Goat Anti-Mouse Alexa Fluor 568 conjugated	ThermoFisher	A-11004 RRID:AB_2534072
Goat Anti-Rabbit Alexa Fluor 488 conjugated	ThermoFisher	A-11008 RRID:AB_143165
Chemicals, peptides, and recombinant proteins		
Lipopolysaccharide (LPS)	Sigma	L3012
PolyI:C (HMw)	Invivogen	Tirl-pic
Dexamethasone	Sigma	D4902
Alexa Fluor 488 conjugated Phalloidin	ThermoFisher	A12379
Protein A/G agarose beads	ThermoFisher	20,421
Tris	Sigma	648310-M
Sodium Chloride (NaCl)	Sigma	S9888
EDTA	Sigma	E9884
Nonidet P40 (NP40)	Sigma	21–3277
Sodium deoxycholate	Sigma	D5670
SDS	Sigma	L3771
10x Protease Inhibitor Cocktail cOmplete	Sigma	CO-RO
10x Phosphatase Inhibitor Cocktail PhosSTOP	Sigma	PHOSS-RO
Critical commercial assays		
Mouse SP-A (SFTPA) ELISA detection kit	Novus Biologicals	NBP2-77693
Mouse Albumin Detection Kit, Albuwell	Ethos Biosciences	1011
Human SP-A (SFTPA) ELISA detection Kit	Biovendor	RD191139200R
Human SP-D (SFTPD) ELISA detection Kit	Biovendor	RD194059101
Human IL6 ELISA detection kit	R&D Systems	D6050
Human TNFα ELISA detection Kit	R&D Systems	DTA00D
Experimental models: Cell lines		
Human Podocytes	Obtained from Dr Moin Saleem. University of Bristol	N/A
RPMI 1640	ThermoFisher	11,875
Fetal Bovine Serum	ThermoFisher	A3160402

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pen/Strep	ThermoFisher	15,140,122
Insulin/Transferrin/Selenium	ThermoFisher	41,400,045
Experimental models: Organisms/strains		
"Wild type" C57BL/6J mice	The Jackson Laboratory	#000664 RRID:IMSR_JAX:000,664
Oligonucleotides		
Primers for human IL6 (*il6*) Forward	5′-TCCTACCCCAATTTCCAATGCTC-3′	N/A
Primers for human IL6 (*il65*) Reverse	5′-TTGGATGGTCTTGGTCCTTAGCC-3′	N/A
Primers for human TNFα (*tnfa*) Forward	5′-CCCCAAAGGGATGAGAAGTT-3′	N/A
Primers for human TNFα (*tnfa*) Reverse	5′-CTCCTCCACTTGGTGGTTTG-3′	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed and will be fulfilled by the Lead Contact, Miguel A. Lanaspa (lanaspa@ohsu.edu).

Materials availability

Mouse lines generated in this study are available for any researcher upon reasonable request.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human study

Subjects

Seventeen children (mean age 8, range 3–18 years, 9 boys and 8 girls) were enrolled in the study at the Rocky Mountain Pediatric Kidney Center (Denver, Colorado) of which 14 had SSNS (5 fully steroid sensitive and nine steroid-dependent) and 3 had SRNS. Most children were on corticosteroids and/or other immunosuppressive agents at time of enrollment. Subjects were studied during both relapse and remission. Six had kidney biopsies of which 4 had normal appearing glomeruli, one had mesangial IgM deposits, and one had focal segmental glomerulosclerosis (FSGS). We compared results from urine samples from three healthy volunteers. For the tissue culture studies in Figures 4 and 5, SRNS subjects were excluded and the first six patients that were better age matched (Pt1 to Pt6) with our control analyzed. The study was approved by the Institutional Review Board of the University of Colorado. Informed consents were obtained from both the children and the parents.

Definitions

Nephrotic syndrome (and/or relapse of nephrotic syndrome) was defined as urinary protein creatinine ratio >2.0 mg/mg or 3 + or greater by urine dipstick- or >3 g of protein in 24-h urine collection), serum albumin ≤3.5 g/d, and edema. Remission was defined as negative proteinuria by dipstick or urine protein/creatinine ratio <0.2. Subjects who had been nephrotic but exhibited a sudden drop in proteinuria followed by its resolution within days were considered patients in early remission. The nephrotic syndrome was considered steroid-sensitive if the subject underwent complete remission following 4 weeks with high dose corticosteroid therapy (Lombel et al., 2013). Steroid-dependency was defined as a subtype of SSNS in which subjects underwent remission but relapsed while being weaned from corticosteroid therapy

([Lombel et al., 2013](#)). Steroid-resistant nephrotic syndrome (SRNS) was defined as persistent proteinuria and edema despite corticosteroid therapy.

Study approval

All animal experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals ([National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011](#)). The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado (Aurora, CO).

Animal study

Approximately 8 week old male and female mice (Jackson Labs, C57BL/6J, Cat#000664, RRID:IMSR_JAX:000,664) were used. Mice were subjected to intranasal instillation (by pipette) of lipopolysaccharide (LPS, SIGMA Cat# L3012) and poly I:C (600 μg, Invivogen, Cat# Tirl-pic) mixture (LPS/polyI:C) at a dose of 80 and 240 μg, respectively. This dose is well tolerated by the mice, and mice were kept in metabolic cages for 18 h to allow timed collection of urine. Saline mice received the vehicle (saline) only while unmanipulated mice did not undergo any procedure. Nasal Instillation were given for three consecutive days and tissues collected 3 h after final instillation.

The serum and urine of the mice was assessed for SP-A by ELISA (NOVUS Biologicals, Cat# NBP2-77693). Urine albumin was measured by ELISA (mouse Albuwell kit, Ethos Biosciences, Cat# 1011) and SIRPα (cat# 13,379, Cell Signaling RRID:AB_2798196) by western blot.

The kidneys were fixed in methylcarnoy's solution, and paraffin embedded sections (4 μm) were stained by periodic acid-Schiff (PAS). Cryostat (4 μm) sections were stained by immunofluorescence with anti-SIRPα (cat#13379, Cell Signaling that recognizes both human and mouse RRID:AB_2798196), anti-synaptopodin (cat#sc-21537 Santa Cruz Biotechnology, RRID:AB_2201166) and SP-A (Santa Cruz cat # sc-13977, RRID:AB_66194) followed by respective anti-rabbit Alexa Fluor 488 conjugated (ThermoFisher, A-11008 RRID:AB_143165) or Anti-mouse Alexa Fluor 568 conjugated (ThermoFisher, A-11004 RRID:AB_2534072) secondary antibodies.

METHOD DETAILS

Human studies

Blood samples from patients and control subjects were centrifuged for 5 min at 2400 RPM and serum collected and stored at −80 °C. Urine samples from patients and controls were stored at −80 °C until samples were analyzed. Surfactant Proteins A and D (SP-A and SP-D) levels were measured in serum by ELISA (Biovendor, Cat#s RD194059101 for SP-D and RD191139200R for SP-A) and SP-A was evaluated in urine by western blot (Santa Cruz, cat # sc-13977, RRID:AB_66194). Western blots of human urine were evaluated using SP-A (ThermoFisher, PA5-79986, RRID:AB_2747101) and SIRPα (cat#13379, Cell Signaling RRID:AB_2798196) antibodies respectively and loading 1 mg of creatinine per lane. Urinary albumin and creatinine were measured by autoanalyzer (VETACE, Alfa Wasserman).

Human podocytes

Human immortalized podocytes (provided by Dr Moin Saleem, University of Bristol) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS, ThermoFisher Cat# A3160402), 1% insulin-transferrin-selenium-A supplement (ThermoeFisher, Cat# 41,400,045), penicillin (100 U/mL) and streptomycin (100 μg/mL, ThermoFisher 15,140,122) at 33 °C in 95% air, 5% CO2 and converted to differentiated podocytes by incubating at 37 °C for 10 days ([Saleem et al., 2002](#); [Shimada et al., 2012](#)). Differentiated cells are placed in 1% FBS growth media for 16 h and then incubated with 15% serum or 15% FBS growth media. Serum is passed through a 0.22 μm syringe filter prior to stimulation. Dexamethasone (SIGMA, D4902) was provided for up to 24 h at a 100 nM concentration. IL6 (R&D, D6050) and TNFα (R&D, DTA00D) levels in cell supernatant were detected by ELISA using manufacturer's protocol.

Total Nephrin was assessed by western blot using an antibody from ThermoFisher (Cat#PA5-25932, RRIB:A_2,543,432) while phosphorylated Nephrin was assessed by western blot employing specific antibodies against tyrosine residues 1173 and 1193 (Abcam Cat# ab80299) and normalized by total actin expression (Cell Signaling, Cat# 4970, RRID:AB_2223172. Actin reorganization was assessed by confocal

microscopy using fluorophore-conjugated (Alexa 488) phalloidin (ThermoFisher, Cat# A12379), a toxin that is directed against polymerized actin or F-actin. The microscopy evaluates for the presence of central stress fibers (observed in healthy podocytes) for evidence of disruption of these central fibers with reorganization of actin cytoskeleton laterally at the cortical ring indicative of podocyte activation (Shimada et al., 2012).

Co-immunoprecipitation

Co-IP studies were performed to evaluate whether SHP2 (PTPN11) shifts from SIRPα to Nephrin. Podocytes were incubated with serum control, or from MCD patients in remission or relapse for 2 h. After 2 h, cells were placed on ice and lysed with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors. After quantification, 500 μg of protein lysate was precleared with agarose beads protein A and protein G (ThermoFisher, Cat# 20,421). The precleared supernatant was incubated overnight at 4°C with rotation with 2 μg of anti-SHP2 antibody (Cell Signaling, Cat# 3752, RRID:AB_2300607). The next day, the immunocomplex is pulled down by incubation with 20 μL agarose beads protein A/G slurry following a series of centrifugation/washes with ice-cold PBS and eluted by boiling the samples in 1X western blot loading buffer.

Immunofluorescence and confocal microscopy in human podocytes

Differentiated podocytes were grown on coverslips and treated with sera from patients in relapse and remission for 6-8 h. After the incubation period, media was removed and cells washed three times with ice-cold PBS. Cells were then fixed with 4% paraformaldehyde and 10 mM glycine, permeabilized with Triton X-0.3% in PBS and incubated with phospho-Nephrin antibody (Abcam Cat# ab80299) and Alexa 568 conjugated (ThermoFisher, A-11004, RRID:AB_2534072) secondary antibody. Actin filaments were stained with phalloidin conjugated with Alexa Fluor 488 (ThermoFisher, Cat# A12379).

Electron microscopy

Transmission Electron Microscopy (TEM) was performed using standard procedures. Briefly, tissue was fixed using 2.5% glutaraldehyde followed by 1% osmium tetroxide then dehydrated in a graded ethanol series prior to embedding in EPON 812 epoxy resin. Ultrathin TEM sections were created and collected on copper grids using a Leica UC7 (Illinois, USA) ultramicrotome. The TEM sections were post-stained with UranyLess and lead citrate prior to imaging performed at 120 kV using a JEOL JEM-1400plus TEM (Tokyo, Japan) equipped with a Gatan Model 832 (California, USA) digital camera.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All numerical data are presented as the mean ± SEM Independent replicates for each data point (n) are identified in figure legends. Data graphics and statistical analysis were performed using Prism 5 (GraphPad). Data without indications were analyzed by one-way ANOVA, Tukey post hoc test. A value of $p < 0.05$ was regarded as statistically significant. Animals were randomly allocated in each group using randomizer (www.randomizer.org). Power calculations for the number of animals assigned to each group were based on our previous publications and designed to observe a greater than 15% difference in proteinuria between groups. IN general, an n of six mice per group was used. No animals were excluded from the study and whenever possible experiments were done in a blond fashion. For example, for data analysis, except for western blot, single samples (plasma, homogenates) were first codified and decoded after determination. Similarly, histological records and scoring were done in a blind fashion.