IMMUNE PROFILE OF GLUCOCORTICOID RESISTANT NEPHROTIC SYNDROME PATIENTS

Thesis

SUBMITTED TO
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By

Akhilesh Kumar Jaiswal

DEPARTMENT OF BIOTECHNOLOGY
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW-226 025
AND

DEPARTMENT OF NEPHROLOGY &
DEPARTMENT OF CLINICAL IMMUNOLOGY
SANJAY GANDHI POSTGRADUATE INSTITUTE OF
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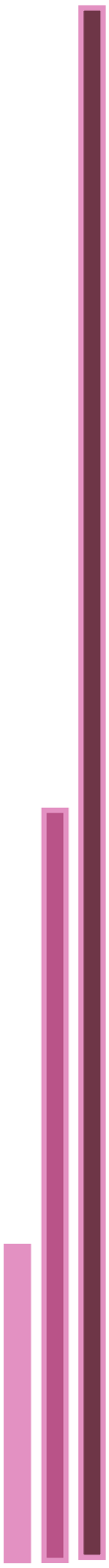

To God, my country and my family

Start by doing what's necessary, then do what's possible;
and suddenly you are doing the impossible.

St. Francis of Assisi

DECLARATION

I, Akhilesh Kumar Jaiswal, declare that the work embodies in the Thesis entitled ***"Immune profile of glucocorticoid resistant Nephrotic Syndrome patients"*** has been carried out by me, under Supervision of Dr. D. R. Modi, Associate Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow and joint Co-supervision of Prof. Narayan Prasad, Department of Nephrology and Prof. Vikas Agarwal, Department of Clinical Immunology of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow.

The work included in this thesis has not been submitted for any other degree and unless otherwise stated, is all original. I have duly acknowledged all the sources used by me in the preparation of the thesis.

Lucknow

Date:

Akhilesh Kumar Jaiswal
Research Scholar
(Enrolment No. 478/11)
Department of Biotechnology,
B.B.A. (Central), University, Lucknow.

Dated: August, 2016

CERTIFICATE

This is to certify that the thesis entitled ***"Immune profile of glucocorticoid resistant Nephrotic Syndrome patients"*** submitted by Akhilesh Kumar Jaiswal is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the **Doctor of Philosophy (Ph.D.)** *regulation-1999 as amended in 2010* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Prof. D. R. Modi
(Supervisor)
Department of Biotechnology
B.B.A. University,
Lucknow

Prof. M. Y. Khan
(Head of Department)
Department of Biotechnology
B.B.A. University,
Lucknow

Prof. Narayan Prasad
(Co-Supervisor)
Department of Nephrology
SGPGIMS, Lucknow

Prof. Vikas Agarwal
(Co-Supervisor)
Department of Clinical Immunology
SGPGIMS, Lucknow

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Akhilesh K. Jaiswal

Contents

Abbreviations

µl	Microliter
µm	Micro Molar
ACE	Angiotensin-Converting Enzyme
APCs	Antigen Presenting Cells
ARBs	Angiotensin II Receptor Blockers
ATP	Adenosine Triphosphate
EDTA	Ethelene Diamine Tetra Acetic Acid
Teff	Effector T cells
ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron Microscope
ER	Endoplasmic Reticulum
ESRD	End-Stage Renal Disease
FBS	Fetal Bovine Serum
FP	Foot Processes
FSC	Forward Light Scatter
FSGS	Focal Segmental Glomerulosclerosis
GBM	Glomerular Basement Membrane
GC	Glucocorticoid
GR	Glucocorticoid Receptor
HEPES	1-(5- Isoquinoline Sulfonyl)-2- Methyl Piperazine
HRP	Horseradish Peroxidase
IFN	Interferon
IL	Interleukin
ISKDC	International Study of Kidney Diseases In Children
LTT	Lymphocyte Transformation Test

MCNS	Minimal Change Nephrotic Syndrome
MDR	Multidrug Resistance
mg	Milligram
ml	Milliliter
MTT	3-[4, 5-Dimethylthiazol-2-Yl]-2, 5-Diphenyltetrazolium Bromide
NF-kB	Nuclear Factor –Kappa B
ng	Nanogram
NK	Natural Killer Cells
NS	Nephrotic Syndrome
PBMCs	Peripheral Blood Mononuclear Cells
PBLs	Peripheral blood lymphocytes
PBS	Phosphate Buffered Saline
P-gp	P-Glycoprotein
PMA	4-Phorbol 12-Myristate 13-Acetate
Tregs	Regulatory T cells
SD	Standard Deviation
SD	Slit Diaphragms
SNP	Single Nucleotide Polymorphism
SRNS	Steroid Resistant Nephrotic Syndrome
SSNS	Steroid Sensitive Nephrotic Syndrome
suPAR	Soluble Urokinase Receptor
Th	T Helper
TMB	Tetramethyl Benzidine
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Introduction

Introduction

International Study of Kidney Diseases in Children (ISKDC) has harmonized most of the clinical knowledge about the childhood idiopathic nephrotic syndrome (NS) since 1967 (ISKDC, 1978; ISKDC, 1979; ISKDC, 1981). ISKDC has also tried to standardize the definitions of various clinical aspects of this syndrome. NS is clinically characterized by the rapid onset of edema, proteinuria, hypoalbuminemia, and hyperlipidemia. The annual incidences of NS are 2-7 cases per 100,000 children and a prevalence of nearly 16 cases per 100,000 children (Eddy AA *et al.,* 2003). Minimal change nephrotic syndrome (MCNS) is the primary histopathological feature of NS which accounts for 90% of NS in children and also for 10–15% of NS cases in adults (Webb NJ *et al.,* 1996; Nachman PH *et al.,* 2008; Hodson EM, 2003). Histolopathologically MCNS is characterized only by podocyte foot process (FP) effacement observed under electron microscope (EM), where the expression of glomerular proteins (ie. podocin, nephrin, α-actinin-4, TRPC6, actin, myosin II, talin, vinculin, α3/β1-integrin and dystroglycans) (Drenckhahn D *et al.,* 1988; Mundel P *et al.,* 1991; Adler S, 1992, Regele HM *et al.,* 2000; Wernerson A *et al.,* 2003; Hingorani SR *et al.,* 2004) are unchanged. Podocyte the final barrier to urinary protein loss, are terminally differentiated cells that line the outer aspect of the glomerular basement membrane (GBM) that maintain podocyte FP and interposed slit diaphragms (SD) (Tryggvason K *et al.,* 2001; Simic I *et al.,* 2013).

Although all NS patients seem to be quite homogenous in biochemical alterations and clinical manifestations, substantial differences are observed within the context of response to steroid and disease relapse pattern. Clinical response to steroid therapy is one of the most important prognostic factors of the disease (Bagga A *et al.,* 2008; Sumant S *et al.,* 2012). Approximately 60–80% of patients who show initial steroid response subsequently become nonresponsive to steroid called secondary non responder. The steroid non responder and frequently relapsing patients progress to later stages of the disease, leading to chronic glomerular sclerosis (Paul SK *et al.,* 2014; Working Group for National Survey on Status of Diagnosis and Treatment of Childhood Renal Diseases, 2014; Ezaki J *et al.,* 2015). Steroid resistant nephrotic syndrome (SRNS) accounts for approximately 15% of all end-stage renal disease (ESRD) in children (Warady BA *et al.,* 1997).

The mechanisms involved in resistance to the steroid therapy in NS may be due to (i) changes in histological characteristics of patients from MCNS to focal segmental glomerulosclerosis (FSGS), a condition with poor response to steroid (ii) alteration in T-cell subpopulation and persistent high levels of inflammatory cytokines (Giuliana L *et al., 2002;* Kanai T *et al.,* 2010; Schnaper HW *et al.,* 1989; Araya C *et al.,* 2009; Yap HK *et al.,* 1999), (iii) podocytes injury which may not be reversed after steroid therapy (Barisoni L *et al., 2007;* Regele HM *et al., 2000)* and (iv) change in pharmacokinetics of the drug is another evolving concept in drug resistance in NS.

Immune dysregulation in MCNS

In 1974, Shalhoub proposed that MCNS is a disorder of CD+ T cell dysfunction resulting in increased lymphocyte derived glomerular permeability factor that leads to alter podocyte ultrastructure.(Shalhoub R, 1974) Hypothesis was supported by the absence of humoral components in glomeruli, responsiveness to steroids, the association of remission following measles infection, and the association of MCNS with Hodgkin's disease.

For several decades, efforts have been made to search for the role of circulating factor(s) in MCNS. It has been observed that soluble factors produced from cultured T cells isolated from patients with MCNS lead to transient proteinuria when injected into rats (Koyama A *et al.,* 1991). It has also been observed that plasma and peripheral blood lymphocytes (PBLs) from NS patients decrease human podocyte synthesis of glycosaminoglycans in vitro. (Birmele B *et al.,* 2001) Moreover, imbalances between CD4+/CD8+/natural killer (NK) cells has been also demonstrated in NS.(Giuliana L *et al., 2002;* Fiser RT *et al.,* 1991).

The $CD4^+$ T helper cells are categorized as effector cells (Th1, Th2 and Th17) and regulatory T cells (Tregs). Imbalance in peripheral blood regulatory and effector T cells (Teff) are linked to cell mediated immune response and may be associated with steroid response in NS.

Giuliana L *et al.* have also suggested that monitoring of Th1/Th2 balance would be useful in evaluating the response to steroid therapy. They have observed

higher levels of soluble interleukin-2 (IL-2) receptors (sIL-2R), IL-2, IFN-γ and TNF-α cytokines during relapse and resistance (Giuliana L *et al., 2002*). In contrast to this observation, Kaneko et al have shown no skewing of Th1/Th2 balance. (Kaneko K *et al.*, 2002). Another study reported predominance of Th2 activity in steroid sensitive nephrotic syndrome (SSNS) and expansion of peripheral activated memory (CD45RO+CD4+CD25+) and suppressor-inducer (CD45RA+CD4+CD25+) T cells in SRNS patients suggesting Th1 and Th2 cell activity. Kanai T *et al.* showed Th2 cells and their cytokines play a predominant role in the pathogenesis of childhood NS (Kanai T *et al.*, 2010). Le Berre *et al.* have shown that the induction of Tregs attenuates idiopathic NS in rats (Le Berre *et al.,* 2009). Araya *et al.* have shown that Tregs suppressor mechanism is deficient and thereby enhance the cytokine release by Teff cells leading to proteinuria and NS (Araya C *et al.,* 2009).

Normally, the expression and release of cytokines by T cells is transient, due to the suppressive effect of active Tregs cells on the Teff cells (Taylor PA *et al.,* 2004; Finger EB *et al.,* 2002; Oaks MK *et al.,* 2000). Recently, it has also been reported that there is an increase in the number of Th17 cells and/or decrease in Tregs in patients with MCD and the dynamic interaction between Th17 and Tregs may be important in the development of NS (Liu LL *et al.,* 2011; Xiao SS *et al.,* 2009; Li YY *et al.,* 2016). However, the role of newly discovered IL-17 and IFN-γ double positive Th17 pathogenic cells are associated with glomerulonephritis, never been seen in NS (Ruihua Z *et al.,* 2013). The Th1, Th2, Tregs, Th17 and pathogenic Th17 cell response has not been studied collectively in NS patients with different phenotypic presentation with sustained remission, relapsing, steroid dependant and resistant course of the disease.

The pharmacogenomics aspect of steroid resistance in NS has also not been elucidated in depth. The factor that modulates the disease response to pharmacological interventions, such as the expression of P-glycoprotein (P-gp) is evolving (Ieiri I *et al.,* 2004). P-gp, a 170kd protein is encoded by the MDR1 gene (ABCB1 ATP-binding cassette, sub-family B, member 1) is located on human chromosome 7q21.12 and play role in response to drugs. (Juliano RL *et al.,* 1976) P-gp works as an energy-dependent efflux pump that plays an important role in the bioavailability and cell-toxicity limitation of a wide range of substances, drugs,

xenobiotics, glucocorticoids, immunosuppressors, and others (Webster JI *et al.,* 2002; Pauli-Magnus C *et al.,* 2004). P-gp is expressed abundantly in many tissues including intestine, blood-brain barrier, liver, kidney, and on peripheral blood mononuclear cells (PBMCs), macrophages, dendritic cells and T and B lymphocytes at varying levels (Klimecki WT *et al.,* 1994; Fung KL *et al.,* 2009; Pechandová K *et al.,* 2006). Resistance to the therapeutic effects of steroid due to increased P-gp expression has been studied in patients with ulcerative colitis (Ho GT *et al.,* 2005), inflammatory bowel diseases (Brant S *et al.,* 2003), and rheumatoid arthritis (Kirkham BW *et al.,* 1991). Over expression of P-gp may be accountable for the poor response to steroid therapy in NS. P-gp overexpression on lymphocytes, the target cells of steroid therapy, leads to efflux of steroid from inside to outside of the cells contributing to poor response to therapy (Wasilewska A *et al., 2006*). MDR1 gene single nucleotide polymorphisms C3435T, C1236T and G2677T/A has been observed to be associated with altered drug disposition and steroid resistance (Wasilewska A *et al.,* 2007; Funaki S *et al.,* 2008; Hyun JC *et al.,* 2011). We have shown strong association of G2677T/A SNPs with steroid resistance in NS patients (Jafar T *et al.,* 2011). Overexpression of P-gp on lymphocytes is associated with poor response to steroid or steroid dependence in NS (Wasilewska A *et al.,* 2006, 2006). Yossef *et al.* has also observed a strong positive correlation between IL2 cytokine and MDR-1 gene expression in SRNS patients and pointed out elimination of glucocorticoid by P-gp as a possible cause of steroid resistance (Yossef DM *et al.,* 2011). The expression of P-gp on the membrane of pluripotent stem cells, dendritic cells, $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, suggest that P-gp may influence cell-mediated immune responses (Frank MH *et al.,* 2001; Shirasaka Y *et al.,* 2006; Grude P *et al.,* 2002). Recently it has shown that high P-gp expressions on pathogenic TH17 cells are refractory to steroid in inflammatory diseases (Radha R *et al.,* 2014). Therefore, we have decided to study the role of P-gp expression on different T cells and its relation with response to steroid therapy in NS patients.

Review of Literature

Definition of Nephrotic Syndrome

NS is one of the most common kidney diseases in children. It is characterized by heavy proteinuria (daily urinary protein ≥3.0–3.5 g/day/1.73m2 BSA in adults (Konder C *et al.,* 2009) or urine protein–creatinine ratio ≥2000 mg/g or ≥300 mg/dL or 3+ protein on urine dipstick in children (KADIGO, 2012) and hypoalbuminemia. Excessive release of serum proteins into urine results in hypoalbuminemia (decrease in serum albumin), edema (swelling of tissues due to fluid accumulation), hypercoagulable state (increased ability of blood clotting) and increased susceptibility to infection.

Classification and etiology of nephrotic syndrome

NS has been classified in to primary or idiopathic NS, and secondary nephrotic syndrome relates to a more clearly defined underlying disease process. Idiopathic NS accounts for 90% of cases of NS in children

Primary Nephrotic Syndrome

- Minimal change nephrotic syndrome (MCNS)
- Mesangial proliferative glomerulonephritis
- Focal segmental glomerulosclerosis (FSGS)
- Immune-complex glomerulonephritis such as IgA nephropathy
- Membranoproliferative glomerulonephritis I-II-III
- Membranous nephropathy
- Congenital nephrosis

Secondary Nephrotic Syndrome

- Bacterial infections - acute post streptococcal nephritis, shunt nephritis and syphilis
- Viral infections - CMV, HBV, HCV and HIV
- Protozoal infections - malaria and toxoplasmosis
- Cardiac and vascular - congestive heart failure, constrictive pericarditis, renal disease and vein thrombosis

- Systemic diseases - amyloidosis, anaphylactoid purpura and diabetes mellitus
- Hereditary disorders- Alport syndrome, nail-patella syndrome, congenital nephrotic syndrome of the Finnish type, familial forms of focal and segmental glomeruloscelrosis
- Drug-induced disorders - nephrotoxins, heavy metals and trimethadione
- Allergens - bee stings, poison ivy and snake venom
- Neoplastic diseases – Hodgkin'sdisease and malignant melanoma

According to the study, 84.5% of all children with NS have histologic features that are compatible with MCNS, 9.5% have focal segmental glomerulosclerosis (FSGS), 2.5% have mesangial proliferation and 3.5%have membranous nephropathy or other etiologies (ISKDC 1979, 81, 82). In children, the major cause of NS is minimal change disease and it occurs frequently between 18 months and 4 years of age, whereas in adults, the most common form of glomerulopathy causing nephrotic syndrome is membranous GN, followed by FSGS.

Synonyms

MCNS is also known as childhood nephrosis, steroid responsive glomerulopathy, nil disease, foot-process disease, lipoid nephrosis and minimal-change nephropathy.

Incidence and Prevalence of MCNS in India and the World

The annual incidence of childhood NS is 2-7 cases per 100,000 in children younger than 16 years and cumulative prevalence rate is approximately 16 cases per 100,000 individuals (Eddy AA *et al., 2003*). The earliest age of NS has been reported in a 7-day old infant (Chang JW *et al.,* 2003). Recent studies suggest that age of disease onset influences the clinical outcomes (i.e., frequency of relapses) and the time to reach long-term remission in childhood SSNS. Age of less than 4 years at onset of SSNS is associated with greater likelihood for frequent relapses and a greater time interwal to attain long-term remission (Kabuki N *et al.,* 1998). MCNS is more common in boys. Almost two thirds of patients with MCNS are boys (ISKDC, 1981; Salcedo JR *et al.,* 1993). MCNS might be over-represented in some populations (e.g., in India), compared to American and European populations (Bhimma R *et al.,* 1997).

Age	3 months to <6 years	6 to <16 years	16 to <35 years	≥35 years
MCNS	87%	53%	25%	15%

Table 1: Influence of age on prevalence of patients with MCNS

MCD is more common in Hispanics, Asians, Arabs and Caucasians than in African–Americans (Bonilla-Felix M *et al.*, 1999; Sharples PM *et al.* 1985; Inguli E *et al.* 1991). In United States of America, diabetic nephropathy with nephrotic syndrome is most common, at an estimated rate of at least 50 cases per million populations. A study from New Zealand found the incidence of nephrotic syndrome to be almost 20 cases per million children under age 15 years (Wong W *et al., 2007*).

Histological characteristics of MCNS

The histological features on light microscopy and immunofluorescence study (IF) do not directly contribute in diagnosis of MCNS. However, the normal morphology on light microscopy and absent immunoglobulin deposit on IF study in presence of nephrotic phenotype suggest diagnosis of MCD. The ultrastructural changes on electron microscopy contribute in diagnosis of MCD.

Light Microscopy

MCNS has glomeruli which appear to be normal with normal capillary walls and normal cellularity on Light microscope (Fig.1). Therefore this disease is also known as minimal or nil lesion disease.

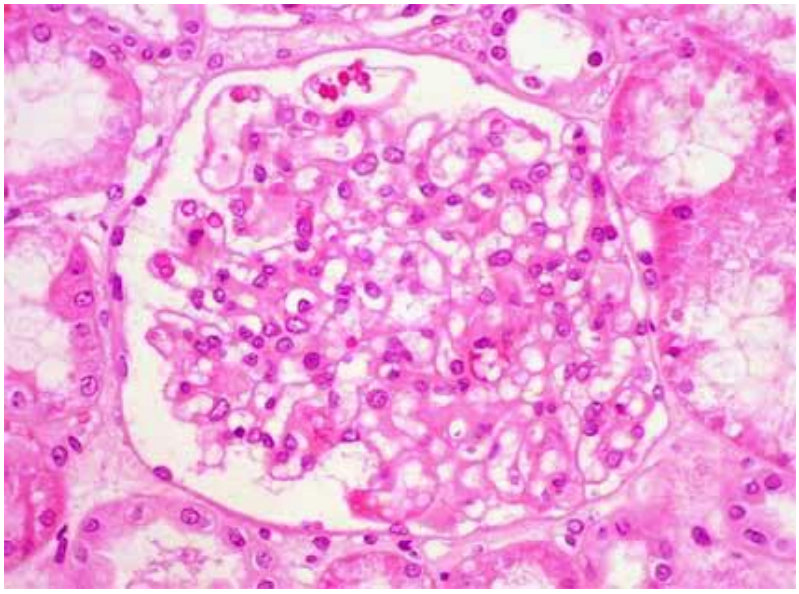

Figure 1: Minimal change disease. *See tuft cellularity and the normal aspect of the glomerulus. Immunofluorescence was negative. (H&E, X400)* (Barisoni L *et al.*, 1999)

Immunoflorescence findings

Immunoglobulins and complement are absent on immunofluorescent analysis of cortical kidney sections in most cases. Some patients with MCNS have mesangial deposition of IgM and prominent mesangial hypercellularity on renal biopsy (Cohen AH *et al.*, 1978) which might be due to the result of passive IgM entrapment and disturbed mesangial macromolecular transport in nephrotic patients (Nadasdy T *et al.*, 2nd edition. 1994). On the other hand, MCNS patients presenting with mesangial IgM deposits have shown association with poor response to steroid therapy (Bhasin HK *et al.,* 1978; Allen WR *et al.,* 1982) and a higher tendency to develop FSGS when compared with MCNS patients whose biopsies are negative for IgM (Zeis PM *et al.,* 2001).

Ultrastructural changes on Electron Microscopy

On Electron microscopy analysis, there is diffuse effacement of the visceral epithelial cell (podocyte) foot processes (Fig. 2 &3) (Roselli S *et al.,* 2004).

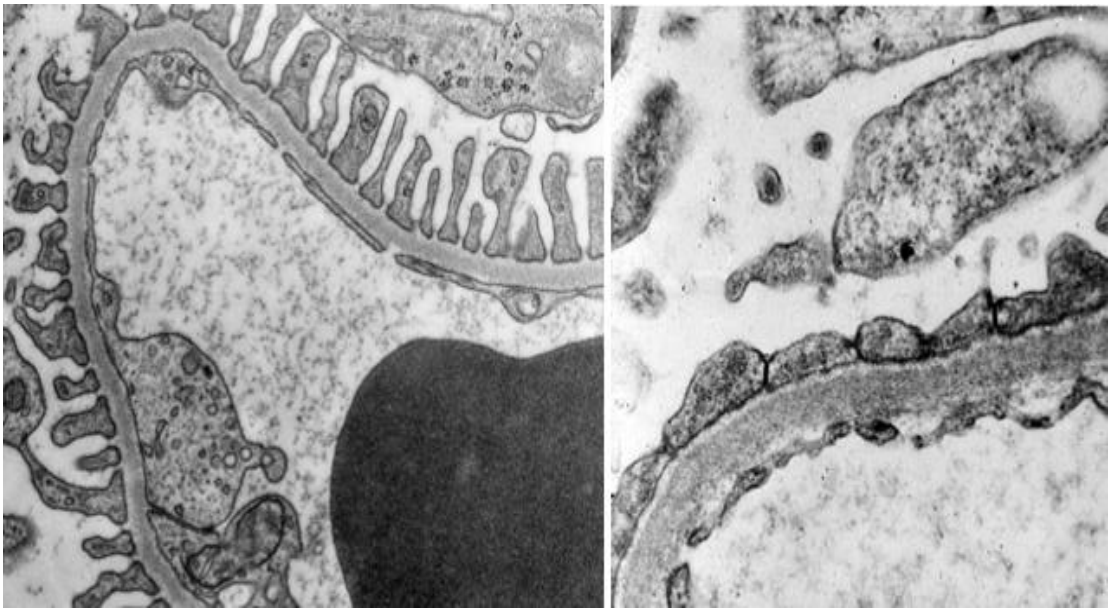

Figure 2: Electron microscopy. On the left, normal aspect with the podocyte foot processes attached to the glomerular basement membrane. On the right, minimal change disease with effacement of foot processes**.**

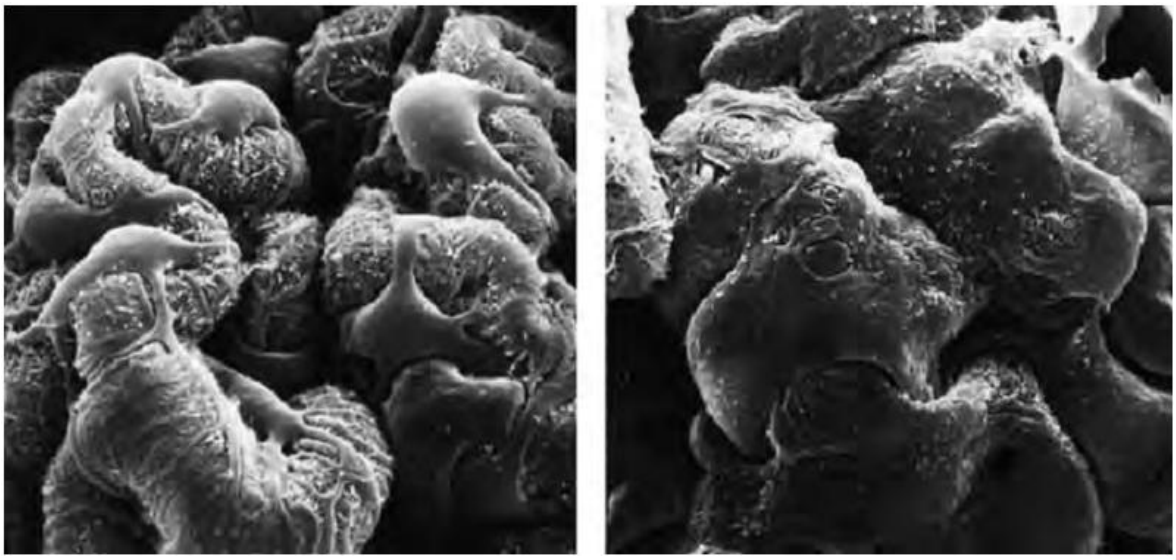

Figure 3: Scanning electron microscopy showing the normal aspect of podocytes with their foot processes on theleft and their effacement in minimal change disease on the right.(Elis D Avner Pediatric Nephrology 7th edition)

Management of Nephrotic Syndrome

Glucocorticoids in Nephrotic Syndrome

Steroids are used to treat NS since 1940's. Steroid response is one of the best prognostic markers for the MCNS. Oral prednisolone used as first line therapy in the treatment of idiopathic nephrotic syndrome. Previously the main two regimes debated were the modified ISKDC regime; and the longer initial steroid induction regime proposed and studied by Ueda et al. and Ksiazek et al. who showed a 2 year relapse free rate of 50% for the long initial prednisolone dose versus 27.3% for the modified ISKDC regime (Ueda N *et al.*, 1988; Ksiazek J *et al.*, 1995).

Modified ISKDC regime

Prednisolone dosage at:

- 60 mg/m2/day (maximum 80 mg/day) for 4 weeks
- 40 mg/m2/48 hours for 4 weeks only.

Long initial prednisolone regime:

Prednisolone dosage at:

- 60 mg/m2/day (maximum 80 mg/day) for 4 weeks
- 40 mg/m2/48 hours for 4 weeks.
- Reduced by 25% monthly over the next 4 months

Treatment of relapse

Prednisolone at 60 mg/m2/day (maximum 80 mg) is to be given until remission defined as urine dipstix is trace or nil for 3 consecutive days after which the prednisolone dose is reduced to 40 mg/m2/48 hours for 4 weeks.

KDIGO guidelines

We recommend that oral prednisone be administered as a single daily dose starting at 60 mg/m2 /d or 2 mg/kg/d to a maximum 60 mg/d. We recommend that daily oral prednisone be given for 4–6 weeks followed by alternate-day medication as a single daily dose starting at 40 mg/m2 or 1.5 mg/kg (maximum 40 mg on alternate days) and continued for 2–5 months with tapering of the dose. (KDIGO, 2012)

Immunosuppressive effect of Glucocorticoids

Glucocorticoid (GC) exerts anti-inflammatory effect by inducing apoptosis of lymphoid cells (Agnes E., 2011). GCs passively diffuse into the cell and bind to the Glucocorticoid receptor (GR) and dissociation of heat shock protein complex (Hsps). Later GS-GR complex gets translocate into the nucleus and competes with NFkB, AP1, STAT5, CREB molecule. Furthermore, it induces the apoptosis of T cells. Also GR can upregulate the Bcl- 2 family protein like Bim and Puma which causes the activation of Caspase-3, -8 and -9 and cathepsin B, which are involved in apoptosis (Herold MJ *et al.*, 2006).

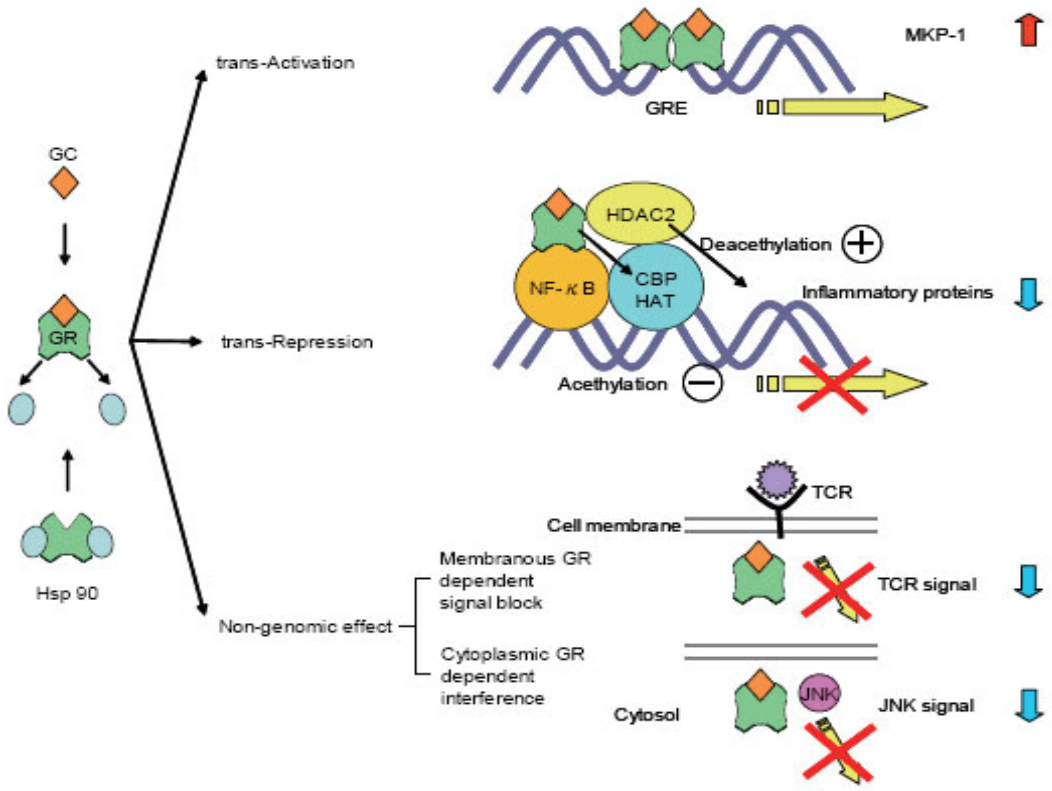

Figure 4: Machanism immune regulation by glucocorticoid (Rossum EF *et al.* 2006).

GC also inhibits the TCR signalling and reduces the effector T cell activity. By inhibition of various inflammatory transcription factors, GC reduces the production of inflammatory cytokines IL-1 beta and TNF-alpha and the 'immunomodulatory' cytokines IL-2, IL-3, IL-4, IL-5, IL-12 and IFN-gamma, as well as of IL-6, IL-8 and the growth factor GM-CSF (Brattsand R *et al.*, 1996). GC also induces the anti-inflammatory molecules like Mapkinases phosphatases (MKP1), which also regulates the activation effector T cell (Herold MJ *et al.*, 2006).

Steroids restore Pododcyte function

MCNS is associated with effacement of foot process of podocyte and steroid therapy restores the podocyte function. Rapid endoplasmic reticulum (ER) stress leads to formation of under-glycosylated nephrin, which remains in the ER as a complex with the chaperones calreticulin/calnexin. Dexamethasone treatment restores synthesis of fully glycosylated nephrin via stimulation of adenosine triphosphate (ATP) production in an energy-depleted cell model, suggesting reversibility of slit diaphragm injuries and foot process effacement (Fujii Y *et al.*, 2006).

Diuretic therapy

It is beneficial for patients with edema. Furosemide may improve edema; but their administration requires expert opinion because of contraction in plasma volume that can lead to hypovolemic shock with aggressive therapy. Metolazone shows additive effectiveness in combination with furosemide to treat resistant edema.

Antihypertensive therapy:

Hypertension is only observed in 20% cases of MCNS. Sometime hypertensions respond to diuretics. Angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) are usually given to reduce proteinuria. But in these settings, they can worsen the kidney function.

Pathogenesis of MCNS

Genetics

Nephrotic Syndrome is considered as infrequent hereditary disorder. However, 3–6% of the cases with NS have an affected sibling (White RH, 1973; Moncrieff MW *et al.,* 1973; Mattoo TK *et al.,* 1990). The cases of SRNS diagnosed in the first year life have at least 66% genetic disorder (Hinkes BG *et al.,* 2007).

Congenital nephrotic syndrome which usually manifests soon after birth is associated with genetic defects. The genes encoding for the slit diaphragm protein are usually affected in these variants of congenital nephrotic syndrome. NPHS1 encodes nephrin, main component of slit diaphragm. Sixteen percentages of the patients have mutated alleles in Finnish population (Kestila M *et al.,* 1998). NPHS2, encoding podocin is responsible for most of infantile SRNS cases. Recessive mutations in this gene account for 42% of familial and 10% of sporadic cases of childhood-onset SRNS (Boute N *et al.,* 2000). Mutations in the ACTN4 and TRPC6 genes, encoding a-actinin-4 (Kaplan JM *et al.,* 2000) and the transient receptor potential cation channel 6 (TRPC6) (Winn MP *et al.,* 2005), respectively, have been involved in this form of NS.

MCNS and the immune system

The immune dysregulation in MCNS remain debatable. This study focuses on the immunological aspects of MCNS. The exact pathogenesis of MCNS remains unknown, and poses one of the most puzzling enigmas in nephrology. Back in 1974, Shalhoub postulated that the abnormality of T cells resulted in the production of a lymphokine(s) that are toxic to the glomerular basement membrane (GBM), resulting in increased glomerular permeability to protein (Shalhoub, 1974). This hypothesis was supported on four clinical observations; (1) remission of MCNS with measles; (2) susceptibility to pneumococcal infection; (3) remissions induced by steroids, and prolonged by cyclophosphamide; and, (4) occurrence of similar glomerular changes in Hodgkin's disease. However, subsequently the studies related to immune dysregulation in NS made the pathogenesis more complex to understand.

Role of circulating factors in MCNS

Later, absence of lymphocytic infiltrates in NS kidney biopsy in SSNS and recurrence of NS after transplantation in cases of FSGS suggested that circulating factors may be responsible for inducing proteinuria in both MCNS and FSGS (Mauer SM *et al.,* 1979, Ingulli E *et al., 1991*). Koyama *et al.* had prepared the T cell hybridoma of MCD relapsing patients and injected into rats which induces glomerular podocyte fusion and proteinuria (Koyama A *et al.,* 1991). Later studies showed the inclusion of interleukin (IL)-2, soluble IL-2 receptor, interferon-gamma (Daniel V *et al.,* 1997), IL-4 (Neuhaus TJ *et al.,* 1995), IL-12 (Lin CY *et al.,* 2004), IL-18 (Matsumoto K *et al.,* 2001), tumor necrosis factor (TNF)-α (Suranyi MG *et al.,* 1993) and vascular endothelial growth factor (VEGF) (Matsumoto K *et al.,* 2001) play role in pathogenesis of NS. Sahali *et al* had reported the increased DNA binding activity of NF-kB. NF-kBa regulates the expression of cytokines which were upregulated during relapses of MCNS. This was associated with down regulation of the regulatory protein IkBa which inactivates NF-kB when they bind with each other (Sahali D *et al.,* 2001). Increased level of IL-4 (Cho BS *et al.,* 1999) and IL-8 (Garin EH *et al.,* 1994) have been detected in mitogen-stimulated PBMCs derived from children with SSNS. Increased expression of IL-4 mRNA was associated with decreased IFN-γ RNA in some children while IL-8 was shown *in vitro* to increase sulphation of the GBM, a process that leads to alteration in the charge selectivity of

the GBM usually observed in MCNS cases. Some studies have also shown that these cytokines can directly induce NS pathology. VEGF increases the permeability of capillaries by promoting release of nitric oxide and later induces endothelial cell fenestration. Injection of VEGF did not induced proteinuria in rats (Webb NJ *et al.,* 1999). Similarly IL- 13-transfected rats develop proteinuria and shows phenotype like MCD (Lai KW *et al.,* 2007) while patients of asthma, psoriasis and allergic dermatitis who had high level of IL-13 did not develop proteinuria. However, infusion of TNF-α induces urinary albumin excretion in rat (Laflam PF *et al.,* 2006) and MCNS patients treated with infliximab (chimeric TNF-α monoclonal antibody) induces remission (Raveh D *et al.,* 2004). Soluble urokinase receptor (suPAR) has recently been identified as circulating factor that may cause FSGS (Wei C *et al.,* 2011). However, Spinale *et al* observed there is no role of suPAR in proteinuria or FSGS (Spinale JM *et al.,* 2015).

Cellular Immunity in MCNS

Role of T cell in pathogenesis of NS

Glomerular injury plays key role in renal insufficiency and aberrantly stimulated immune system causes initiation and progression of glomerular diseases. Lymphocyte Transformation Test (LTT) by mitogens in NS showed that lymphocyte proliferation gets impaired in relapse, while lymphocyte responsiveness to mitogens remain normal during remission (Gupta S *et al.,* 1985, Taube D *et al.,* 1981, Minchin MA *et al.,* 1980, Tomizawa S *et al.,* 1979).

Studies on lymphocyte sub-populations

Th1 and Th2 cells in NS

Th1 cells typically produce IFN-γ, TNF and IL-2 which induce cytotoxicity and inflammatory reactions. In 1959, Hardwicke et al had reported that NS patients have pollen hypersensitivity. Similarly allergies developed due to vaccinations (Kuzemko *et al.,* 1972), food and insect stings (Tareyena IE *et al.,* 1982) have been shown to be associated with NS. Incidence of atopy was reported higher in patients with idiopathic NS than in healthy subjects, ranging from 17–40% in MCNS patients compared with 10–23% in age-matched control subjects (Groshong T *et al.,* 1973).

By contrast, Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 which are associated with the regulation of strong antibody and allergic responses (Abbas AK *et al.*, 1996). In asthma, cytokines IL-10 and IL-13, produced by activated Th2 lymphocytes, act directly on pulmonary fibroblasts and bronchial epithelium and thereby cause an important part of the phenotype.

Atopic dermatitis and allergy is common in MCNS patients, which show importance of Th2 response in Nephrotic syndrome. Later studies have shown that increased Th2 population (Davin JC *et al.,* 2011; Kanai T *et al.,* 2010; Van den *et al.,* 2004) and high titre of serum IgE and IgG4 in MCNS patients (Yokoyama H *et al.,* 1985; Warshaw BL *et al.,* 1989, Kimata H *et al.,* 1995) which suggest that MCNS is Th2 mediated disease. Kaneko *et al.* reported no significant difference in T cell subsets like Th0, Th1 and Th2 or the ratio of Th1/Th2 in relapse, remission and normal control population (Kaneko K *et al.,* 2002). However increase in memory T-cells (CD45RO+CD4+, CD45RO+CD8+), activated CD4+T-cells, CD3+CD25+T-cells (IL-2 receptor positive T-cells), natural killer cells (CD16+) and subset of B-lymphocytes have been reported to be associated with relapse of MCNS patients (Yan K *et al.,* 1998; Daniel V *et al.,* 1997; Neuhaus TJ *et al.,* 1995; Kobayashi K *et al.,* 1994; Tani Y *et al.,* 1982). However, studies during relapse of MCNS have also shown decrease in total count of CD4+ and CD8+ T-cells (Daniel V *et al.,* 1997; Hulton SA *et al.,* 1994; Ozaki T *et al.,* 1992; Fiser RT *et al.,* 1991).

Role of T regulatory cells in Nephrotic syndrome

Tregs induces peripheral tolerance against self-antigens. Transcription factor Foxp3 controls Treg development and function. Dysfunctional mutation in Foxp3 gene leads to development of autoimmunity called as Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome.

Tregs produce cytokines IL-10, IL-35 and TGFβ1 which suppress the activation of different effector T cells. It kills effector T cells by producing Granzyme b and perforin or upregulating tryptophan metabolite. Tregs modulates the activation, differentiation, and effector functions of Teff cells and antigen presenting cells (APCs), by inducing their apoptosis (Caridade *et al.,* 2013).

Reference	Disease	Population	Immunopathogenic findings
Benz K et al 2010	MCD or FSGS	38 children	More infiltrating T cells when compared to controls but reduced number of infiltrating Foxp3+ T cells in both MCD and FSGS patients
Bertelli R et al 2011	INS	41 children	Decreased numbers of peripheral CD39+Foxp3+ Treg cells and impaired ATP catabolism
Araya C et al 2009	MCD	21 Adults	Similar number of Foxp3+ Treg cells compared to controls but reduced suppressive function during active disease
Liu LL et al 2011	MCD	25 adults	Increased circulating Th17 cells over Treg cells when compared to controls, which correlated with severity of proteinuria. Ratios reverted upon steroid therapy in most patients
Xiao SS et al 2009	INS	36 children	] Increased circulating Th17 cells over Treg cells when compared to controls, together with increased intra renal IL-17 expression
Hashimura Y et al 2009	MCD + IPEX	5-year-old boy	The occurrence of MCD in IPEX syndrome, where Treg cells are deficient, suggests a possible pathogenic association

Table 2: Studies supporting Treg cell involvement in the immunopathogenesis of minimal change disease.

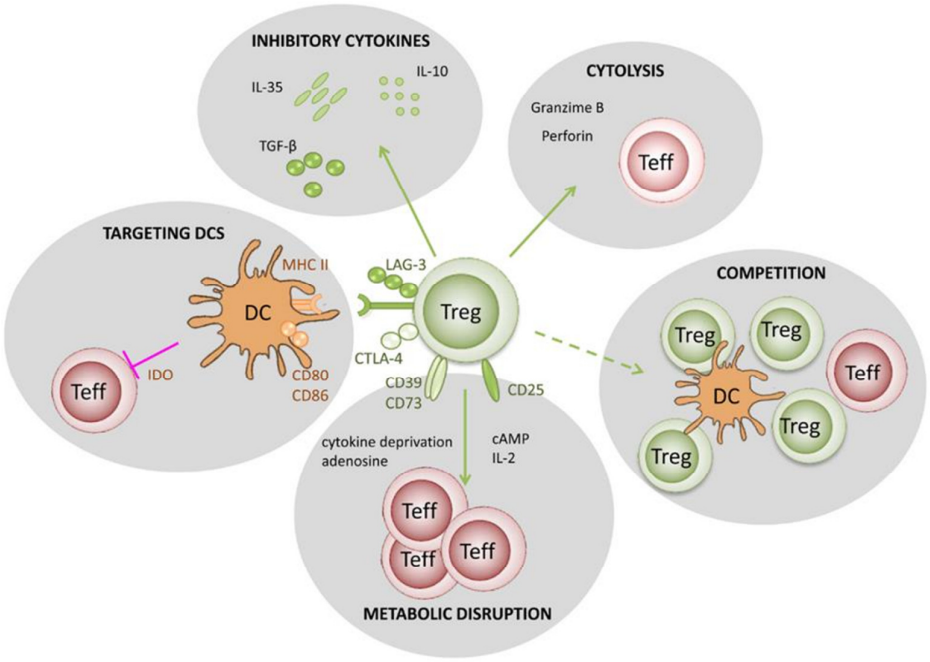

Figure 5: Mechanism of immune regulation by regulatory T cell (Caridade et al. 2013)

Impaired regulatory T cell function leads to prolonged CD80 expression on podocytes, which is major player in glomerular injury (Abdel-Hafez M *et al.,* 2009, Ishimoto T *et al.,* 2011). Normally, the expression and release of cytokines by T cells is transient, due to the suppressive effect of active Tregs cells on the Teff cells (Taylor PA *et al.,* 2004; Finger *et al.,* 2002; Oaks MK *et al.,* 2000). One of the study reported that frequency of Tregs cells was significantly lower in patients with NS than in normal controls and that it was markedly decreased in patients with NS compared to patients with isolated hematuria (Xiao SS *et al.,* 2009). Increased infiltration of effector T cell and decreased $Foxp3^+$ Tregs in MCNS has been reported (136 Benz et al. 2010). Bertelli *et al* reported that cytokines produced by effector T cell induces release of ATP, a pro-inflamatory molecules which is hydrolysed by CD39+Foxp3+ Tregs cells. Increase in effector cell cytokine and reduced number of CD39+Foxp3+ Tregs cells in NS results into increase in ATP, which induces podocyte injury (Bertelli R *et al.,* 2011). Another study has shown that number of Foxp3+ Tregs remains similar during relapse and remission of patients but their suppressive potential are reduced (Araya C *et al.,* 2009). In rat model of NS, treatment with LF15 reduced the proteinuria which was attenuated by Tregs (Le berre *et al.,* 2009). We have also found increased frequency of T regulatory cells in remission state of NS (Prasad N *et al.,* 2015). (Table.2)

Th17

Th17 cells have emerged as an important mediator in inflammatory and autoimmune diseases. These cells have been characterized as preferential producers of interleukin (IL-17A; also known as IL-17), IL-17F, IL-21, IL-22, and IL-26 in humans (Ouyang W *et al.,* 2008). Retinoid orphan nuclear receptor (RORc), which encodes the human ortholog of mouse RORrt, is a key regulator of Th17-cell lineage differentiation (Ivanov II *et al.,* 2006). Interleukin-1β, IL-6, and IL-23 are the inducers and TGF-β is the inhibitor of Th17 differentiation in humans (Wilson NJ *et al.,* 2007). Th17-related cytokines such as IL-17, IL-6 and IL-1β have been found in patients with, MCNS. Moreover, the increased expression of IL-17 in kidney biopsy tissue may suggest that an increased number of cells that mainly produce IL-17 may play a role in the NS microenvironment (Xiao SS *et al.* 2009). Th17/IL-17 may contribute to the pathogenesis of NS by decreasing the podocalyxin level, which is required for formation/maintenance of foot processes (Doyonnas R *et al.,* 2001). IL-17 induced decreased level of podocalyxin which induces podocyte apoptosis (Wang L *et al.,* 2013). A few studies suggested that increased circulating Th17 over Tregs increases the severity of NS which reverts upon steroid therapy (Liu LL *et al.,* 2011, Xiao SS *et al.* 2009).

Podocyte: New player in pathogenesis of NS

Various genetic studies support that mutation in podocyte genes leads to NS. Podocytes are terminal differentiated epithelial cells present on outer side of glomerular basement membrane (GBM) and form the barrier for urinary protein loss by forming slit diaphragms. Podocyte foot processes (FPs) forms the slit diaphragms (SDs). These SDs acts as main selective permeable barrier of kidney (Tryggvason K *et al.,* 2001). Podocyte FPs contains contractile and dynamic apparatus consisting of actin, myosin II, α-actinin-4, talin, vinculin, and synaptopodin (Drenckhahn D *et al.,* 1988; Mundel P *et al.,* 1991). The FPs are anchored to the GBM via α3/β1-integrin (Adler S *et al.,* 1992) and dystroglycans. Our knowledge of SD structure is based on genetic studies of familial NS, which led to identification of SD proteins such as podocin, nephrin, α-actinin-4, and TRPC6. The genes for these proteins may be mutated in inherited NS (Orth SR *et al.,* 1998). Urinary protein loss is due to defective SD between podocytes and this is due to reduced expression of nephrin protein, which further leads to detachment in FSGS cases (Tojo A *et al.,* 2012). However, it still

remains a mystery that how albumin diffuse across the effaced podocyte FPs in MCD. In MCD 80% of the podocyte are effaced. The effacement of podocyte is due to reduced expression of tight junction (Lahdenkari AT *et al.*, 2004).

Role of CD80 expression on podocytes in NS

CD80 is a costimulatory molecule of T-cells and regulate the T-cell response. During interaction with CD28, T cell response is activated, while binding of CD80 to CTLA4 terminates the T cell response (Wing K *et al.*, 2008). MCD is associated with pronounced expression of CD80 on podocytes and increased urinary excretion of CD80 (Garin EH *et al.*, 2009). In LPS induced MCD model, increased CD80 expression on podocyte leads to effacement of podocyte (Reiser J *et al.*, 2004). Also IL-13 (Yap HK *et al.*, 1999) and TLR3 ligand induced the expression of CD80 molecule. CTLA-4 and IL-10 inhibits the CD80 expression (Wing K *et al.*, 2008), and resolves proteinuria. MCD patients with dysfunctional Tregs, have upregulated CD80 on podocyte and persistent proteinuria. The urinary soluble CD80/CTLA-4 ratio was >100-fold higher in MCD relapse patients as compared to patients in remission (Garin *et al.*, 2009).

Non-responsiveness of steroid therapy

As per ISKDC, NS in children is defined as proteinuria of 40 mg/m2/hr or spot urine protein (mg)/ creatinine (mg) ration of 2 in first morning urine sample. The remission of NS is defined by urinary protein excretion < 4 mg/m2/hour or urine dipstix nil/trace for 3 consecutive days. The relapse is defined by urinary protein excretion > 40 mg/m2/hour or urine dipstix ++ or more for 3 consecutive days. Frequent relapse is defined by 2 or more relapses within 6 months of initial response or four or more relapses within 12 months period. The steroid dependence is defined by two consecutive relapses occurring during the period of steroid tapering or within 14 days of its cessation. Relapses are treated with daily prednisone 60 mg/m2 until remission will be achieved, followed by 40 mg/m2 on alternate days for 4 weeks and then gradual tapering of steroid. In steroid-dependent patients, maintenance alternate day prednisone is given. The alternate day dose is gradually tapered-off to determine each patient's individual threshold at which relapse occurred. The steroid resistant is defined as no response to therapy after 8 weeks of high dose (60 mg/m2) of prednisolone therapy.

Role of P-glycoprotein in response to steroid therapy

P-gp upregulation has been reported to be associated with resistance to a variety of drugs including glucocorticoids (Bates SE *et al.,* 1996). P-gp is expressed in almost all the tissues including lymphocytes, the main targets of pharmacotherapy in NS (Coon JS *et al.,* 1991, Wacher VJ *et al.,* 1995). P-gp play an important role in protecting host tissue from toxic side effects and the overexpression of P-gp results in the reduction of the concentration of peptides, alkaloids, steroids, immunosuppressive drugs, and calcium channel blockers (Tsuruo T *et al.,* 1993; Ueda K *et al.,* 1987; Ueda K *et al.,* 1992).The treatment resistance with increased expression of P-gp is reported in many diseases including malignancies, systemic lupus erythematosus, inflammatory bowel disease and other autoimmune diseases (Beck WT *et al.,* 1996; Gottesman MM *et al.,* 2002; Farrel RJ *et al.,* 2003; Richaud- Patin *et al.,* 2004).

P-gp is a 170-kD product coded by multidrug resistance-1 (MDR-1) gene is a component of biological barriers belonging to the ATP binding cassette superfamily of transport proteins (Dilger K *et al.,* 2004; Juliano RL *et al.,* 1976). P-gp is richly expressed in most of the tissues, in the haemopoietic system and peripheral blood lymphocytes, which are the putative targets of pharmacotherapy in NS (Coon JS *et al.,* 1991; Wacher VJ *et al.,* 1993).

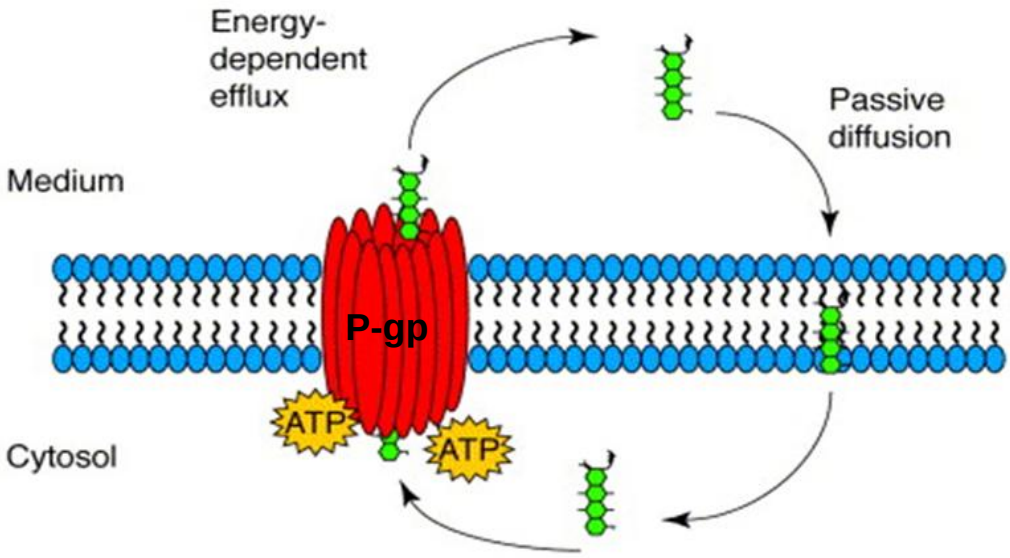

Figure 6: Active transport of substrate by P-glycoprotein molecule in energy-dependent manner using ATP. (Johnstone *et al.* 2000)

While studying the P-gp expression on children with nephrotic syndrome in relation to their clinical response to steroid it has been found that elevated levels of P-gp are associated with diseased group (Wasilewska A *et al.,* 2006). However MDR-1 expression in lymphocytes has been reported to be negatively correlated with response to prednisone, cyclophosphamide and cyclosporine A in NS children (Stachowski J *et al.,* 2000). However, the serial monitoring of P-gp expression on lymphocytes in different phenotypes of NS relapsing, resistant and during remission has not been study in any single study.

P-gp is expressed abundantly in many tissues including intestine, blood-brain barrier, liver, kidney, placenta, and on PBMCs, macrophages, natural killer, dendritic cells and T and B lymphocytes at varying levels where it exerts its protective effects (Klimecki WT *et al.,* 1994; Fung KL *et al.,* 2009; Pechandová K *et al.,* 2006).

A large number of studies have reported that certain MDR-1 single nucleotide polymorphisms (SNPs) are associated with altered drug disposition (Ieiri I *et al.,* 2004; Woodahl EL *et al.,* 2004). MDR1 single nucleotide polymorphisms C3435T, C1236T and G2677T/A are the most widely investigated and associated with altered drug disposition in NS (Jafar T *et al.,* 2011). The putative genetic regulation of MDR-1gene expression and P-gp function has yet not been clearly studied in nephrotic patients. The genetic polymorphisms reported within the MDR-1 sequence, showed strong linkage disequilibrium (Soranzo N *et al.,* 2004). In a study it has been reported that 3435 T allele is a marker of steroid response (Zheng H *et al.,* 2002), and it has been found that children homozygous for the T allele were more likely to be weaned of steroids one year after transplantation than patients with 3435 CC and 2677 GG genetic polymorphism. However, in another Japanese study the femoral head osteonecrosis after renal transplantation reported to be weakly associated with TT genotype in positions 3435 and 2677 (Asano T *et al.,* 2003). More recently it has been reported that TT genotype required longer duration of therapy for proteinuria cessation after the oral steroid initiation (Wasilewska A *et al.,* 2004), and a trend for association with relapse rate was also observed. These results support the belief for relationship between the TT genotype and P-gp expression. It was stated that individuals who are homozygous T/T or A/A genotype for the G2677T/A polymorphism also had lower placental P-gp expression (Wasilewska A *et al.,* 2004).

The C3435T polymorphism is located in the exon 26 of the MDR-1 gene and is silent polymorphism causing no amino acid changes (Ile/Ile). However, in many studies it was shown that individuals having the homozygous T/T genotype have lower P-gp expression in various normal tissues (Tanabe M *et al.,* 2001; Ameyaw MM *et al.,* 2001; Hitzl M *et al.,* 2001). Recent studies have shown that T/T genotype minimize P-gp activity in a substrate-dependent manner (Salama NN *et al.,* 2006).

Yossef et al. had observed a strong positive correlation between IL2 cytokine and MDR-1 gene expression in SRNS patients and pointed out that elimination of glucocorticoid by P-gp as a possible cause of steroid resistance(Youssef DM *et al.,* 2011) The expression of P-gp on the membrane of pluripotent stem cells, monocytes, dendritic cells, CD4+ and CD8+ T lymphocytes, NK cells, and B lymphocytes, suggest that P-gp may influence cell-mediated immune responses (Frank MH *et al.,* 2001; Shirasaka Y *et al.,* 2006; Grude P *et al.,* 2002). Recent studies have shown that higher P-gp expression on pathogenic TH17 cells is refractory to glucocorticoids in inflammatory diseases (Radha R *et al.,* 2014). Thus, it was worth studying the role of P-gp expression on different T cells in response to steroid therapy in NS patients.

Aims and Objectives

Aims and Objectives

The aim of the study was to investigate the role of CD4+T cell subsets and P-glycoprotein efflux pump in pathogenesis and steroid resistance of NS.

Objectives

I. To demonstrate the frequency of different T cell and their respective cytokine profiles in patients with sustained remission, during relapse and steroid resistant NS.

II. To demonstrate the expression of P-gp in NS patients after steroid therapy and correlate its expression with alteration in the frequency of different T cell.

Materials & Methods

Patients and Healthy Controls

From the prevalent patients of childhood biopsy proven MCD (n=105), 35 were in sustained remission, 35 during relapse and remaining 35 SRNS patients were randomly selected for inclusion in the study. Of them 3 patients from remission group, 7patient from relapse group and 5 patients from SRNS group were excluded because they did not consent for inclusion in the study and 3 patients from relapse group 2 patients in SRNS has family history of NS. Thus, 32 (boys 29, median age 6 years, range3-16 years) NS patients in sustained remission, 25 (boys 21, median age 7 years, range4-15 years) patients during relapse, and 28 (boys 26, median age 7.5 years, range6-16 years) SRNS patients remained for the study purpose. Fifteen (boys 13, median age 9 years, range 5-15 years) healthy children of same ethnicity were included as control in the study. All patients had renal biopsy and tissue histology was examined on light microscopy, immunofluorescence and electron microscopy; and patients with MCNS only were included in the study.

Inclusion Criteria:

Childhood onset NS (ISKDC definition) with biopsy proven MCNS

Age 2-16 years

Informed consent from patient's parent/ guardian

Exclusion criteria:

Viral infections such as HBV, HCV and HIV

Family history of NS

Abnormal complement/ low complement levels

Findings suggestive of secondary glomerular disease on renal biopsy

The definitions of NS, remission, relapse and steroid resistance were based on established criteria according to the International Study for Kidney Diseases in Children. (ISKDC, 1978)NS in children was defined as proteinuria of 40 mg/m^2/h or,

ratio of 2 for spot urine protein (milligram)/creatinine (milligram) in the first morning urine sample with hypo-albuminemia (serum albumin <2.5 g/dL) and presence of edema. Remission of NS was defined by urinary protein excretion <4 mg/m^2/h or urine dipstick nil/trace for three consecutive days and patients were defined in sustained remission if remission persists for at least of 6 months after stopping steroid. Relapse was defined as urinary protein excretion >40 mg/m2/h or urine dipstick ++ or more for three consecutive days. Primary steroid resistance was defined as unresponsiveness of 60 mg/m^2 body surface area per day for 4 weeks of prednisolone therapy. Secondary steroid resistance was defined as no response to 4 weeks of daily prednisone therapy at a dose of 60 mg/m^2/day in a child previously known to have a steroid-sensitive course. With regard to SRNS, patients enrolled in this study did not have any (i) underlying secondary causes, they were negative for HBSAg surface antigen seropositivity, anti-HCV seropositivity and HIV seropositivity and had normal serum complements (C3 and C4) levels. An informed consent was obtained from a parent or guardian of both patients and controls when participant <15 years and from the participant when age is >15 years as per institute guidelines. This study was approved by the Institute Ethics Committee, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, INDIA (PGI/DIR/RC/186/2011).

Sample collection

Total 8 ml peripheral blood was collected from patients and controls. Of which 5 ml in heparinized vial for flowcytometer experiments, and peripheral blood mononuclear cells (PBMCs) isolation for culture experiment and 3 ml in plain vial for serum collection. Serum sample were stored at -80^0C in aliquots till analysis. Clinical assessments such as relapse of disease, SRNS were performed by a nephrologist.

Analysis of T cell phenotype and P-gp expression by Flowcytometry

Flowcytometry: basic principle

Flowcytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity and relative fluorescence intensity.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

- The fluidics system transports particles in a stream to the laser beam for interrogation.
- The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
- The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

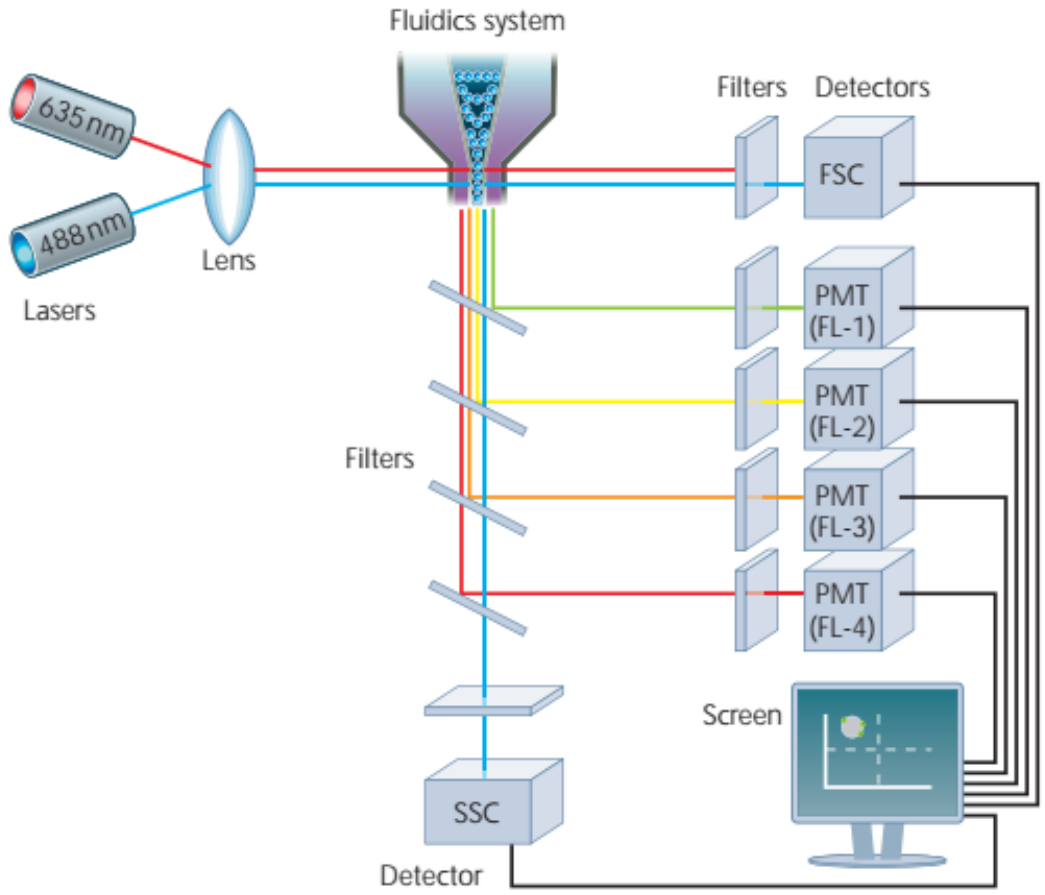

Figure 7: Schematic overview of a typical flow cytometer setup

If there a fluorochrome labeled monoclonal antibody (mAb) associated with the cell, it becomes excited by the laser and a fluorescent emission results. The resulting signals are processed to gather information about the relative size of the cell (forward light scatter; FSC), its shape or internal complexity (side light scatter; SSC) as well as a diversity of cellular structures and antigens (fluorescence).

Enumeration of T cell phenotype and P-gp expression from whole blood

For analysis of Th1, Th2, Th17 and pathogenic Th17 cells, whole blood was stimulated with mitogen, while for Tregs unstimulated blood was used. Further whole blood was stained with their specific markers (Appendix I).

Intracellular staining for Th1, Th2and Th17 lymphocytes

Helper T cell phenotyping was done according to previously described study (Duramad P *et al.*, 2004) Whole blood was diluted 1:1 with RPMI 1640 culture media and stimulated with phorbol 12-myristate 13-acetate; PMA (20ng/ml; Sigma Aldrich, Saint Louis, Missouri, USA) and ionomycin (1µg/ml; Sigma Aldrich, Saint Louis, Missouri, USA) for 5hours at 37^0 C. Monensin (2 µM; BD Biosciences, San Diego, CA) was also added for the final 2h of activation as a protein transport inhibitor**.** FITC-conjugated Mouse Anti-Human CD4 and PE-conjugated Mouse Anti-Human P-gp20µleach mAb were added in 100 µl of stimulated blood for surface staining of CD4and P-gp for 30 minute at room temperature**.** After surface staining, RBCs were lysed by incubation in 1ml 1X BD FACS lysing solution for 10 minutes. Cells were then washed, fixed and permiabilize with Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instruction. After washing, Alexa Fluor 647-conjugated Mouse Anti-Human IFN-γ mAb for Th1, APC-conjugated Mouse Anti-Human IL-4mAb for Th2 and PerCP-Cy5.5-conjugated Mouse Anti-Human IL-17AmAb for Th17 cells were added for intracellular cytokine staining. FITC-conjugated Mouse IgG1-k, PE-conjugated Mouse IgG1-k, Alexa Fluor 647-conjugated Mouse IgG1-k, PerCP-Cy5.5-conjugated MouseIgG1-k and Allophycocyanin-conjugated Mouse IgG1-k isotypes were used as control. At least 10,000 lymphocytes were acquired on BD FACS Calibur (Becton Dickinson, Mount View, CA, USA) for each sample and analyzed with FlowJo (Ashland, OR, USA).Th1 and Th17 cells were analyzed in the same tube whereas for the Th2 cells a separate tube was used.

Intracellular staining for regulatory T lymphocytes

For the frequency analysis of T regulatory cell, 100µl whole blood was incubated with a cocktail of 3 mAb directed to CD4 (FITC), CD25 (PerCP-Cy 5.5) and P-gp (PE) 20µl each for 30 minute at room temperature. Then RBCs were lysed with 1ml

1X BD FACS lysing solution for 10 minutes. For intracellular staining of FoxP3, cells were subsequently fixed and permeabilized with BD Human FoxP3 Buffer Set according to the manufacturer's guidelines before 5µl Alexa Fluor 647-conjugated Mouse Anti-Human FoxP3mAb was added. Isotype-matched antibodies were used as controls (Muthu R *et al.,* 2011). A minimum 50,000 events in lymphocyte counts were acquired on a FACS Calibur (Becton Dickinson, CA, USA) flowcytometry and analyzed with FlowJo (Ashland, OR, USA).

Gating strategy of Flowcytometric data analysis

Whole blood is stained for analysis of T helper subsets. Lymphocyte population were gated according to size and granularity on Dot plot. FITC conjugated CD4+ cells were gated in FL1 channel. Tregs were characterised by CD25+ and FoxP3+ expression (Appendix I). CD4+CD25+ FoxP3+ Tregs were analysed further for P-gp expression. For Th1, Th2 and Th17 cells, we analysed the CD4+ T cells expressing IFN-γ, Il-4 and IL-17 respectively. For the pathogenic Th17 cell we analysed according to IL-17 and IFN-γ double positive CD4+ T cells. After gating double positive cells we then analysed for P-gp expression in FL2 channel. (Figure 8)

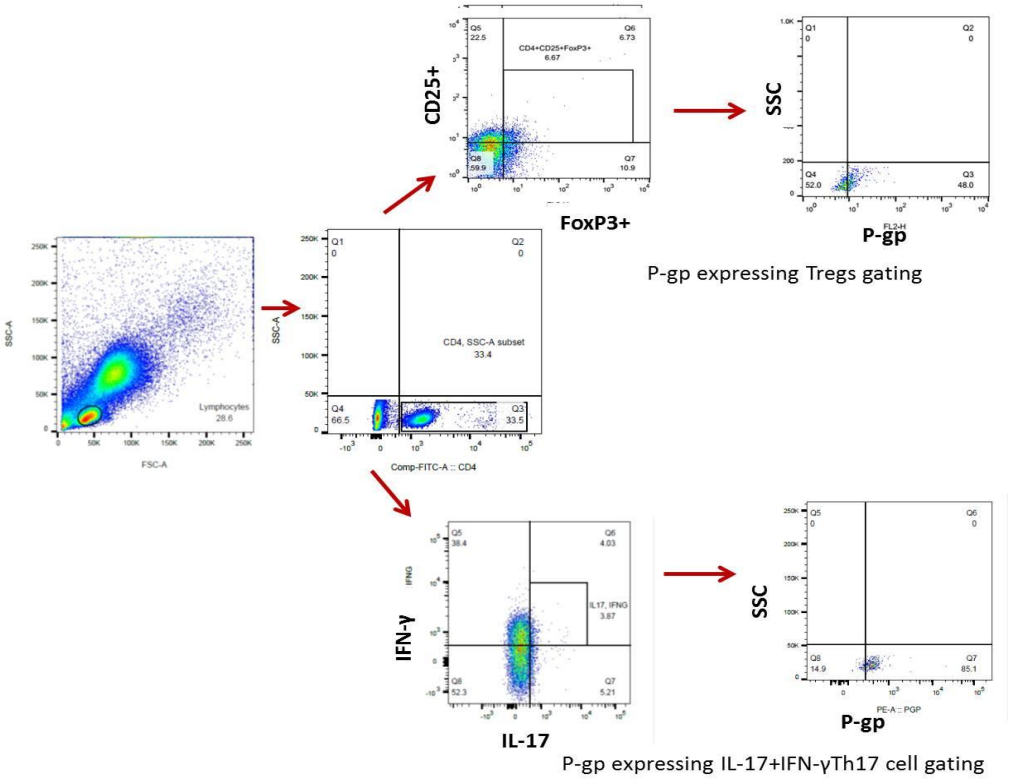

Figure 8: Gating strategy for analysis of different T cell subsets with P-gp expression

Serum cytokine analysis by Enzyme-linked immunosorbent assay (ELISA)

The serum of patients and controls were assayed for IFN-γ, IL-4, IL-6, TNF-α, IL-10, TGF-β (Becton Dickinson, USA) and IL-17A (R & D System, USA) by sandwich enzyme-linked immunosorbent assay using commercial kits as per manufacturer's protocol. Absorbance was measured at 450 nm within 30 minutes of stopping reaction in a microtiter plate reader (Biorad Laboratories, USA). Concentration of the samples were calculated according to respective standards of the kit by plotting graph on the excel sheet.

In vitro production of cytokines by PBMCs

The mitogen stimulated PBMCs were cultured and supernatant was analyzed for various cytokines as has been described previously (Giuliana L *et al.,* 2002; Schnaper HW *et al.,* 1989l; Yap HK *et al.,* 1999).

Separation of PBMCs

PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St Louis, USA. Heparinized blood was diluted with equal volume of RPMI 1640 containing Hepes (25 mM), gentamicin (50pg/ml) and10% heat-inactivated fetal calf serum (FCS). Diluted sample were carefully layered upon Histopaque-1077 in a ratio of 3:1 and the tube was centrifuged at 1800 rpm for 30 minutes. The interface containing mononuclear cells was collected and washed thrice at 1500 rpm for 15 minutes, 1200 rpm for 12 minutes and finally at 1000 rpm for 10 minutes. Finally cell pellet was suspended in 1 ml RPMI 1640 and cells were counted using Neubauer's chamber and used for further experiments.

Optimization of Cell Viability Assay for PBMCs culture

Cell viability was assessed by mitochondria-dependent reduction of a yellow tetrazolium dye, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] (Sigma, USA) to insoluble purple formazan by dehydrogenases (Mosmann TR *et al.,* 1983). PBMCs were plated in 96-well tissue culture plate in

triplicate at a concentration of 10^5cells/well and stimulated with PMA (1, 10, 50, 100, 500ng/ml) and ionomycin (0.1, 1, 5, 10 µg/ml). Following incubation at 37^0C in 5% CO_2 for 24 h, medium was discarded and 10 µl of MTT stock solution (5 mg/ml) and 100 µl of fresh RPMI was added to each well. Following 3-4 hours of incubation, remaining dye was extracted with 100 µl/well of lysis buffer (10% SDS (w/v), 45% dimethyl formamide (v/v) adjusted to pH 4.5 by glacial acid). The optical density of the resulting supernatant was measured photometrically at 570 nm. The respective median effective concentration (EC50) values for the test items were determined from the percentage of the test item relative to the negative control. All the calculations were done using only media as negative control and dose of maximum stimulation as 100%. Concentration of PMA (50 ng/ml) and ionomycin (1 µg/ml) was sublethal and had shown effective stimulation of the cells. The above concentration of PMA and ionomycin was taken for the stimulation of PBMCs.

Stimulation of PBMCs

Cells were resuspended at 1×10^6cells/ml in RPMI 1640 supplemented with2 mM of extra glutamine and 10% FCS. The viability of the cells was checked by trypan blue. In vitro culture of the PBMCs was performed with and without mitogen PMA(50 ng/ml)and ionomycin (1 µg/ml) in a flat-bottom sixwell culture plate at 37^0C, 5% CO2, 100% humidity. Culture supernatants were harvested after 24 h and stored at -80^0C till analysis.

Quantification of supernatant cytokines by ELISA

Cytokine levels in culture supernatants were quantified by commercially available ELISA kits for human IFN-γ, IL-4, IL-6, TNF-α, IL-10, TGF-β(Becton Dickinson, USA) and IL-17A (R & D System, USA)as per manufacturer's protocol. Briefly, monoclonal capture antibody diluted in sodium carbonate buffer (0.1 M, pH 9.5, Appendix-II) at desired concentration was added to micro wells (Nunc, Maxisorb, Denmark) was incubated overnight at 4^0C. The plates were washed thrice with0.05% Tween-20 in PBS and blocked with 300 µl of 10% FBS (Gibco, NZ) in PBS for one hour. Standard and culture supernatant were loaded on to the well and incubated for 2 hours. Samples were stimulated with H_2SO_4 to activate the latent TGF-β. Plates were washed again in wash buffer five times and cytokines were

detected by addition of desired concentration of horseradish peroxidase-conjugated streptavidin labelled antibodies for 1 hour. Plates were again washed in wash buffer seven times and colour was developed by adding 100 µl of tetramethyl benzidine (TMB) (BD Biosciences, CA, U SA) for 15-20 min. Enzyme-substrate reaction was stopped by adding 50 µl of 2N H_2SO_4. Absorbance was measured at 450 nm within 30minutes of stopping reaction in a microtiter plate reader (Biorad Laboratories, USA).Concentration of above cytokines was determined from the standard curve generated from readings of standards. The lower detect ion limits for the individual assays are as follows: IFN-γ, 4.7pg/ml; IL-4, 7.8pg/ml; IL-6, 4.8pg/ml; TNF-α, 7.8pg/ml; IL-17A, 15.6pg/ml; IL-10, 7.8pg/ml and TGF-β, 125pg/ml.

FITC Mouse Anti-Human CD4, PE Mouse Anti-Human P-gp, Alexa Fluor 647 Mouse Anti-Human IFN-γ, APC Mouse Anti-Human IL-4, PerCP-Cy5.5 Mouse Anti-Human IL-17A, PerCP-Cy5.5 Mouse Anti-Human CD25, Alexa Fluor 647 Mouse Anti-Human FoxP3 monoclonal antibodies and FITC Mouse IgG1-k, PE Mouse IgG1-k, Alexa Fluor 647 Mouse IgG1-k, PerCP-Cy5.5 Mouse IgG1-k and APC Mouse IgG1-k isotypes were purchased from BD Biosciences, San Diego, CA, USA. BD FACS Lysing Solution was purchased from BD Biosciences, San Jose, USA. BD Cytofix/Cytoperm Plus kit was purchased from BD Biosciences, San Diego, CA, USA. RPMI 1640, PMA and ionomycin were purchased from Sigma, Saint Louis, Missouri, USA. FCS was purchased from Gibco, South America.Histopaque-1077was purchased from Sigma Aldrich, Saint Louis, MO, USA. Human IFN-γ, IL-4, IL-6, TNF-α, IL-10, TGF-β elisa kit were purchased from Becton Dickinson, San Diego, CA92121, USA and IL-17A from R & D System, Minneapolis, MN USA.

Statistical Analysis

Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc. Chicago, USA). Data are expressed as mean ± standard deviation or median with range. Means within the groups were analysed using the one-way analysis of variance (ANOVA) with Bonferroni correction for parametric values. Pearson's correlation test was used to analyse the correlation between variables. $P<0.05$ was considered as significant.

Results

Demographic characteristics of study populations

Total 85 patients with NS and 15 healthy controls were included in the study. Of the 85 patients with MCNS, 32 NS patients in sustained remission (males 29, median age 6 years, range 3-16 years), 25 NS patient during relapse (males 21, median age 7 years, range 4-15 years) and 28 SRNS patients (males 26, median age 7.5 years, range 6-16 years)were included in study. Fifteen healthy children (males 13, median age 9 years, range 5-15 years) of same ethnicity were included as control. The mean age of onset was significantly higher for SRNS (9.73±4.65years) than for SSNS (5.87±4.78years) and for relapse (5.13±4.25years) patients. The mean BMI was higher in SRNS (17.71±2.92) and relapse (17.65±2.84) than that of SSNS (16.15±1.47) patients. (Table 3)

Demography	SSNS (N=32)	Relapse (25)	SRNS (N=28)	Controls (N=15)	P-value
Male	29 (90.6%)	21(84%)	26 (92.85%)	13 (86.7%)	
Age (years)	6 (3-16)	7 (4-15)	7.5 (6-16)	9 (5-15)	
BMI	16.15±1.47	17.65±2.84	17.71±2.92	–	0.015
Age of Onset	5.87±4.78	5.13±4.25	9.73±4.65	–	0.003

Table 3: Demographic profiles of patients and healthy controls included in the study

Biochemical parameters of patients

The biochemical profiles of patients with SSNS, during relapse and with SRNS are summarized in Table 4 .The mean level of serum creatinine was significantly higher in patients with SRNS and during relapse than that of patients with SSNS. The serum protein and serum albumin level were higher in patients with SSNS and relapse than that of patients with SRNS. The mean levels of hemoglobin and blood ureanitrogen among SSNS, relapse and SRNS patients were similar. The spot urine protein/creatinine ratio, triglyceride and total cholesterol were also higher during relapse and SRNS patients that SSNS patient.

Characteristics	SSNS (N=32)	Relapse (25)	SRNS (N=28)	P-value
Hb(g/dL)	12.94±1.19	12.15±1.38	12.46±1.59	0.834
Serum Creatinin (mg/dL)	0.73±0.36	0.92±0.26	0.95±0.26	0.034
Serum Protein (g/dL)	6.53±1.08	3.83±0.89	3.69±0.89	0.001
Serum Albumin (g/dL)	4.67±0.91	2.27±0.84	2.53±0.84	0.006
Blood urea nitrogen(mg/dL)	11.40±6.26	15.93±6.48	17.90±9.60	0.436
urine protein/creatinine ratio	1.34±0.28	2.68±0.47	2.95±0.36	0.001
Triglyceride (mg/dL)	188.43±65.55	346.52±153.97	546.67±360.82	0.027
Total Cholesterol (mg/dL)	201.50±50.63	326.76±138.7	418.0±183.67	0.032

Table 4: Clinical parameters of SSNS, Relapse and SRNS patients included in the study.

Circulating Treg, Th1, Th2, Th17 and pathogenic Th17 in CD4+ cells in SSNS, Relapse, SRNS and Controls

Frequency of CD4+ lymphocytes

The percentage of CD4+ lymphocytes was 30.5±5.11, 28.6±4.8, 25.65±7.3 and 32.0±6.02 in SSNS, during relapse, SRNS and healthy control respectively. (Figure 9)

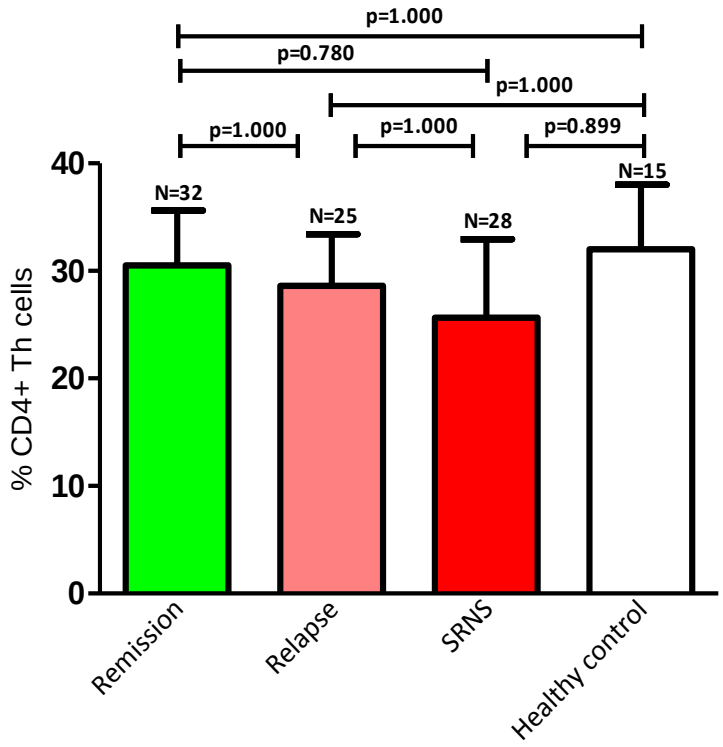

Figure 9: CD+ lymphocytes population.

Frequency ofCD4+CD25+FoxP3+ Treg

The percentage of CD4+CD25+FoxP3+ Treg were significantly greater in patients with sustained remission (7.22±4.99) as compared to patients during relapse (3.77±3.48; P=0.02) and SRNS patients (3.85±3.21; P=0.019); however the percentage of CD4+CD25+FoxP3+ Treg was similar to that of the healthy control (7.80±5.50; P=1.00). (Figure 10)

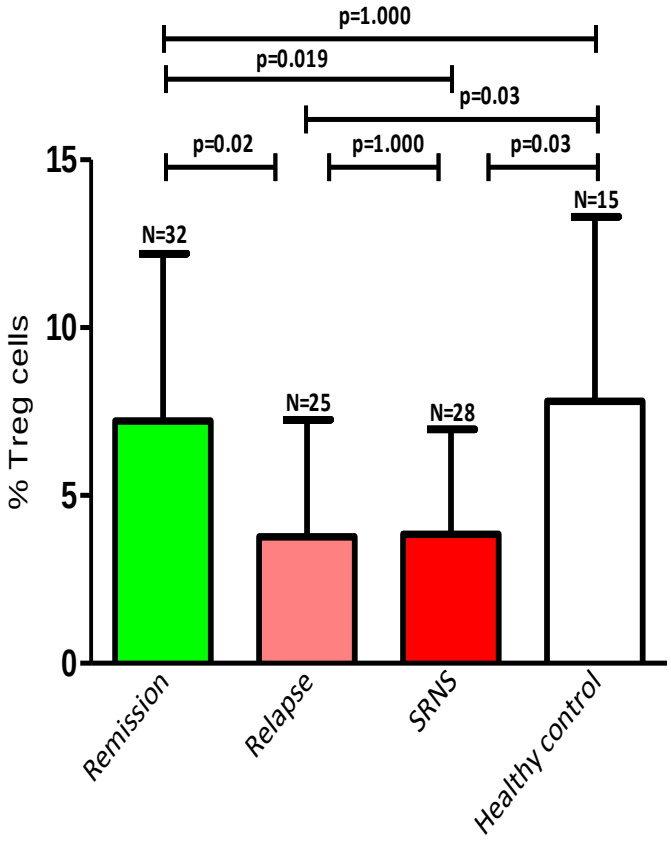

Figure 10: CD4+CD25+FoxP3+ Tregs lymphocytes population.

Frequency of CD4+IFN-γ+ Th1 cells

The percentage of CD4+IFN-γ+ Th1 cells was significantly lesser in patients with sustained remission (10.62±5.11) as compared to that of patients during relapse (16.38±10.03; P=0.025); SRNS patients (18.28±7.30; P=0.001); and in control (13.97±6.02) subjects. However the percentage of CD4+IFN-γ+ Th1 cells was not statistically different amongst patients during relapse, SRNS and healthy controls as shown in Flowcytometer plot of CD+IFN-γ+ staining. (Figure 11)

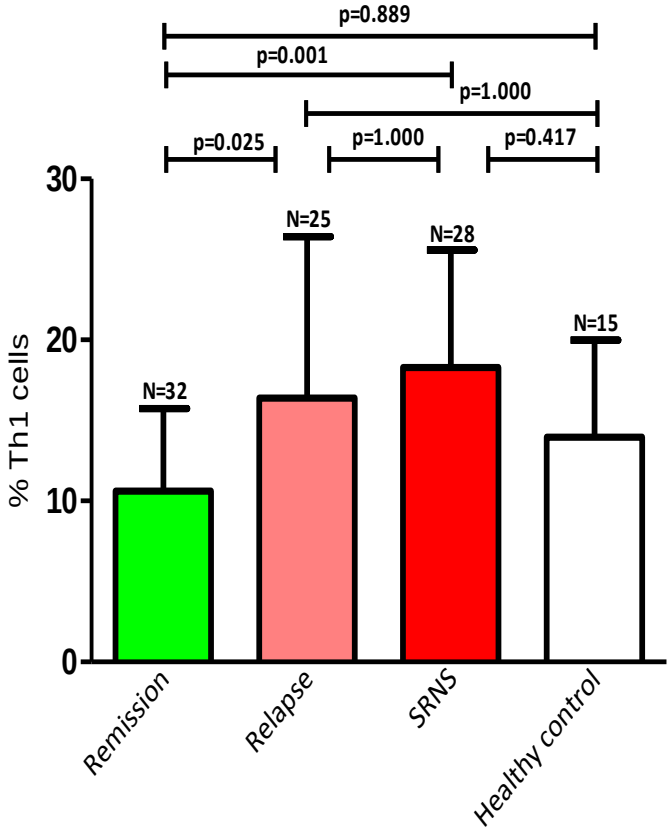

Figure 11: CD4+IFN-γ+ Th1 lymphocytes population.

Frequency of CD4+IL-4+ Th2 cells

The percentage of CD4+IL-4+ Th2 cells in patients with sustained remission (4.89±2.98) was significantly lower than that of patients during relapse (9.79±5.14; P=0.001) or SRNS patients (7.35±1.64; P=0.032); and however it was similar to that of control subjects (4.29±2.41; P=1.000). (Figure 12)

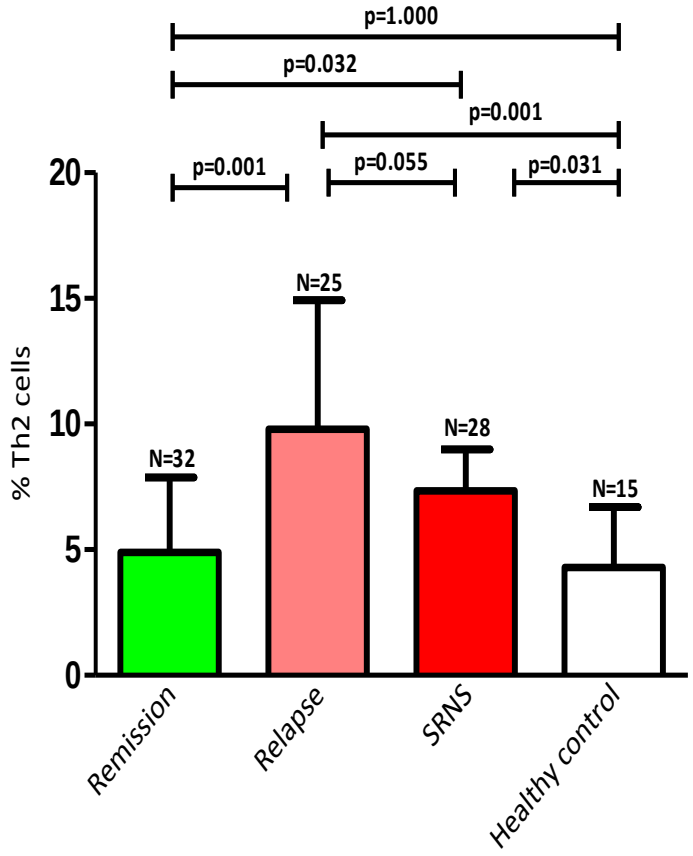

Figure 12: CD4+IL-4+ Th2 lymphocytes population.

Frequency of CD4+IL-17+ Th17

The percentage of CD4+IL-17+ Th17 cells in patients with sustained remission (2.50±1.19) was significantly lower than that of patients during relapse (3.58±1.14; P=0.043) and SRNS patients (3.76±2.24; P=0.006); and with control subjects (1.16±0.60; P=0.033). (Figure 13)

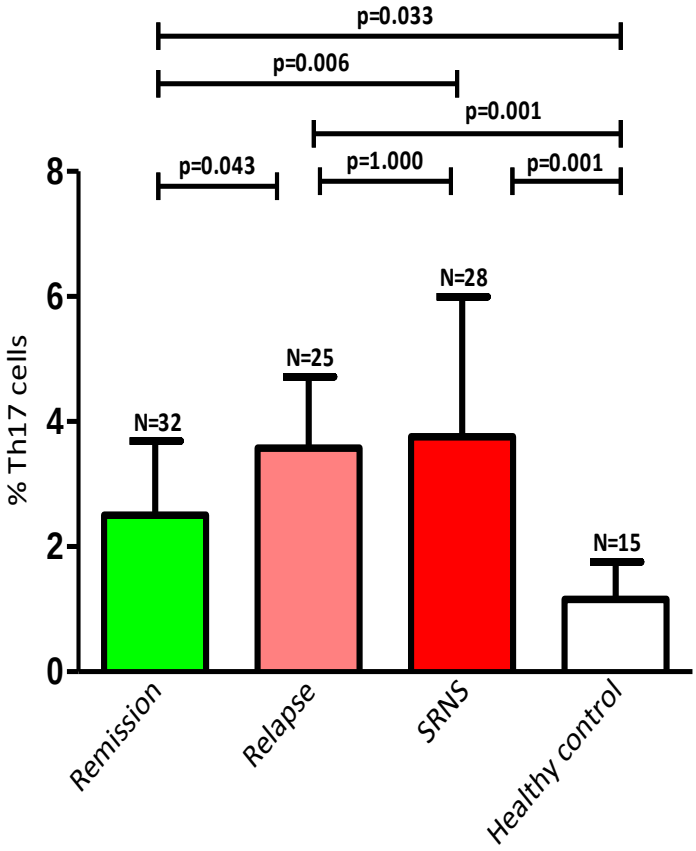

Figure 13: CD4+IL-17+ Th17 lymphocytes population.

Frequency of pathogenic CD4+IFN-γ+IL-17+ pathogenic Th17 cells

The percentage of double positive CD4+IFN-γ+IL-17+ pathogenic Th17 cells in patients with sustained remission (0.95±0.21) was significantly lower than that of patients during relapse (3.05±1.68; P=0.001) or SRNS patients (2.86±1.23; P=0.001); and with control subjects (0.32±0.09; P=0.033). (Figure 14)

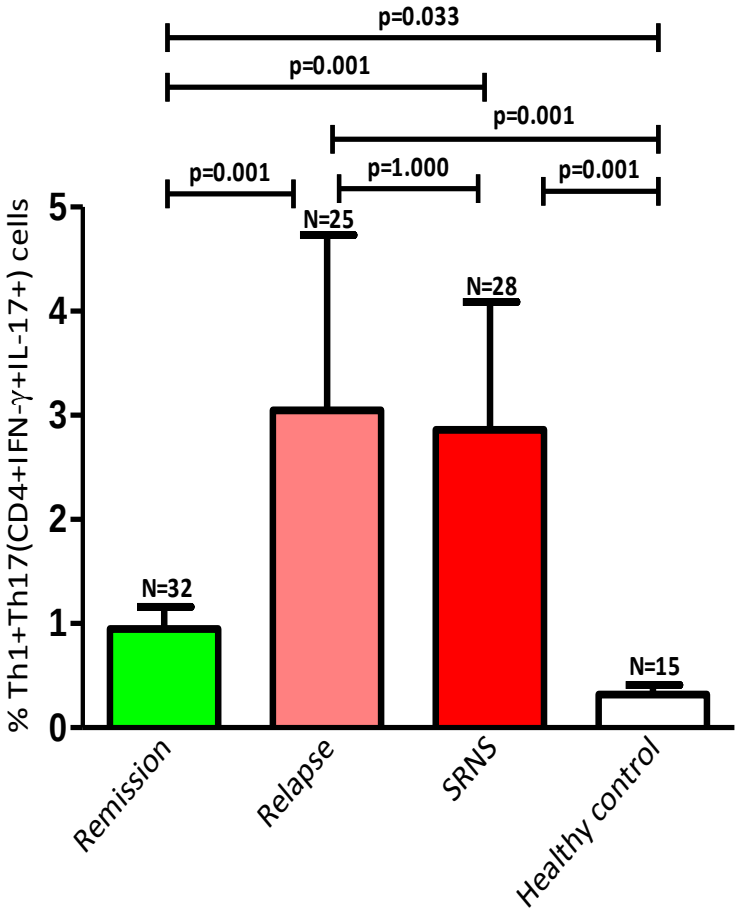

Figure 14: CD4+IFN-γ+IL-17+ pathogenic Th17 lymphocytes population.

Ratio of Treg/Th17 cells in the peripheral blood of different groups

Ratio of Treg/Th17cells in patients with sustained remission (2.96±1.42) was significantly higher than that of patients during relapse (1.34±1.75; P=0.013) and SRNS (1.44±1.13; P=0.017); and it was much lower than that of control subjects (7.14±3.36;P=0.001) (Figure 15).

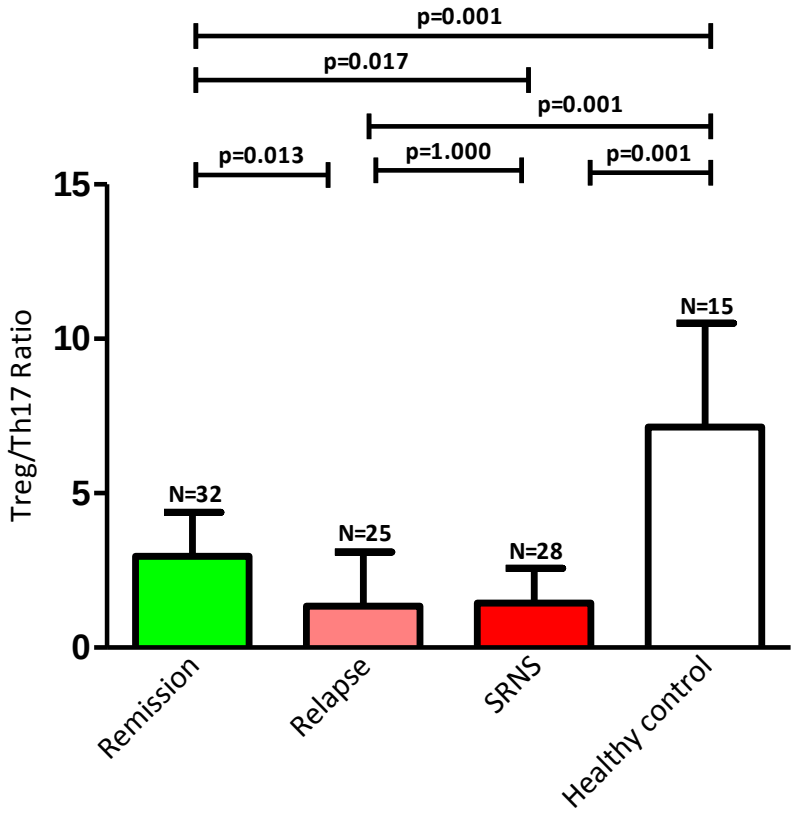

Figure 15: Ratio of Treg/Th17 cells in the peripheral blood of different groups

ELISA for Serum cytokine levels in different groups of patients

Levels of the cytokines IFN-γ, IL-4, IL-6, TNF-α, IL-10, TGF-β (Becton Dickinson, USA) and IL-17A in serum samples were found below the detection limits of the various ELISA kits in most of the patient.

Cytokine levels in PBMCs culture supernatant of SSNS, Relapse, SRNS and healthy control

There was no production of cytokines from unstimulated PBMCs after culture. The cytokines profile of stimulated cultured PBMCs is shown in Table 5. The production of IL-10 and TGF-β1 was significantly increased during remission as compared to the relapse values and were similar to healthy controls. The production of both the cytokines decreased in SRNS. In contrast, secretion of IFN-ϒ, IL-4, IL-6, TNF-αand IL-17decreased significantly during remission and was comparable to healthy controls. The secretion of inflammatory cytokines increased significantly at the time of relapse and in SRNS patients. (Figure 16)

Cytokines (pg/ml)	Remission	Relapse	Resistant	Control
IL-10	59.20±28.36	19.29±14.53	27.31±19.65	43.05±33.64
TGF-β	1975.71±837.44	1053.75±604.74	1115.12±632.21	2202.6±1197.54
IFN-γ	284±250.75	497.44±303.18	609.09±334.78	183.95±237.40
TNF-a	13±4.46	36.15±11.55	30.68±11.27	11.9±4.13
IL-4	86.1±67.94	261.56±95.43	202.28±123.47	55.52±50.01
IL-17	43.59±20.09	72.17±21.58	85.24±30.73	21.88±4.31
IL-6	9.40±4.84	16.53±8.67	21.89±9.36	5.88±0.72

Table 5: Cytokine levels in PBMCs culture supernatant

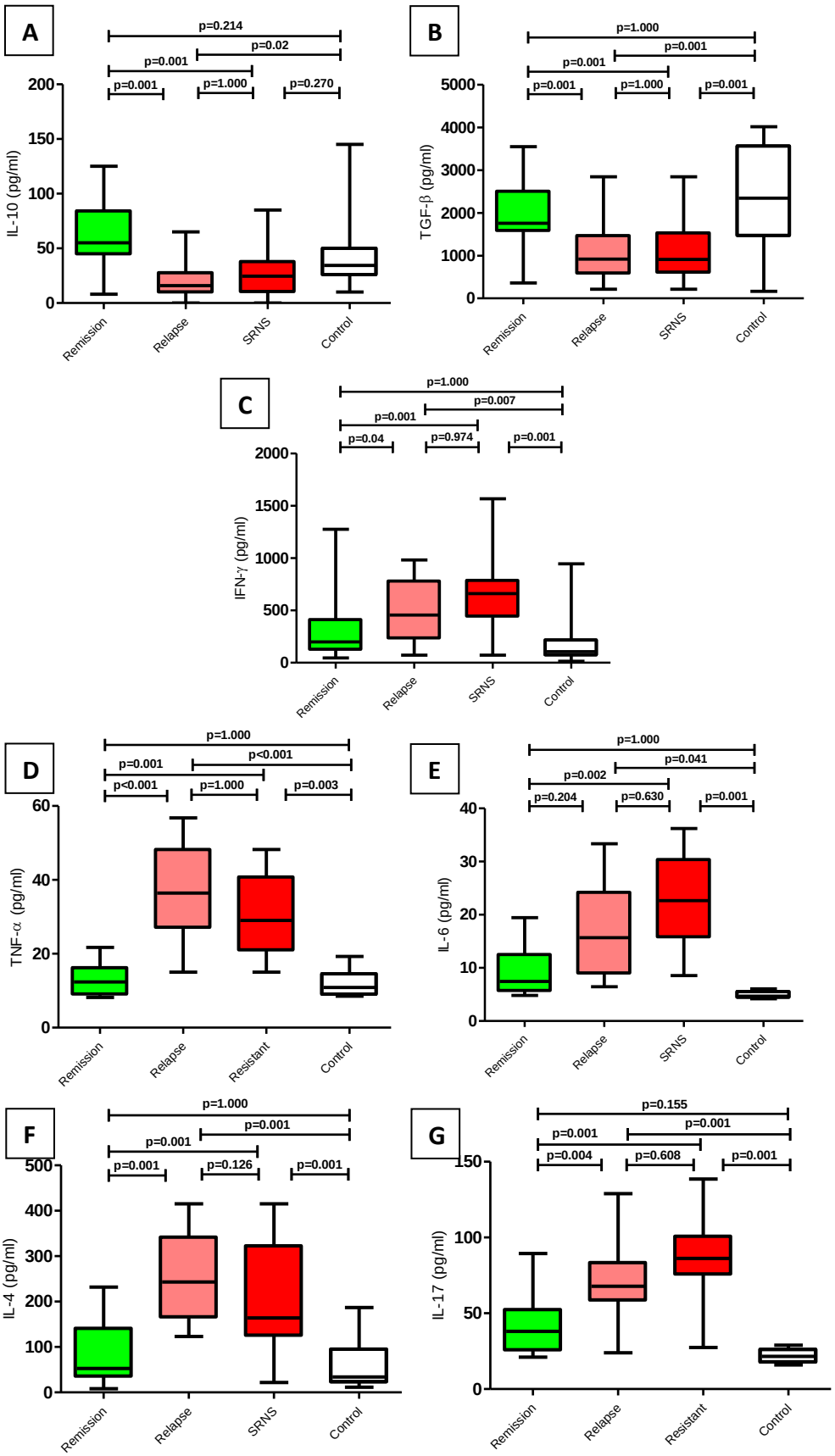

Figure 16: Box plot showing mean cytokine levels with range in SSNS, Relapse, SRNS and Control. Cytokines IL-10, (A); TGF-β (B); IFN-γ, (C); TNF-α, (D); IL-6, (E); IL-4, (F); IL-17, (G)

P-gp expression on lymphocytes in SSNS, Relapse, SRNS and Control

The percentage of lymphocytes positive for P-gp expression was significantly lesser in patients with SSNS as compared to that of patients during relapse, and that of SRNS patients. However, it was similar to that of control subjects. P-gp expression on lymphocytes was not different between patients during relapse and SRNS patients. On analysing the absolute expression of P-gp molecules on the lymphocytes (the product of RFI and percentage of positive cells), it was observed that the absolute expression was significantly greater in patients during relapse and SRNS patients as compared to that of patients with SSNS. The absolute expression of P-gp molecules was also greater in patients during relapse and SRNS patients as compared to that of healthy controls. The absolute expression of P-gp in patients with SSNS and healthy controls was similar and the differences between relapse and SRNS were also not significant. (Table 6, Figure 17 A, B)**.**

Characteristics	**Remission**	**Relapse**	**SRNS**	**Control**
% P-gp positive cells	4.52±2.47	10.79±7.72	11.93±5.50	3.90±2.16
RFI	6.21±2.06	8.50±2.61	8.26±1.50	6.80±2.63
D-value	0.37±0.16	0.40±0.18	0.48±0.28	0.28±0.20
RFI×% P-gp positive cells	34.64±31.23	83.22±54.80	100.93±55.75	33.24±25.74

Table 6: Percentage of P-gp positive peripheral blood lymphocytes and absolute expression of P-gp in SSNS, Relapse, SRNS and Controls

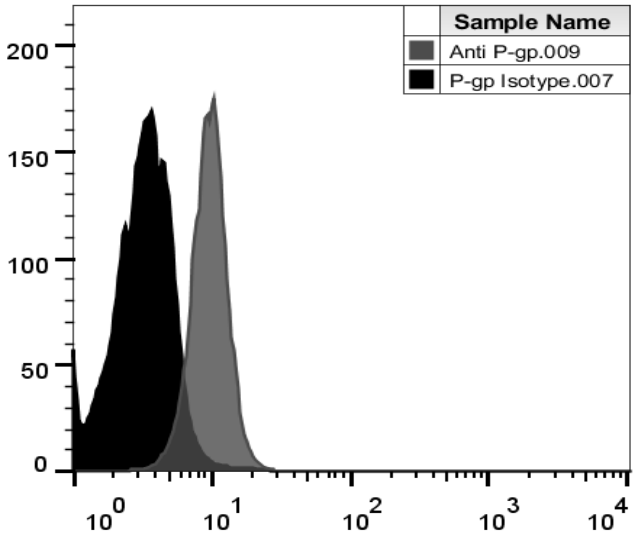

Figure 17, A: Relative fluorescence intensity (RFI) in one of the representative sample from a patient with SRNS

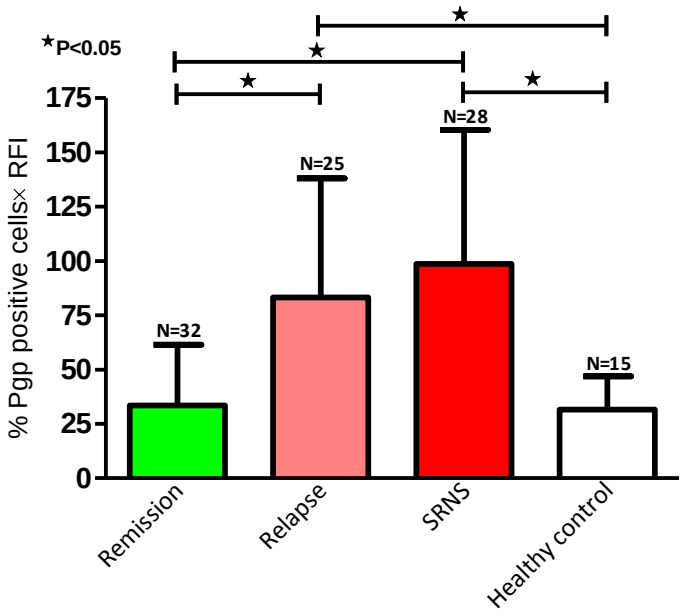

Figure 17, B: Bar diagram show absolute P-gp expression in SSNS, Relapse, SRNS and healthy control

P-gp expression on peripheral blood Treg, Th1, Th2, Th17 and pathogenic Th17CD4+ lymphocytes

The frequency of Tregs expressing P-gp in healthy control, SSNS and during relapse was 28.07%, 33.51% and 51.98%respectively, however 57.14% Tregs in SRNS also showed P-gp expression. The percentage positivity of P-gp on Teff (Th1,Th2, Th17 and pathogenic Th17) cells was significantly high during relapse and in SRNS patients as compared to control subjects and SSNS patients. Highest percentage of P-gp positivity was observed on pathogenic (double positive IFN-γ and IL-17) population of Th17 lymphocyte in SRNS patients (95.8%). (Table 7)

	SSNS	Relapse	SRNS	Control
% of P-gp expressing Tregs	33.51%	51.98%	57.14%	28.07%
% of P-gp expressing Th1	45.0%	72.28%	72.37%	30.99%
% of P-gp expressing Th2	53.78%	60.06%	72.51%	34.49%
% of P-gp expressing Th17	30.27%	72.06%	78.45%	15.51%
% Pgp+IFN-γ+IL-17+	30.1%	93.44%	95.8%	15.62%

Table 7: Percentage positivity for P-gp on different T cells in different group of study population.

Correlation between P-gp expression with alteration in the frequency of different T cell subsets.

We observed that P-gp expression correlated negatively with the regulatory CD4+CD25+FoxP3+Tregs (r= -0.545, p= 0.002). However correlate positively with inflammatory CD4+IL-4+Th2, (r= 0.418, p= 0.015); CD4+IL-17+Th17, (r= -0.507, p= 0.003) and CD4+ IFN-γ+ IL-17+Th17, (r= 0.739, p= 0.001) lymphocytes. There was no association between P-gp expression and CD4+IFN-γ+Th1 cells. (Table 8)

Pearson correlation with absolute P-gp (P-gp×RFI)		
Circulating T cell frequency	**r^2**	**P-value**
CD4+CD25+FoxP3+Treg	-0.545	0.002*
CD4+IFN-γ+Th1	0.149	0.399
CD4+IL-4+Th2	0.418	0.015*
CD4+IL-17+Th17	0.507	0.003*
CD4+ IFN-γ+ IL-17+Th17	0.739	0.001*

Table 8: Pearson correlation between P-gp expression and different T cell frequency

Correlation between absolute P-gp and different cytokines

The correlation between absolute P-gp and different cytokines are shown in table. The absolute P-gp expression was negatively correlated with regulatory cytokines (IL-10 and TGF-β), and positively correlated with inflammatory cytokine (IFN-γ, IL-6, TNF-α, IL-4, IL17) levels. Strongest positive correlation between absolute P-gp with serum IL-17 cytokine was observed. (Table 9)

Pearson correlation with absolute P-gp (P-gp×RFI)		
Serum Cytokine level	**r^2**	**P-value**
TGF-β	-0.483	0.001
IL-10	-0.627	0.001
IFN-γ	0.464	0.001
IL-4	0.563	0.001
TNF-α	0.420	0.008
IL-6	0.495	0.002
IL-17	0.812	0.001

Table 9: Pearson correlation between P-gp expression and concentration of cytokines in mitogen stimulated *in vitro* culture supernatant

Discussion

Discussion

Nephrotic syndrome is a complex disease, clinically characterized by proteinuria, hypoalbuminemia, edema, hypercoagulable state and increased susceptibility to infection. MCNS is most common type of NS and characterized by diffuse effacement of podocyte under electron microscope. The first thing that goes wrong is that the glomeruli become leaky for protein. Initially Shallhoub *et al*., hypothesized that T cell and their secreted circulating factor cause leakiness of glomeruli and excretion of plasma proteins in urine (Shallhoub *et al.*, 1974). As protein is lost into the urine, the liver can't keep up making new protein and the blood protein level falls leading to decreased intravascular oncotic pressure and leaking of blood fluid to extravascular compartment which makes body puffy, and simultaneous salt and water retention from kidney makes the situation worse.

For treating NS, the mainstay therapy is corticosteroid which blunt the T cell mediated immune system. Majority of children suffering from MCNS shows effective response to steroid therapy, again emphasizing the role of T cells in MCNS. However, it is still not clear that which type of T cells are predominantly involved in pathogenesis of NS. Some studies believed that altered number and activity of Tregs, (Bennett L *et al.*, 2001; Araya C *et al.*, 2009; Le Berre *et al.*, 2009), and some believe increased number and activity of Th2 cells (Davin JC *et al.*, 2011; Kanai T *et al.*, 2010) or the altered ratio of Th1/Th2 (Kaneko K *et al.*, 2002) plays role in pathogenesis of MCNS. Recent studies emphasize that Th17 and or Th17/Treg imbalance leads to glomerular damage (Liu LL *et al.*, 2011; Xiao SS *et al.*, 2009; Wang L *et al.*, 2013).

Steroid is the cheapest, easily available, first line of therapy used for treatment MCNS. Steroid protects from inflammatory diseases by reducing inflammatory cells and there cytokines and simultaneously increases the anti-inflammatory cytokine response (Woroniecki RP *et al.*, 2006; Brattsand R *et al.*, 1996). It has been also observed that protective effect of steroid is mediated by restoration of podocyte function which is usually altered in MCNS (Fujii Y *et al.*, 2006). Majority of patients responds to steroid, however approximately 10% shows resistance to steroid and known as primary non responder and approximately 60% of initial responsive

patient shows frequently relapsing and steroid dependence course during treatment for longer duration. Of them approximately 10% of patients do not show response to steroid therapy during course of the disease, they are called secondary non responder. The relapsing patients require repeated course of treatment with steroid predisposing them for steroid toxicity. High dose of steroid induces malfunctioning of musculoskeletal, endocrine, cardiovascular, central nervous systems and bone resorption.

It is still not understood that what mechanism and immune dysregulation drives the resistance and relapse of the disease. Is it the increased number or altered population of T cell subsets regulates the steroid responsiveness or the pharmacological factor (i.e. overexpression of P-gp) regulating effective availability of steroid plays role in resistant and relapsing state of NS. Therefore, we felt that there is need to study the changes in immune profiles of patients in various phenotypes of nephrotic syndrome who remain in sustained remission, who relapses and those who show resistance to steroid during therapy. It might be possible that drug resistance might be due to efflux of drug by P-gp. P-gp upregulation has been reported to be associated with resistance to a variety of drugs including glucocorticoids (Bates SE *et al.*, 1996). P-gp is expressed in nearly all the tissues including lymphocytes, the main targets of pharmacotherapy in NS (Coon JS *et al.*, 1991; Wacher VJ *et al.*, 1995). Therefore P-gp expression on lymphocytes is putative target of therapy in MCNS is worth exploring.

In this study, we collectively evaluated the T cell subsets and their cytokines producing ability in NS subgroups. We tried to show the changes in Th1, Th2, Tregs, and Th17 cells in different phenotypes of MCNS and to predict the factors which lead to altered response to steroid in these patients. We found that Th2, and Th17 cells are dysregulated in SRNS and relapse patients as compared to SSNS and healthy control. The frequency of effector T cells was higher in resistant group as compared to relapsing group of patients. Tregs, major regulatory cells were down regulated in relapse and resistant group of patients as compared to healthy control and remission group of patients. Analysis of cytokines IFN-γ, Il-4, IL-10, IL-17, TGF-β and TNF-α in culture supernatant of mitogen stimulated NS PBMCs was done to evaluate the functionality. Level of inflammatory cytokines IL-4, IL-6, IL-17, IFN-γ and TNFα

were highly upregulated in culture supernatant of PBMC in resistant and relapse group as compared to patients in remission and healthy control. However anti-inflammatory cytokines IL-10 and TGF-β were down regulated in resistant and relapse group as compared to patients in remission and healthy control.

Our study revealed similar frequency of Th1 cells in healthy control and NS patients and their subgroups while frequency of Th2 cells was higher in NS patients as compared to healthy controls. These finding suggested the predominant role of Th2 over Th1 in NS. Previous studies on association of atopic dermatitis with Th2 and nephrotic syndrome boosted the findings of our study. Clinical response of steroid therapy in NS also goes hand in hand with Th2 population. Th2 cells were higher in resistant and relapse patients. In contrast to our study, Kaneko *et al.* reported that no significant skewing in T helper cells subset like Th1 and Th2 or the ratio of Th1/Th2 in relapse, remission and normal controls population (Kaneko K *et al., 2002*). But subsequent studies supported our study that MCNS patients have significantly higher Th2 population as compared to healthy control (Davin JC *et al.,* 2011; Kanai T *et al.,* 2010; Van den *et al.,* 2004). The relatively less studied area in NS patients is role of Th17 and their cytokines. Our study finding suggested that Th17 cells are upregulated in NS as compared to healthy group. Th17 frequency was higher in resistant and relapse group of patients as compared to remission and healthy control, which suggest a possible role of Th17 in NS. Our study was supported by findings of Wang et al who have also shown that Th17 frequency was higher in NS patients as compared to healthy control (Wang L *et al.,* 2013). Our study again support the findings of Shao et al who observed that CD4 to Th17 conversion was high and responsible for increased level of IL-17 in NS as compared to healthy control (Xiao SS *et al.,* 2009).

We have observed that Tregs frequency was reduced in NS patients as compared to healthy control. Frequency of Tregs was lower in resistant and relapse group as compared to remission group and healthy controls. Frequency of Treg was significantly lower in patients with NS than that of normal controls and that it was markedly decreased in patients with NS compared to patients with isolated hematuria (Xiao SS *et al.,* 2009). Tregs modulates the activation, differentiation, and effector functions of Th1, Th2, Th17 cells and APCs, or inducing their apoptosis (Caridade M *et al.,* 2013). In our study, Tregs/Th17 ratio was high in NS as compared to healthy

control. Also ratio of Treg/Th17 was high in resistant and relapse patients compared to remission and healthy control. Similar to our study, Liu et al. have shown that Th17/Treg is altered in MCNS compared to healthy control (Liu L *et al.,* 2011).

During our study of Th17 cells, we also found for the first time in NS a rare pathogenic population of Th17 which express IFN-γ and IL-17 both, which is higher in NS as compared to healthy control. Frequency of pathogenic Th17 was high in resistant and relapse group as compared to remission and healthy control. It has been observed that pathogenic Th17 is involved in organ damage compared to non-pathogenic Th17 (Zhang R *et al.,* 2013). We have first time studied the frequency of pathogenic Th17 cells in NS patients.

In our study, on mitogen stimulation of PBMC from NS patients produced more inflammatory cytokines i.e. IFN-γ, IL-4, IL-6, IL-17 and TNFα as compared to control. Suranyi *et al.* showed TNFα but not IFN-γ upregulation in NS (Suranyi MG *et al.,* 1993). IFN-γ, IL-4 and TNF-α were highly elevated in resistant and relapse patients as compared to remission and healthy control (Giuliana L *et al.,* 2002). Il-6 & IL-17 was upregulated in NS as compared to healthy control (Xiao SS *et al.,* 2009). IL-17 induces the reduced expression of podocalyxin level and inducing podocyte apoptosis (Wang L *et al.,* 2013) suggesting role of IL-17 in NS.

We have also analysed the levels of anti-inflammatory cytokines, IL-10 & TGF-β in culture supernatant of mitogen stimulated PBMC and we found reduced level of IL-10 and TGF-β in resistant and relapse patients as compared to remission and healthy control. IL-10 and TGF-β are inhibitory to Th17 cells (Caridade M *et al.,* 2013) again emphasizing the protective role of these anti-inflammatory cytokines in maintaining remission of patients.

P-glycoprotein and steroid resistance:

Pharmacogenomics in drug resistance is evolving concept. Besides changes in histologic characteristics of the disease, it is possible that steroid resistance might be contributed by alteration in drug pharmacokinetic and pharmacodynamic.

P-gp a 170-kD product coded by multidrug resistance-1 (MDR-1) gene (Dilger K *et al.,* 2004; Juliano RL *et al.,* 1976), plays important role in protecting host tissue from toxic side effects and the overexpression of P-gp results in the reduction of the concentration of peptides, alkaloids, steroids, immunosuppressive drugs, and calcium channel blockers (Tsuruo T *et al.,* 1993; Ueda K *et al.,* 1987; Ueda K *et al.,* 1992). The treatment resistance because of P-gp expression has been reported in many diseases including malignancies, systemic lupus erythematosus, inflammatory bowel disease and other autoimmune diseases (Beck WT *et al.,* 1996; Gottesman MM *et al.,* 2002; List AF *et al.,* 2002; Farrel RJ *et al.,* 2003; Richaud-Patin *et al.,* 2004). P-gp is expressed on lymphocytic membrane, a putative target of steroid (Coon JS *et al.,* 1991, Wacher VJ *et al.,* 1995)and acts as an efflux pump removing substrate drug steroid from inside to outside of cells, thus preventing to act on receptors. We also found that frequency of P-gp expressing lymphocytes was significantly high in NS patients as compared to healthy control. Similar to our observation, Wasilewska et.al has also observed that association of P-gp expression with clinical response during treatment of NS (Wasilewska A *et al.,* 2006). Our study carries merits of monitoring P-gp expression in different phenotypes of NS like SRNS, during relapse and SSNS. P-gp expression was higher in resistant and relapse group of patients as compared to patients in remission and healthy controls. In our study, P-gp which is the one of important protein involved in drug resistance were highly upregulated in PBMC of NS patients compared to healthy controls. Overall P-gp expressing inflammatory T cell Th1, Th2 and Th17 were high in resistant and relapsed patients followed by patients in remission and healthy control. P-gp expressing Tregs were higher in remission and healthy control compared to resistant and relapse group of patients. We have also demonstrated the expression of P-gp on different types of lymphocytes separately. In relapse and resistant patients P-gp expression was high on effector T cell i.e. Th1, Th2, Th17 and pathogenic Th17 cells as compared to remission and healthy control. We have first time demonstrated the P-gp expression on subtypes of lymphocyte. However, we have also found higher P-gp expression on Tregs in patients in remission as compared to relapse and SRNS patients which was possibly due to upregulation of Tregs population because of steroid therapy. Karagiannidis *et al*. have observed that Tregs frequency is upregulated during steroid therapy in treatment of bronchial asthma and similar observations has been reported by Braitch

et al. during treatment of multiple sclerosis. (Karagiannidis C *et al*., 2004; Braitch M *et al*., 2009)

Steroid increases and maintains Tregs function via inhibiting transcription factor i.e. NFkB, Ap-1 and NFAT involved in inflammatory cytokine production and also checks their plasticity (Safinia N *et al*., 2015). It is possible that upregulation of P-gp may led to reduction in effective steroid concentration and increases the Tregs plasticity into other effector T cells. However, it remained to be established in our study. It has been demonstrated that P-gp expression and function can be repressed by different compounds such as cyclosporine-A, and tacrolimus (Hauser IA *et al*., 1998; Hidetoshi A *et al*., 2001). This could be the purpose why many relapsing NS patients react to calcineurin inhibitors. In the present study we have successfully validated that P-gp expression was negatively correlated with immunosuppressive cytokines and positively correlated with proinflammatory cytokines. (Prasad N *et al*., 2015). The negative correlation of P-gp expression on PBMCs with IL-10 and TGF-b and positive correlation with cytokines IFN-γ and IL-4 in our previous study advocated that P-gp expression is upregulated on PBLs in pro-inflammatory state. We assume that during remission, increased Tregs may have mopped up IL-2, a cytokine that affects the mRNA synthesis by MDR-1 gene and lead to decreased expression of P-gp on PBMCs. Natural Tregs constitutively express CD25, the high affinity receptor for IL-2 cytokines. Tregs by mopping up IL-2 suppress Teff cells proliferation and differentiation and/or activate Teff cells apoptosis. Amplified Tregs during remission in the present study may have counter balanced the effect of Teff cells (Th1 and Th2), however, we have not studied the levels of IL-2. (Tang Q *et al*., 2008). Another recent study has clearly demonstrated that P-gp positive Th17 cells may be important mediators particularly in setting of steroid resistant. (Radha R *et al*., 2014)

P-gp expression increases on pathogenic T cells in diseased condition. P-gp has been recently targeted as the molecule responsible for poor response to many chemotherapeutic agents. Increased P-gp expression could be one of the possible aspects responsible for relapse besides other immunological and nonimmunological factors. Our present study had suggested that P-gp plays a major role in resistance and relapse of disease during steroid treatment. Monitoring of P-gp expression may predict steroid resistance or relapsing patients. Steroid is the substrate of the P-gp and

calcineurin inhibitors is both substrate and inhibitor of P-gp, therefore it is possible that those patients who are resistant to steroid alone, responds to calcineurin inhibitors if added in treatment regimen. Non-responsive patients may be treated with other therapies instead of using higher dose of steroid, which have toxic effect. Rituximab and Tacrolimus with low dose steroid had shown effectiveness in resistant NS (Bock ME *et al*., 2013; Gulati A *et al*., 2008; Kim J *et al*., 2012). Tacrolimus and other calcineurin inhibitors may be used as steroid sparing agents in this scenario.

Limitations of our study

MCNS is considered to be a podocytopathy and we have not studied the effect of steroid and expression of P-gp on podocytes in our study. It remains to be explored in further study.

Conclusion

Conclusion

To conclude, there is immune dysregulation in peripheral blood in NS. Frequency of the T effector cells; Th2 and Th17 and IL-17+IFNγ+ Th17 cells and their cytokines were upregulated and Tregs and their regulatory cytokines were down regulated in SRNS and relapse as compared to SSNS patients.

In addition, P-gp expression on T effector cells was upregulated in relapse and SRNS patients and downregulated in SSNS patients. Increased expression of P-gp was strongly associated with inflammatory cytokines levels. P-gp expression was negatively associated with regulatory cytokines; TGF B and IL-10. The correlation coefficient for association of P-gp expression was highest with Il-17. The role of P-gp inhibitors in management of NS patients need to be explored.

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Appendix-I

Markers	Fluorochrome-conjugated Antibodies for P-gp expressing CD4+ T cells (T helper) subsets			
	Th1	**Th2**	**Th17**	**Tregs**
CD4 (surface staining)	FITC Anti-Human CD4	FITC Anti-Human CD4	FITC Anti-Human CD4	FITC Anti-Human CD4
CD25 (surface staining)				PerCP-Cy5.5 Anti-Human CD25
P-glycoprotein (surface staining)	PE Anti-Human P-gp	PE Anti-Human P-gp	PE Anti-Human P-gp	PE Anti-Human P-gp
Cytokines (intracellular staining)	Alexa Fluor 647 Anti-Human IFN-γ	APC Anti-Human IL-4	PerCP-Cy5.5 Anti-Human IL-17A	
Transcription Factor (intracellular staining)				Alexa Fluor 647 Anti-Human FoxP3

Fluorochrome-conjugated mAB used to analyze P-gp expressing CD4+ T cells (T helper) subsets.

Appendix-II

Reagents for PBMCs culture

Phosphate buffered saline (PBS) 1X [0.15M]

Sodium chloride (NaCl)	8.0g
Disodium hydrogen phosphate (Na2HPO4)	1.15g
Potassium dihydrogen phosphate (KH2PO4)	0.2g
Potassium Chloride (KCL)	0.2g

Dissolved in TDW to the final volume of 1 liter and pH was adjusted to 7.2. the solution was autoclaved and stored at 4^0C.

Complete RPMI-1640 (c-RPMI)

RPMI-1640 (GibcoBRL)	10.4g
HEPES (SRL)	6.0g
L-glutamine (SIGMA)	0.258g
Sodium pyruvate (GibcoBRL)	0.11g
Sodium bicarbonate (SIGMA)	2.0g
Antibiotic-antimycotic (100X)	10.0ml
Heat inactivated FBS	10%

Dissolved in 1 liter TDW final volume, pH was adjusted to 7.2, filtered through 0.2 millipore filter and stored at 4^0C.

ELISA buffers

Coating buffer [Carbonate bicarbonate buffer (pH 9.6, 100ml)]

Sodium bicarbonate (SIGMA)	0.293g
Sodium carbonate (SIGMA)	0.159g
Wash buffer [PBS-T]	0.05% Tween-20 in PBS.
Stop solution	2N H_2SO_4

Appendix-III

AN8-V1/SGSOP 03/V1

Consent Form (English)

Study Title **"Immune profile of glucocorticoid resistant Nephrotic Syndrome patients"**

Study Number_____

Subject's Full Name _____

Date of Birth/Age_____

Address _____

_____

1. I confirm that I have read and understood the information document dated _____for the above study and have had the opportunity to ask questions.
   OR I have been explained the nature of the study by the Investigator and had the opportunity to ask questions.

2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.
3. I understand that the sponsor of the clinical trial/project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. However, I understand that my Identity will not be revealed in any information released to third parties or published.
4. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).
5. I permit the use of stored sample (tissue/blood) for future research. **Yes [ ] No [ ]**
6. I agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____

Signatory's Name_____Date_____

Signature of the Investigator_____Date_____

Study Investigator's Name _____

Signature of the Witness _____Date_____

Name of the Witness _____

Received a signed copy of Participant Information Document and Consent Form.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____ Date_____

Appendix-IV

Immune profile of Glucocorticoid resistant Nephrotic Syndrome patients

Case Report Performa

Serial No.: CR No.: Date of visit:

Name: Age/Sex/Race:

Address:

Phone number:

History

Age of Onset:
Edema: Y/N Hematuria: Microscopic/ Gross/cola color urine

Hypertension: Oliguria:

Associated systemic symptoms:

Associated renal failure:

Family history of renal disease:
Infections:

Drug	Dose	Duration	Response

Examination

Height: Weight: BMI: BP:
PR: Respiratory rate:
CVS: Chest:
eGFR:

Investigations

Blood

Hb: TLC: DLC:

Platelets: ESR: CRP:

S. Creatinine: S.Albumin/Protein:

S.Urea: ANA: IgG:

Serum Total Cholesterol:

Triglyceride:

LDL/HDL/VLDL:

Serum protein/ albumin: Serum Alkaline Phosphatase:

Serum Complement C3 C4

Other complements HIV/HCV/HBV:

Urine

Albumin/Protein: Microscopy:

24 hr urine protein/creatinine: Spot urine protein/creatinine ratio:

Kidney histology:

Light microscopy:

No. of glomeruli and glomerular changes:

Interstitial: no fibrosis/fibrosis (% cortical area)

Tubules: no atrophy/atrophy (% atrophy)

Other remarks

Impression: MCD/FSGS/MGN/ MPGN/C3 glomerulopathy/ IgA nephropathy/ others name

IF study: no deposit/ deposit of .....

Impression of IF study:

Electron microscopy changes: **Final diagnosis**

List of Publications

From Thesis work:

1. **A Jaiswal**, N Prasad, V Agarwal, B Yadav, D Tripathi, M Rai, M Nath, RK Sharma, DR Modi. Regulatory and effector T cells changes in remission and resistant state of childhood nephrotic syndrome. Indian J Nephrol. 2014; 24(6): 349–355.
2. Narayan Prasad, **Akhilesh K Jaiswal**, Vikas Agarwal, Brijesh Yadav. Differential alteration in peripheral T-regulatory and T-effector cells with change in P-glycoprotein expression in Childhood Nephrotic Syndrome: A longitudinal study. Cytokine 2015; 72(2): 190–196.
3. **Akhilesh K Jaiswal**, Narayan Prasad,Vikas Agarwal, Dinesh R Modi. IL-17/IFN-g double-positive Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoid in childhood Minimal Change Disease. NDT (submitted)
4. **Akhilesh K Jaiswal**, Narayan Prasad,Vikas Agarwal, Dinesh R Modi, Mohit Rai. Alteration Of Th17/Treg Ratio Affects Steroid Response In Childhood Minimal Change Disease. Pediatri Nephrol (submitted)

From Other work:

1. Narayan Prasad, **Akhilesh Jaiswal**, Vikas Agarwal et **al.** FGF23 is associated with early post-transplant hypophosphataemia and normalizes faster than iPTH in living donor renal transplant recipients: a longitudinal follow-up study. Clinical Kidney Journal 2016 : 065.
2. Yadav B, Prasad N, Agrawal V, **Jaiswal A**, Agrawal V. Urinary Kidney injury molecule-1 can predict delayed graft function in living donor renal allograft recipients. Nephrology (Carlton). 2015 Nov;20(11):801-6.
3. Yadav B, Prasad N, Agrawal V, Jain M, Agarwal V, **Jaiswal A**, Bhadauria D, Sharma RK, Gupta A. T-bet-positive mononuclear cell infiltration is associated with transplant glomerulopathy and interstitial fibrosis and tubular atrophy in renal allograft recipients. Exp Clin Transplant. 2015;13(2):145-51.
4. Prasad N, Gurjer D, Bhadauria D, Gupta A, Srivastava A, Kaul A, **Jaiswal A**, Yadav B, Yadav S, Sharma RK. Is basiliximab induction, a novel risk factor for new onset diabetes after transplantation for living donor renal allograft recipients? Nephrology (Carlton). 2014; 19(4):244-50.
5. Harsh Vardhan, Narayan Prasad, **Akhilesh Jaiswal**, Brijesh Yadav. Outcomes of living donor renal transplant recipients with and without basiliximab induction: A long-term follow-up study. Indian Journal of Transplantation 2014; 8:44-50.

Original Article

Regulatory and effector T cells changes in remission and resistant state of childhood nephrotic syndrome

A. Jaiswal, N. Prasad, V. Agarwal[1], B. Yadav, D. Tripathy[1], M. Rai, M. Nath, R. K. Sharma, D. R. Modi[2]

Departments of Nephrology, [1]Clinical Immunology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, [2]Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

ABSTRACT

Idiopathic minimal change disease is a disorder of T-cell dysfunction. The relative predominance of regulatory T cells (Tregs), Th1, and Th2 cells in nephrotic syndrome (NS) remains controversial. Imbalance in peripheral blood regulatory and effector T cells (Teff) are linked to cell mediated immune response and may be associated with steroid response in NS. Peripheral blood CD4 + CD25 + FoxP3 + (Tregs), CD4 + IFN-γ^+ (Th1), and CD4 + IL-4 + (Th2) lymphocytes were analyzed in 22 steroid-sensitive NS (SSNS) patients in sustained remission, 21 steroid-resistant NS (SRNS) and 14 healthy controls. The absolute percentage values and ratio of Th1/Tregs, Th2/Tregs, and Th1/Th2 were compared between SSNS, SRNS and control subjects. The percentage of Tregs was lower in SRNS patients (P = 0.001) compared with that of SSNS and healthy control. The percentage of Th1 cells was higher in SRNS (P = 0.001) compared to that of SSNS patients; however, it was similar to healthy controls (P = 1.00). The percentage of Th2 cells in SRNS (P = 0.001) was higher as compared to SSNS and controls. The ratio of Th1/Treg cells in SRNS (P = 0.001) was higher as compared to SSNS patients and controls. The ratio of Th2/Treg was also higher in SRNS as compared to SSNS and controls. The ratio of Th1/Th2 cells in SSNS, SRNS, and healthy controls were similar. The cytokines secretion complemented the change in different T-cell subtypes in SSNS, SRNS and healthy controls. However, the IFN-γ secretion in healthy controles was low inspite of similar percentage of Th1 cells among SRNS cases. We conclude that greater ratio of Tregs compared to that Th1 and Th2 favor steroid sensitivity and reverse ratio results in to SRNS. The difference in ratio is related to pathogenesis or it can be used as marker to predict steroid responsiveness needs further evaluation.

Key words: Childhood nephrotic syndrome, effector T cells, regulatory T cells

Introduction

Idiopathic nephrotic syndrome (NS) is the commonest primary glomerular disease in children. One of the most important prognostic factors of the disease is steroid response. Approximately, 60-80% of steroid responsive patients experience relapses, and some remains steroid dependent or become steroid resistant.[1]

Address for correspondence:
Dr. Narayan Prasad, Additional Professor, Department of Nephrology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow - 226 014, Uttar Pradesh, India.
E-mail: narayan.nephro@gmail.com,

Access this article online	
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Idiopathic minimal change disease (MCD) is a disorder of T-cell dysfunction. Until the last decade, the dominant paradigm in NS was an imbalance between Th1 and Th2 cytokines and it was proposed that the cytokines secreted from activated Th2 cells, increase the glomerular permeability resulting into NS.[2] These discrepancies have been pointed out by Lama *et.al.*[3] One of the study showed that Th2 plays a predominant role in the Th1/Th2 imbalance hypothesis in childhood NS,[4,5] while another study revealed no skewing of Th1/Th2 balance[6] and possibly another subset of T cells the nonhelper regulatory T cells plays a role in NS. The nonhelper Tregs actively suppress the activation of the immune system, and effector T cells.[7-9] In 2009, Le Berre *et al.*, have shown that the induction of Tregs attenuates idiopathic NS in rats,[7] and subsequently, Araya *et al.,* have shown that Tregs suppressor mechanism is deficient in these cells and thereby enhance the cytokine release by Teff cells.[6] Recently, it has also been reported that other effector helper T cells also plays a potential role in the pathogenesis of MCD.[9,10] We aimed this study with the hypothesis that the decreased Tregs may result into

activation of Teff cells, which secrete proinflammatory cytokines resulting into persistent proteinuria in SRNS, and increased Tregs result into SSNS.

Methods

Patients and healthy controls

A total of 64 subjects (50 patients and 14 healthy subjects) were included in the study. Of the 50 patients with childhood idiopathic NS, 25 were NS patients in sustained remission for at least 6 months (SSNS) without a steroid; and 25 were steroid resistant nephrotic syndrome (SRNS) patients. Of them, three from remission group and three from SRNS group were excluded because they did not consent for inclusion and one in SRNS had family history of NS. Thus, 22 (males 18, age 8.52 ± 5.8 years) SSNS and 21 (males 20, age 11.7 ± 3.8 years) SRNS patients remained in the study. Fourteen (males 11, 10.6 ± 4.3 years) healthy children of same ethnicity were included as control. All SRNS patients had biopsy proven MCD. Children of <2 years and >16 years; and those with a family history of NS were excluded from the study. Definitions of glomerular diseases were based on established criteria according to the International Study for Kidney Diseases in Children.[11] NS in children was defined as proteinuria of 40 mg/m^2/h or, ratio of 2 for spot urine protein (mg)/creatinine (mg) in the first morning urine sample with hypoalbuminemia (serum albumin <2.5 g/dl) and presence of edema. Remission of NS was defined by urinary protein excretion <4 mg/m^2/h or urine dipstick nil/trace for three consecutive days, and patients were defined in sustained remission if remission persists for at least of 6 months after stopping steroid, and all SRNS patients were on oral steroid at time of enrolment in the study and blood samples for T cell phenotype and cytokine analysis were taken before starting alternative immunosuppressant. Primary steroid resistance was defined as unresponsiveness of 60 mg/m^2 body surface area per day for 4 weeks of prednisolone therapy. Secondary steroid resistance was defined as no response to 4 weeks of daily prednisone therapy at a dose of 60 mg/m^2/day in a child previously known to have a steroid-sensitive course. With regard to SRNS, patients enrolled in this study did not have any (i) underlying secondary causes, they were negative for hepatitis B surface antigen seropositivity, anti-hepatitis C virus seropositivity and human immunodeficiency virus seropositivity and (ii) had normal serum complement (C3 and C4) levels. An informed consent was obtained from a parent or guardian of both patients and controls when a participant <15 years, and from the participant when age is >15 years as per Institute guidelines. This study was approved by the Institute Ethics Committee, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India (pgi/dir/rc/186/2011).

T-cell phenotype analysis

Whole blood was diluted 1:1 with RPMI 1640 (Sigma Aldrich, St. Louis, USA) culture media and stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma Aldrich, St. Louis, USA) and ionomycin (1 μg/ml; Sigma Aldrich, St. Louis, USA) for 5 h. Monensin (2 μM; BD Biosciences, San Diego, CA, USA) was also added for the final 2 h of activation as a protein transport inhibitor. For surface staining fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD4 and PerCP-Cy5.5 conjugated mouse anti-human CD69 were added for staining of activated CD4 cells. After surface staining, red blood cells (RBCs) were lysed with BD FACS lysing solution. Cells were washed, fixed and then permiabilize with Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instruction. For intracellular cytokine staining with Alexa Fluor 647-conjugated mouse anti-human IFN-γ for Th1 and allophycocyanin-conjugated mouse anti-human IL-4 for Th2.[12] The isotypes FITC-conjugated mouse IgG1k, PerCP-Cy5.5 conjugated mouse anti-human IgG1k, Alexa Fluor 647-conjugated mouse IgG1k and allophycocyanin-conjugated mouse IgG1k were used as a control. Minimum 96% CD69 positive cells were used for phenotypic analysis. At least 10,000 lymphocytes were acquired on BD FACSCalibur (Becton Dickinson, Mount View, CA, USA) for each sample and analyzed with FlowJo (Ashland, OR, USA).

For Treg frequency analysis, whole blood was incubated with a cocktail of 2 mAb directed to CD4 (FITC), and CD25 (PerCP-Cy5.5). RBCs were lysed with BD FACS lysing solution. For intracellular staining of FoxP3, cells were subsequently fixed and permeabilized with BD Human FoxP3 Buffer Set according to the manufacturer's protocol before Alexa Fluor 647-conjugated mouse anti-human FoxP3 was added.[13] Isotype-matched antibodies were used as controls. All the antibodies were purchased from BD biosciences (BD Pharmingen, Sanjose, California, USA). A minimum 50,000 events in lymphocyte counts were acquired on a FACSCalibur (Becton Dickinson, CA, USA) flow cytometry and analyzed with FlowJo (Ashland, OR, USA).The representative plot of CD4 + CD25 + FoxP3 + Treg, CD4 + IFN-γ$^+$ Th1 and CD4 + IL-4 + Th2 lymphocytes in a SSNS, SRNS and control subjects is shown in Figure 1. All the flow cytometric experiments were carried out in duplicate. The coefficient of variation for various markers CD4$^+$, CD25$^+$, FoxP3$^+$, IFN-γ$^+$, and IL-4 was 1.6%, 2.3%, 4.1%, 3.2%, and 2.1% respectively.

Separation of peripheral blood mononuclear cells

Heparinized venous blood was diluted 1:1 with RPMI 1640, containing Hepes (25 mM), gentamicin (50 pg/ml) and 10% heat-inactivated fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMCs) were isolated over Histopaque-1077 (Sigma, St. Louis, MO 63103, USA) and washed three times.

In vitro production of cytokines by peripheral blood mononuclear cells

In vitro production of cytokines from cultured PBMCs was analyzed in 10 subjects in each groups. We measured following cytokines representative of different T cell subtypes secreted from cultured PBMCs; IL-10 and transforming growth factor-β1 (TGF-β1) for Tregs; IFN-γ for Th1 cells; IL-4 for Th2 cells.

Cells were resuspended at 1 × 10^6 cells/ml in RPMI 1640 supplemented with 2 mM of extra glutamine and 10% FCS. The viability of the cells was checked by trypan blue. *In vitro* culture of the PBMCs was performed with mitogen phorbol 12-myristate 13-acetate (50 ng/ml; Sigma Aldrich, St. Louis, MO 63103, USA) and ionomycin (1 μg/ml; Sigma Aldrich, St. Louis, MO 63103, USA) in a flat-bottom six well culture plate at 37°C, 5% CO_2 and 100% humidity. Culture supernatants were harvested after 24 h and stored at −80°C.

Enzyme-linked immunosorbent assay

Cytokine levels in culture supernatants were quantified by commercially available kit for human IL-10, IFN-γ, IL-4, and TGF-β (Becton Dickinson, San Diego, CA 92121, USA). The lower detection limits for the individual assays are as follows: IL-10, 7.8 pg/ml; IFN-γ, 4.7 pg/ml; IL-4, 7.8 pg/ml; and TGF-β, 125 pg/ml.

Statistical analysis

Data are expressed as mean ± standard deviation. Data were analyzed using SPSS statistical software 15.0 (SPSS,

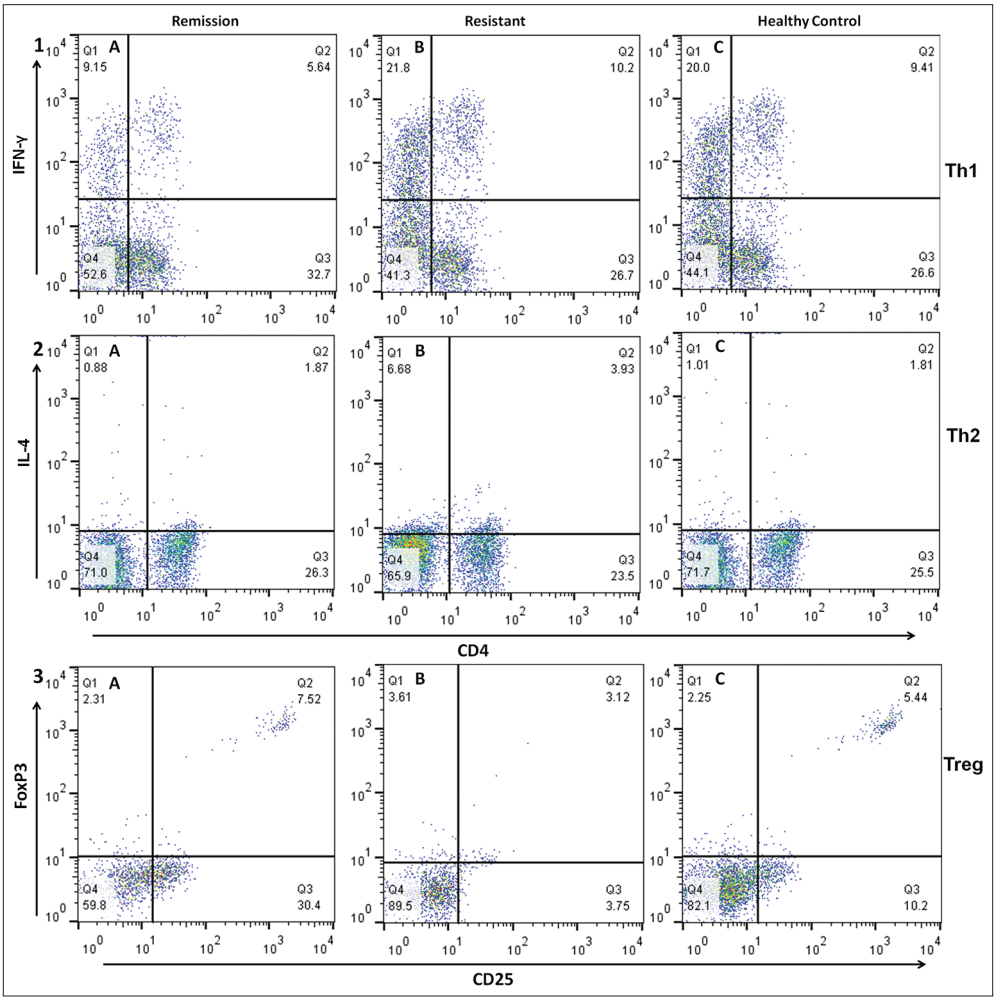

Figure 1: Flow cytometric detection of Th1, Th2, and Treg cells in sustained remission, steroid resistant and healthy control. Stimulated peripheral blood were stained with fluorescein isothiocyanate-conjugated CD4 mAb and AlexaFluor 647 conjugated interferon-γ mAb for Th1 cells (1) and APC conjugated IL-4 mAb for Th2 cells (2) For Treg cells fluorescein isothiocyanate-conjugated CD-4 mAb, PerCP-Cy conjugated CD-25 mAb and AlexaFluor 647 conjugated FoxP3 mAb were used for staining of whole blood (3)

Chicago, IL, USA). The means values in different groups were compared with analysis of variance (ANOVA) for parametric values. The percentage values in different groups were compared using Chi-square test or, Fisher exact test as per application required. $P < 0.05$ were considered as significant.

Results

The percentage of CD4 + CD25 + FoxP3 + Tregs was significantly lower in SRNS patients (3.03 ± 1.18%); $P = 0.001$ as compared to SSNS (7.84 ± 4.26%) and healthy control (7.99 ± 3.12%) [Figure 2a]. The percentage of CD4 + IFN-γ^+ Th1 cells was significantly higher in SRNS (20.24 ± 7.01%; $P = 0.001$) as compared to SSNS (10.37 ± 3.49%), $P = 0.001$; however, it was similar to healthy control (18.38 ± 3.28% ($P = 1.00$) [Figure 2b]. The percentage of CD4 + IL-4 + Th2 cells in SRNS (10.74 ± 5.91%; $P = 0.001$) was significantly higher as compared to SSNS (5.18 ± 3.12%) and controls (4.91 ± 1.24%) [Figure 2c].

The ratio of CD4 + IFN-γ^+ Th1 and CD4 + CD25 + FoxP3 + Treg cells in SRNS (7.65 ± 3.72; $P = 0.001$) was significantly higher as compared to SSNS patients (1.69 ± 1.0) and controls (2.79 ± 1.52) [Figure 3a]. The ratio of CD4 + IL-4 + Th2 and CD4 + CD25 + FoxP3 + Treg was higher in SRNS (10.74 ± 5.91%; $P = 0.001$) as compared to SSNS (5.18 ± 3.12%) and controls (4.91 ± 1.24%) [Figure 3b]. The ratio of CD4 + IFN-γ^+ Th1 and CD4 + IL-4 + Th2 cells in SSNS (3.21 ± 1.50), SRNS (2.85 ± 1.12), and healthy controls (2.79 ± 1.51) were similar [Figure 3c].

Cytokine levels from peripheral blood mononuclear cell culture supernatants of different groups

There was no production of cytokines from unstimulated PBMCs. The cytokines secreted from stimulated cultured PBMCs as shown in Figure 4. There was significantly lower secretion of IL-10 [Figure 4a] and TGF-β [Figure 4b] in PBMCs from SRNS patients as compared to that of SSNS and healthy controls. Significantly increased secretion of IFN-γ [Figure 4c] was observed in SRNS as compared to SSNS and healthy controls. Significantly greater secretion of IL-4 was observed in SRNS as compared to that of SSNS and healthy controls [Figure 4d]. The cytokines secretion complemented the different T cell subtypes in SSNS, SRNS, and healthy controls except the IFN-γ cytokine secretion in healthy controls was low despite the fact that percentage of Th1 cells was similar to SRNS.

Discussion

In the late 1980s, the Th1 and Th2 cell imbalance hypothesis had emerged for all immune mediated diseases, streaming from observations in mice of two subtypes of helper T cells differing in cytokine secretion patterns and other functions. This very concept was applied to human immunity[14,15] and for almost 2 decades, the Th1 and Th2 imbalance in NS remained a major research focus.[16,17] Consequently, Th1/Th2 imbalance concept was raised to the level of paradigm. The dominant paradigm in NS is an imbalance between Th1 and Th2 cytokines such as interleukin 13 (IL-13) a cytokine secreted from activated Th2 cells, increases glomerular permeability resulting into NS.[2]

However, the predominance of Th1/Th2 over each other in pathogenesis of NS still remains controversial and debatable. Kanai *et al.,* have shown that Th2 plays a predominant role amongst the Th1/Th2 ratio in children with NS.[4] Kaneko *et al.,* have shown that there is no skewing of Th1/Th2 balance in childhood NS and that the cardinal immunological abnormality does not lie in helper T cells but in other cells, such as suppressor/cytotoxic T cells.[5] In 2009, Le Berre *et al.*, have shown that the induction of Treg cells attenuates idiopathic NS in rats,[7] and subsequently, Araya *et al.*, have shown that Treg cell suppressor mechanism is deficient in these cells and thereby enhance the cytokine release by Teff cells resulting in to increased permeability and NS.[6]

Our study revealed that Th1 and Th2 cells in SRNS patients were significantly higher than the SSNS patients. However, the ratio of Th1/Th2 was not different in both groups.

In the present study, it was observed that the SSNS patients in remission had greater percentage of circulating Treg and lesser percentage of Th1 and Th2 cells. Further, the percentage of Tregs in SRNS patients was low and percentage of Th1 and Th2 cells was high as compared to SSNS. We also observed that Tregs cytokine profile was significantly higher in the SSNS and healthy controls as compared to that of SRNS. These observations indicate that an imbalance between Treg and Th1 and Th2 cells may be associated with the remission and resistant state of NS. One of the contrasting observations was that the percentage of Th1 cells was similar in control subjects and SRNS patients. However, Th1 cells from healthy controls were functionally less active as confirmed by IFN-γ cytokine secretion by stimulated PBMCs. This could be explained by the effect of functionally active Tregs in healthy controls, which might have suppressed the activity of Th1 cells.

Tregs are a specialized subpopulation of T cells that actively suppress activation of the immune system.[18] In renal transplantation, Treg phenotypically

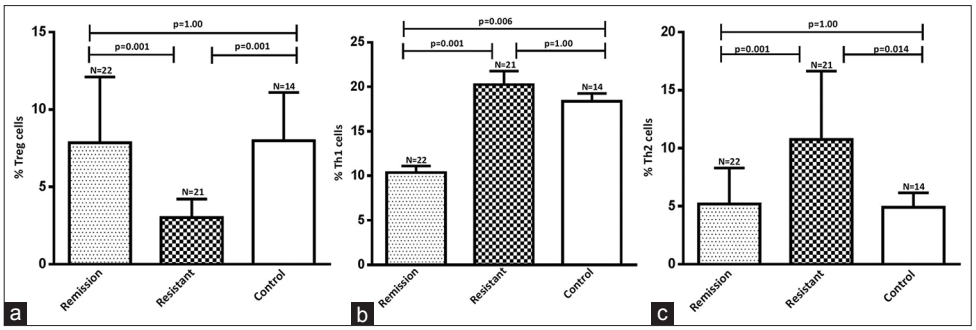

Figure 2: Results are expressed as the percentage of CD4+CD25+FoxP3+ Treg, CD4+IFN-γ+ Th1 and CD4+IL-4+ Th2 cells in CD4+ lymphocytes in blood. Significant decrease in Treg cells in steroid resistant (a) Th1 cells were significantly decreased in remission compare to both resistant and healthy control (b) and (c) shows significant increase in the population of Th2 cells in steroid resistant nephrotic syndrome patients

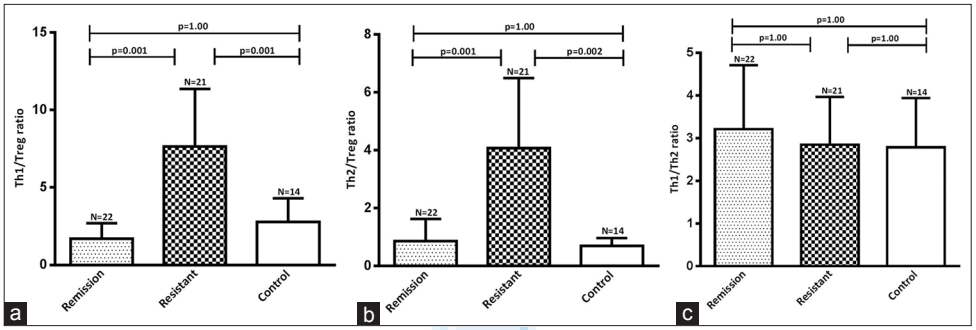

Figure 3: Bar-diagram shows significant increase in the ratio of Th1/Treg (a) and Th2/Treg (b) cells in resistant patients as compared to remission and healthy control whereas no difference in the ratio of Th1/Th2 (c) cells between the groups

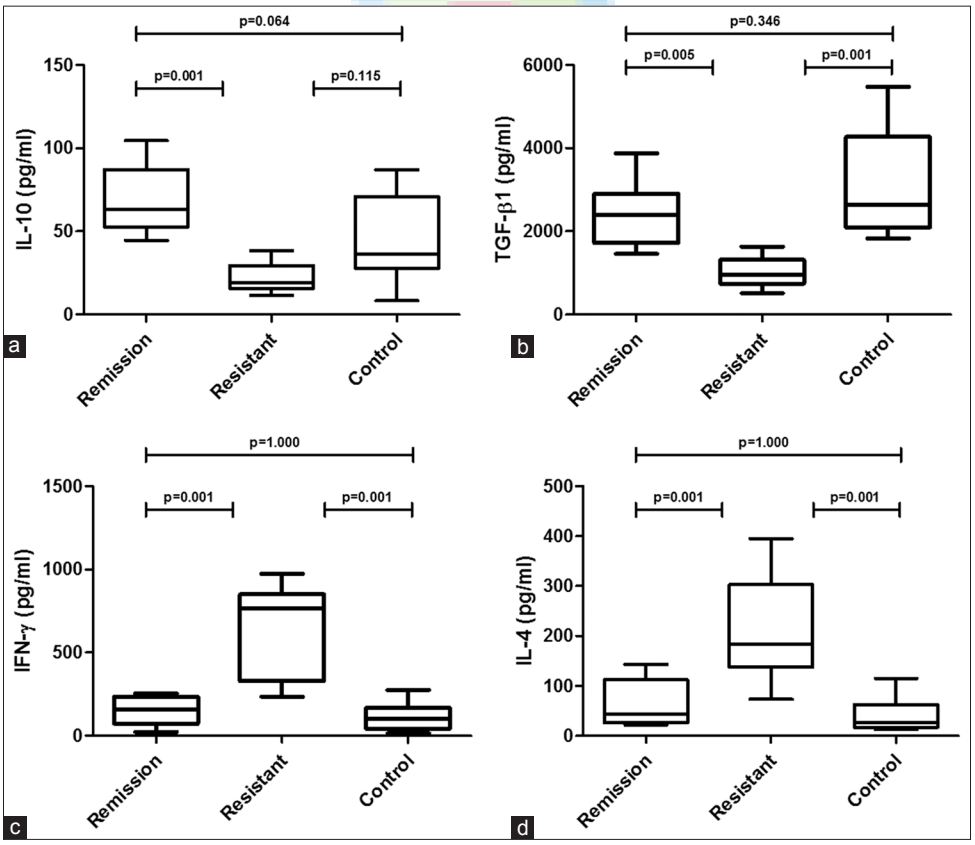

Figure 4: Cytokine levels in peripheral blood mononuclear cell culture supernatants of different groups. Significantly higher production of interleukin-10 (IL-10) (a) and transforming growth factor-β1 (b) whereas significantly decrease production of interferon-γ (c) and IL-4 (d) in remission and control compared to resistant patient after 24 h culture

defind as CD4 + CD25 + FoxP3$^+$,[19,20] are associated with graft tolerance;[21] and circulating Tregs with presence of FoxP3 in the graft is associated with donor-specific hyporesponsiveness in patients after renal transplantation.[22] Treg cells mitigate the deleterious effect of immune system by several mechanisms; promoting graft survival by consuming IL-2, inhibiting mRNA synthesis in nonTreg,[23] by secreting anti-inflammatory cytokines such as TGF-β, IL-10, IL-35, by blocking co-stimulatory signal in Teff cells and also by blocking the granzyme-dependent direct killing by pathogenic Teff cells.[24]

The major strength of our study is that it successfully demonstrated that beyond Th1/Th2 imbalance, it is the imbalance of Treg with Th1 and Th2 which may be associated with SSNS and SRNS state in NS patients. With accumulating evidences of the use of Treg therapy in various autoimmune diseases and transplantation, it is possible that Tregs may also be used for the therapeutic purpose towards treatment of SRNS patients in future.[25]

The major limitation of our study is that we have not studied expression of Th17 cells which has been reported to play a role in immunological imbalances.

Conclusion

Remission and resistant state of NS is associated with an imbalance between Tregs, Th1 and Th2 cells. The ratio of Teff cells (Th1 and Th2) and Tregs was observed to be different in SSNS and SRNS. The greater ratio of Tregs compared to that Th1 and Th2 may result into state of SSNS and reverse ratio results into SRNS. However, the difference in ratio is related to pathogenesis or it can be used either as a marker to differentiate SSNS from SRNS or, to predict steroid responsiveness.

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Differential alteration in peripheral T-regulatory and T-effector cells with change in P-glycoprotein expression in Childhood Nephrotic Syndrome: A longitudinal study

Narayan Prasad [a,*], Akhilesh K Jaiswal [a], Vikas Agarwal [b], Brijesh Yadav [a], Raj Kumar Sharma [a], Mohit Rai [b], Harshit Singh [b], Saurabh Chaturvedi [b], Ajay Singh [a]

[a] Department of Nephrology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India
[b] Department of Clinical Immunology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India

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ABSTRACT

Introduction: Childhood Idiopathic Nephrotic Syndrome (INS) responds to glucocorticoid therapy, however, 60–80% of patients relapse and some of them become steroid non responsive. INS may occur because of T cell dysfunction, abnormal cytokines and podocytopathies which reverse on steroid treatment. The reason of relapses could be imbalances in T cells phenotypes and respective cytokines. Herein, we hypothesize that relapses in INS may occur due to imbalance in T-regulatory and T-effector cell with their respective cytokines and overexpression of P-gp on lymphocytes.

Methods: The frequency of peripheral blood $CD4^+CD25^+FoxP3^+$ Treg, $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}4^+$ Th2 lymphocytes and their respective cytokines and P-gp expression on peripheral blood lymphocytes (PBLs) were analyzed in INS patients at baseline ($n = 26$), during remission ($n = 24$) and at relapse ($n = 15$).

Results: Compared to baseline, the frequency of Tregs was significantly increased at remission and decreased during relapse. In contrast, the frequency of Th1 and Th2 lymphocytes was significantly decreased during remission and increased at the time of relapse. Similarly, expression of P-gp was significantly high at baseline and at the time of relapse as compared to remission. Levels of cytokines IL-10 and TGF-β in the supernatant of stimulated PBMCs was increased during remission and decreased during relapse. In contrast, levels of IFN-γ and IL-4 were decreased during remission and increased at the time of relapse.

Conclusions: Steroid therapy in INS induces decreased P-gp expression on PBLs along with increased frequency and cytokine response of T-regulatory cells, and reduced frequency and respective cytokine response of Th1 and Th2 cells during remission. However, reversal in the frequency and respective cytokines of T-regs, Th1 and Th2, and P-gp expression on PBLs occurs during relapses on follow-up.

1. Introduction

Minimal change disease (MCD) is one of the most common glomerular diseases causing Idiopathic Nephrotic Syndrome (INS) in children. MCD may be associated with T-cell dysfunction [1], increase in cytokines level [2–4] and podocyte injury which may be reversed after steroid therapy [5,6]. The exact cause of this podocytopathy in MCD remains unknown. Podocytopathy in MCD may be idiopathic, genetic; associated with NPHS2 mutation or reactive associated with malignancy or other immunologic stimuli. Non genetic idiopathic and reactive forms of MCD are usually steroid responsive and cell mediated immunity has been invoked as etiologic factor for this form of MCD [1,5]. However the predominant role of different T cell phenotypes; regulatory T cells (Tregs), and effector T cells (Teff) T helper cells (Th1) and T helper cells (Th2) remain poorly understood [7]. The predominance of Th2 phenotype was reported in one study while another study did not report any skewing of Th1/Th2 and suggested a role of Tregs [8]. Araya et al have shown that suppressor function of Tregs cell was deficient in INS that could lead to persistence of pathogenic cytokines released by Teff cells resulting into proteinuria [9]. A case of INS going in to remission following influenza B infection without the need of steroids has been reported. Authors have demonstrated increased Tregs population during remission in the case [10]. Recently, we have observed greater ratio of Tregs/Effector (Teff)

* Corresponding author at: Department of Nephrology, Sanjay Gandhi Postgraduate Institute of medical Sciences, Lucknow 226014, India. Tel.: +91 522 2495187, mobile: +91 9415403140.
E-mail address: narayan.nephro@gmail.com (N. Prasad).

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cells in remission in INS patients and its reversal during treatment resistant state [11].

Approximately 60–80% of steroid responsive INS patients experience relapses of proteinuria on follow up despite initial clinical response to steroids and some of them become steroid non responsive [12]. The treatment of relapsing Nephrotic Syndrome with multiple courses of steroids, cytotoxic agents and calcineurin inhibitors remains a challenging clinical scenario [13]. The reason of poor steroid response could be change in histological pattern from MCD, a condition with podocyte injury without podocytopenia, to FSGS podocyte injury with podocytopenia [14], or changes in pharmacological intervention with overexpression of permeable glycoprotein (P-gp) on lymphocytes which has emerged as one of the major molecule causing poor response to many drugs, peptides, alkaloids, steroids, immunosuppressive drugs, and calcium channel blockers [15]. P-gp is a 170-kD product of the multidrug resistance 1 (MDR-1) gene. Our previous study indicated that homozygous mutant of MDR-1 gene influences steroid responsiveness in NS patients [16]. The MDR-1 gene belongs to the ATP-binding cassette (ABC) energy-dependent transporters [17]. In humans, P-gp is involved in xenobiotics efflux, protecting host tissue from toxic side effects. The overexpression of P-gp causes efflux of steroid from inside of cells to the outside. This limits the concentration of steroids within the cells and their site of action that results in poor response and steroid resistance [18]. P-gp is expressed on the surface of peripheral blood lymphocytes (PBLs), which are the putative targets of pharmacotherapy in INS [19] and the expression of P-gp is regulated by certain cytokines at transcription level which plays role in pathogenesis of INS [20].

Corticosteroids and calcineurin inhibitors are the drugs used for the treatment of NS. It is possible that podocyte itself could be the target of steroid or calcineurin inhibitors in INS as receptors of these drugs has been seen on podocytes. The effect of corticosteroids on lymphocytes and cell mediated immunity in INS is well established and the effect of steroid therapy on PBLs and phenotypic changes in patients during the course of the therapy is easier to monitor than any changes on podocytes. Corticosteroids are the substrate of P-gp and calcineurin inhibitors are substrate as well as inhibitor of P-gp expressed on lymphocytes [19]. The many poor steroid responsive INS patients respond well to calcineurin inhibitors. There is paucity of data on this novel biomarker during the course of the disease. It may help in better monitoring of the disease status. Therefore, we aimed this study to evaluate the alterations in frequency of different peripheral blood T cell phenotypes; Tregs, Th1, Th2 cells, and P-gp expression on PBLs in INS patients receiving steroids at baseline, on achieving remission and at time of relapse.

2. Patients and methods

We longitudinally studied the frequency of CD4+CD25+FoxP3+ Treg, CD4+IFN-γ+ Th1 and CD4+IL-4+ Th2 lymphocytes from whole blood and P-gp expression at baseline ($n = 26$) (before initiating steroid therapy); during remission after stopping steroid for at least 4 weeks (SSNS, $n = 24$); and at the time of relapse before starting immunosuppression ($n = 15$) during follow up. All patients were subjected for the analysis of cytokine production from stimulated PBMCs at baseline, during remission and at the time of relapse. Ten healthy children as controls were also subjected for the different T cell phenotypes, cytokine production and P-gp expression experiments.

Children of less than 2 years and greater than 16 years; and those with a family history of NS were excluded from the study. Definitions of INS, remission, and relapse were based on established criteria according to the International Study for Kidney Diseases in Children [21]. NS in children was defined as proteinuria of 40 mg/m^2/h or, ratio of 2 for spot urine protein (milligram)/creatinine (milligram) in the first morning urine sample with hypo-albuminemia (serum albumin <2.5 g/dL) and presence of edema. All children were treated with prednisolone of 60 mg/m^2 daily for 6 weeks followed by 40 mg/m^2 alternate day for the next 6 weeks. Samples from patients who achieved remission were taken after 4 weeks of stopping steroid treatment. Remission of NS was defined by urinary protein excretion <4 mg/m^2/h or urine dipstix nil/trace for three consecutive days. Relapse was defined as urinary protein excretion >40 mg/m^2/h or urine dipstix ++ or more for three consecutive days. In cases of infection associated relapse, blood sample were not collected if there was any clinical evidence of infection in the patients. In 3 patients with presumed upper respiratory tract viral infection (URTI) associated relapse, blood sample for analysis was collected after 2 weeks of resolution of infection when proteinuria was persisting before staring steroid. Steroid resistance was defined as unresponsiveness of 60 mg/m^2 body surface area per day for 4 weeks of prednisolone therapy. Patients enrolled in this study did not have any (i) underlying secondary causes, they were negative for hepatitis B surface antigen seropositivity, anti hepatitis C virus seropositivity and human immunodeficiency virus seropositivity and had normal serum complement (C3 and C4) levels. An informed consent was obtained from a parent or guardian of both patients and controls when participant <15 years and from the participant when age was >15 years as per institute guidelines. This study was performed in accordance with declaration of Helsinki and approved by the Institute Ethics Committee, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

2.1. T cell phenotype analysis from whole blood

Whole blood was diluted 1:1 with RPMI 1640 (Sigma Aldrich, St. Louis) culture media and stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma Aldrich, St. Louis) and ionomycin (1 μg/ml; Sigma Aldrich, St. Louis) for 5 h. Monensin (2 μM; BD Biosciences, San Diego, CA) was also added for the final 2 h of activation as a protein transport inhibitor. For surface staining, FITC conjugated mouse anti-human CD4 and PerCP-Cy5.5 conjugated mouse anti-human CD69 were added for staining of activated CD4 cells. After surface staining, RBCs were lysed with BD FACS lysing solution. Cells were washed, fixed and then permeabilized with Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instruction for intracellular cytokine staining with Alexa Fluor 647- conjugated mouse anti-human IFN-γ for Th1 and Allophycocyanin (APC)-conjugated mouse anti-human IL-4 for Th2 cells. Isotype-matched antibodies were used as controls in each experiment. At least 10,000 lymphocytes were acquired on BD FACS Calibur (Becton Dickinson, Mount View, CA, USA) for each sample and analyzed with FlowJo software (Ashland, OR, USA).

For T regulatory cells, whole blood was incubated with a cocktail of 2 mAb directed to CD4 (FITC) and CD25 (PerCP-Cy 5.5). RBCs were lysed with BD FACS lysing solution. For intracellular staining of FoxP3, cells were subsequently fixed and permeabilized with BD Human FoxP3 Buffer Set according to the manufacturer's guidelines before Alexa Fluor 647-conjugated Mouse Anti-Human FoxP3 was added. Isotype-matched antibodies were used as controls. All the antibodies were purchased from BD biosciences (BD Pharmingen, Sanjose, California, USA). A minimum 50,000 events in lymphocyte counts were acquired on a FACSCalibur (Becton Dickinson, CA, USA) flow cytometry and analyzed with FlowJo software (Ashland, OR, USA). All fluorescein conjugated monoclonal antibodies were purchased from BD Pharmingen CA, USA.

2.2. Analysis of In vitro production of cytokines

The mitogen stimulated PBMCs were cultured and supernatant was analyzed for various cytokines as has been described

previously [2–4]. Briefly, heparinized venous blood was diluted 1:1 with RPMI 1640 (Sigma Aldrich, 3050 Spruce Street, Saint Louis, MO 63103, USA), containing Hepes (25 mM), gentamicin (50 pg/ml) and 10% heat-inactivated fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMCs) were isolated over Histopaque-1077 (Sigma, St. Louis, MO 63103, USA) and washed three times. Cells were re-suspended at 1×10^6 cells/ml in RPMI 1640 supplemented with 2 mM of extra glutamine and 10% FCS. The viability of the cells was checked by trypan blue. In vitro culture of the PBMCs was performed with mitogen phorbol 12-myristate 13-acetate (50 ng/ml; Sigma Aldrich, St. Louis, MO 63103, USA) and ionomycin (1 μg/ml; Sigma Aldrich, St. Louis, MO 63103, USA) in a flat-bottom six well culture plate at 37°C, 5% CO_2, 100% humidity. Culture supernatants were harvested after 24 h and stored at −80°C till analysis. Cytokine levels in culture supernatants were quantified by commercially available kit for human IL-10, IFN-γ, IL-4 and TGF-β (Becton Dickinson, San Diego, CA92121, USA). The lower detection limits for the individual assays are as follows: IL-10, 7.8 pg/ml; IFN-γ, 4.7 pg/ml; IL-4, 7.8 pg/ml; and TGF-β, 125 pg/ml.

2.3. P-gp expression assay

P-gp expression on lymphocytes was analyzed from whole blood. 50 μl heparinised blood was incubated with 20 μl PE-conjugated human anti-P-gp mAb (BD Pharmingen CA, USA) and 20 μl PE-conjugated matched-isotype control antibody for 30 minutes at room temperature. Then, RBCs were lysed with BD FACS lysing solution and washed twice in PBS. Subsequently, PBMCs were fixed with 0.5% paraformaldehyde and analyzed on a FACSCalibur (Becton Dickinson, CA, USA). At least 10,000 cells were counted and analyzed and separated according to their forward and side scatter characteristics. Results are expressed as the percentage of positive cells and relative fluorescence intensity (RFI).

2.4. Statistical analysis

Data are expressed as mean ± standard deviation. Data was analyzed using SPSS statistical software 15.0 (SPSS, Chicago, IL, USA). The mean values in different groups were compared with analysis of variance (ANOVA) for parametric values and Bonferroni test was used to compare the significance. The percentage values in different groups were compared using chi square test or, Fisher exact test as per required application. P values $\leqslant 0.05$ were considered significant.

3. Results

Of the 26 INS patients (age = 8 ± 4 years, male = 21), 24 patients achieved remission after completion of steroid therapy and 2 remained primary non-responder to steroid. Of the 24 patients who achieved remission, 15 patients experienced relapse of NS on 8.86 ± 3.9 months of follow up.

3.1. Circulating Treg, Th1 and Th2 lymphocytes in INS patients at baseline, during remission and at relapse

The frequency of Tregs was significantly increased in patients who achieved remission (6.82 ± 4.12) as compared to the baseline (1.83 ± 0.84) values and was comparable to controls (5.69 ± 2.34). However, the frequency of Tregs decreased in patients who relapsed (3.03 ± 1.18) and was similar to the baseline values (Fig. 1A). At baseline, the frequency of Tregs in relapsing ($n = 15$) and non-relapsing ($n = 9$) was 1.63 ± 0.69 and 2.30 ± 0.96 respectively ($p = 0.061$). However, Tregs frequency (9.22 ± 5.43) was higher in non-relapsing patients as compared to relapsing patients (5.37 ± 2.26) during remission $p = 0.023$.

The frequency of Th1 (9.9 ± 4.65) cells and Th2 (4.81 ± 1.42) cells decreased significantly during remission as compared to the baseline (Th1, 16.18 ± 7.19; Th2,10.5 ± 4.66) values and were comparable to the healthy controls (Th1, 13.28 ± 9.36; Th2, 2.79 ± 1.51). The frequency increased in patients who relapsed (Th1, 19.83 ± 3.47; Th2, 9.89 ± 5.18) and were similar to the baseline values (Fig. 1B and C).

3.2. Cytokine levels in PBMC culture supernatants at baseline, during remission and at relapse in INS patients and control subjects

There was no production of cytokines from unstimulated PBMCs after culture. The cytokines profile of stimulated cultured PBMCs is shown in Fig. 2. The production of IL-10 (Fig. 2A) and TGF-β1 (Fig. 2B) was significantly increased during remission as compared to the baseline values and were similar to healthy controls. The production of both the cytokines decreased at the time of relapse. In contrast, secretion of IFN-γ (Fig. 2C), and IL-4 (Fig. 2D) decreased significantly during remission and was comparable to healthy controls. The secretion of both the cytokines increased significantly at the time of relapse.

3.3. P-gp expression at baseline, during remission and at relapse

The percentage of P-gp positive lymphocytes and the absolute expression of P-gp (RFI × % P-gp positive cells) were significantly higher at baseline and at relapse in NS patients as compared to their values during remission. The levels of P-gp expression during remission were comparable to controls (Fig. 3 and Table 1). We observed that P-gp expression correlated negatively with the regulatory cytokines IL-10 ($r = -0.671$, $p = 0.001$) and TGF β1

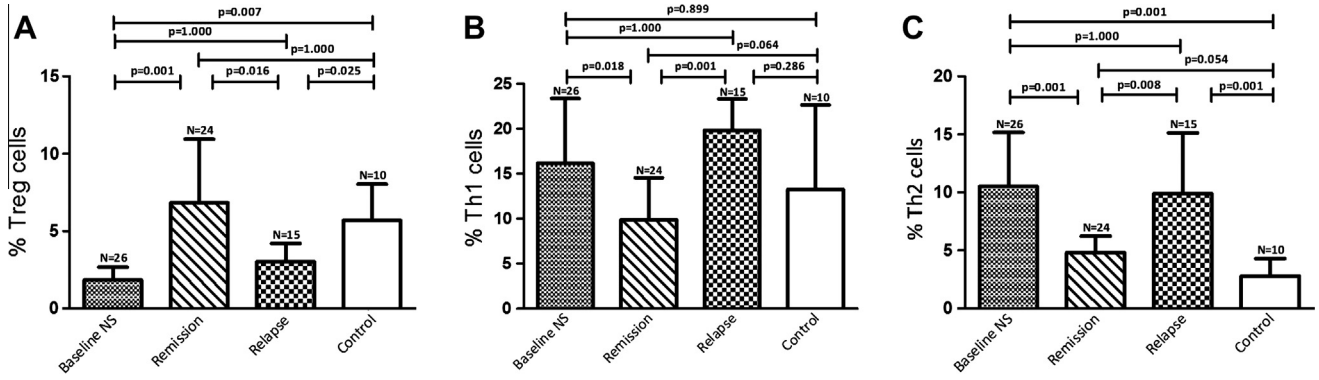

Fig. 1. Results are expressed as the percentage of CD4+CD25+FoxP3+Treg, CD4+IFN-γ+Th1 and CD4+IL-4+Th2 cells in CD4+lymphocyte in blood. Significant increase in Treg cells at remission as compared to the baseline and healthy controls and again decreased after relapse (A). Th1 and Th2 cells were significantly decreased in remission as compared to baseline values and healthy controls and again increased at relapse of NS (B and C).

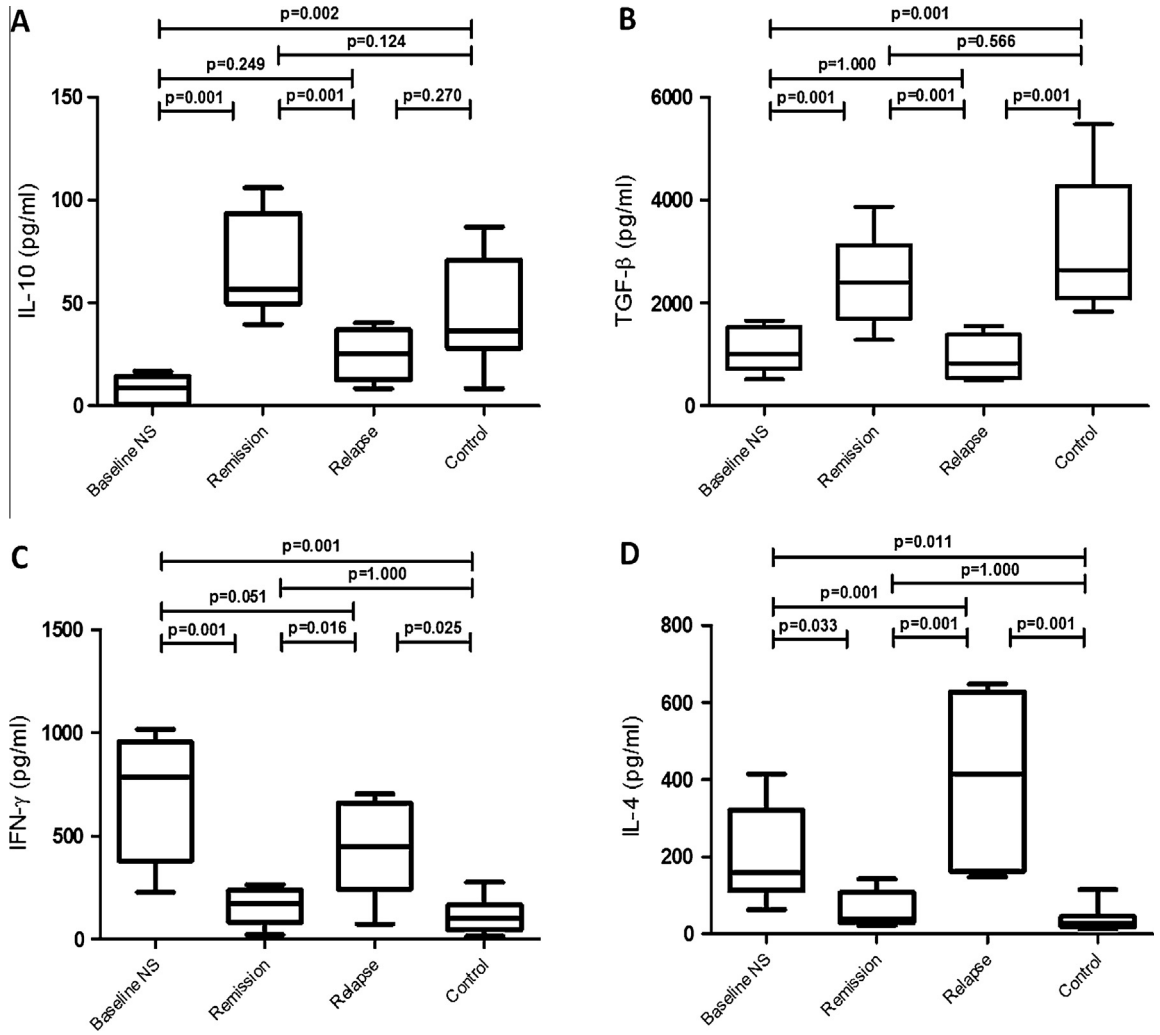

Fig. 2. Cytokine levels in PBMC culture supernatants during follow up. Significantly higher production of IL-10 (A) and TGF-β1 (B) at remission and decreased production at time of relapse compared to control and remission whereas significantly decrease production of IFN-γ (C), and IL-4 (D) in remission and increased production at time of relapse compared to values at remission and control after 24 h culture.

($r = -0.620$, $p = 0.001$) and positively correlated with cytokines, IFN-γ ($r = 0.448$, $p = 0.002$) and IL-4 ($r = 0.523$, $p = 0.001$) (Fig. 4). There was significant negative correlation of Tregs with absolute P-gp expression during remission ($r = -0.575$, $p = 0.003$) and relapse ($r = -0.521$, $p = 0.047$). However, correlation was not significant at baseline ($r = -0.247$, $p = 0.22$).The percentage of P-gp expressing Tregs significantly decreased during remission ($p = 0.002$) and again increased during relapse, however did not reached significance level ($p = 0.10$). The percentage of P-gp expressing Th1 cells decreased significantly during remission ($p = 0.004$) as compared to baseline values and again increased during relapse ($p = 0.001$). The percentage of P-gp expressing Th2 cells decreased significantly during remission ($p = 0.001$) from baseline and again increased during relapse ($p = 0.001$) (Table 2).

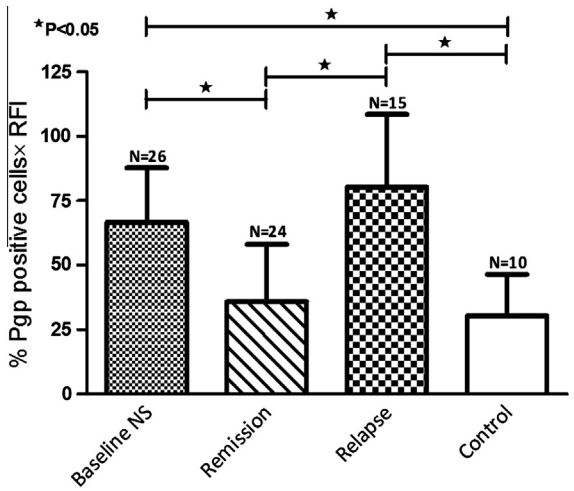

Fig. 3. Absolute P-gp expressions at baseline, during remission, relapse in INS patients and control.

At baseline, the median value of absolute P-gp expression (%) in total steroid responsive patients was 71, and the mean time to achieve remission was 16.8 ± 3.7 days in 13 patients with values ⩾71 and 13.5 ± 1.9 days in 11 patients with values <71 ($P = 0.016$). Moreover, the level of absolute P-gp expression during remission in 15 relapsing patients was significantly higher as compared to 9 non-relapsing patients (44.26 ± 6.09 vs. 21.78 ± 9.81, $p = 0.013$).

3.4. Two primary steroid non responder patients versus 24 steroid responsive patients at baseline

At the baseline in 2 non responder patients, the frequency of Tregs, Th1 and Th2 cells was 1.56 ± 0.02; 21 ± 0.06; and

Table 1
Percentage of P-gp positive peripheral blood lymphocytes and absolute expression of P-gp at baseline, during remission and relapse of Idiopathic Nephrotic Syndrome.

Characteristics	Baseline NS	Remission	Relapse	Control
% P-gp positive cells	8.69 ± 3.62	5.46 ± 3.14	10.02 ± 5.2	4.89 ± 1.73
RFI	8.22 ± 1.82	6.72 ± 1.88	8.65 ± 2.96	7.13 ± 5.26
RFI × % P-gp positive cells	66.59 ± 21.13	35.84 ± 22.26	80.22 ± 28.24	30.41 ± 16.15

P-gp, permeable glycoprotein; RFI, relative fluorescence intensity.

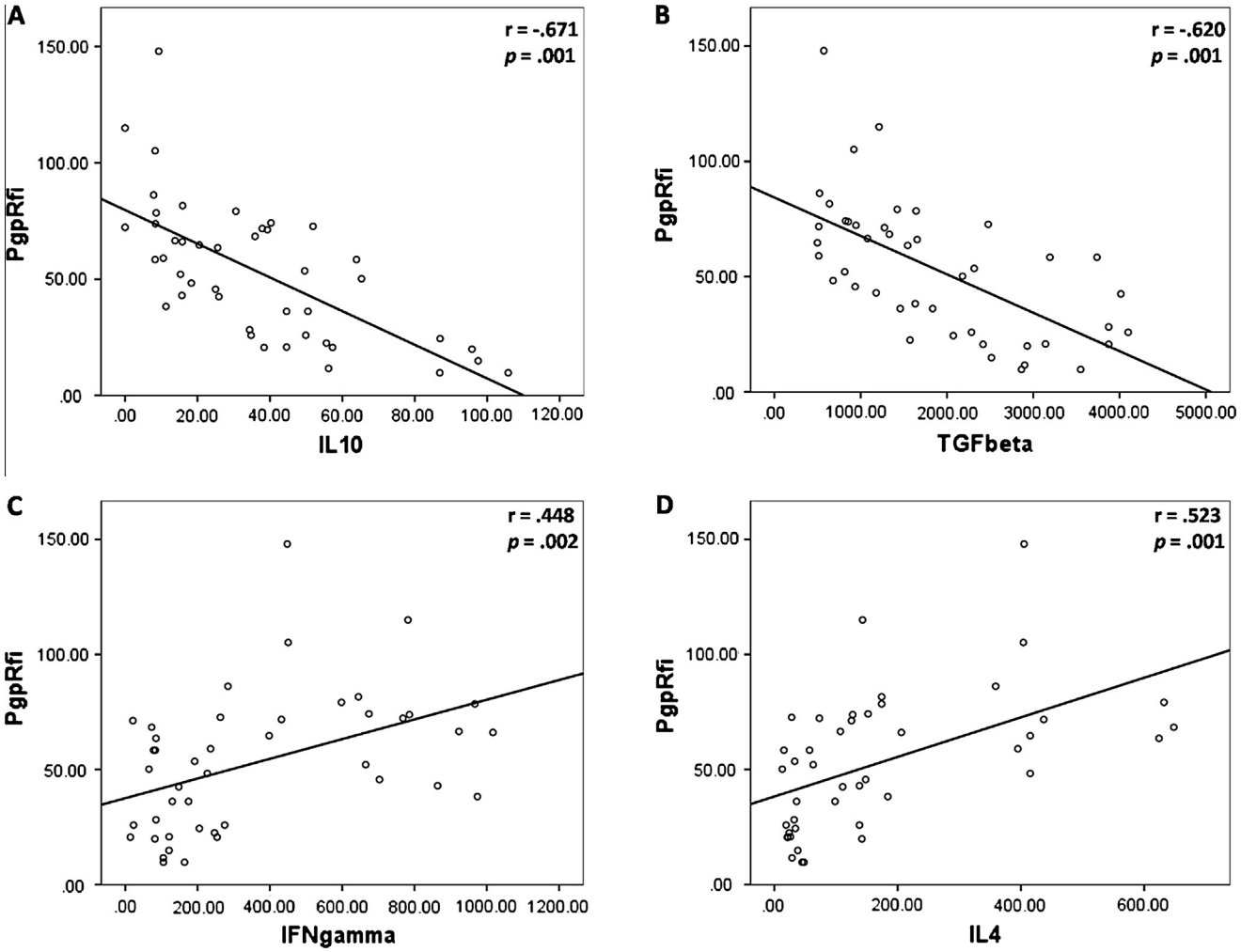

Fig. 4. Correlation of absolute P-gp expression on peripheral blood lymphocytes with cytokines Il-10, TGF-β, IFN-γ and IL-4.

12 ± 0.97, respectively. The cytokine level (pg/ml) of IL-10, TGF-β, IFN-γ and IL-4 was 9.8 ± 3.6; 1021 ± 181.2; 819.5 ± 218.4; and 220 ± 90.5 respectively. In 24 responsive patients, the frequency of T-reg, Th1 and Th2 was 1.62 ± 0.68; 18.2 ± 6.3; and 9.5 ± 5.1 respectively. The cytokine level (Pg/ml) of IL-10, TGF-β, IFN-γ and IL-4 was 10.9 ± 5.8; 1051.7 ± 411.7; 687.9 ± 303.3; and 211 ± 120 respectively. The mean value (%) of absolute expression of P-gp on lymphocytes in 2 non responder patients was 145 ± 16 while it was 85.4 ± 35.7 in 24 steroid responsive patients.

4. Discussion

In the present longitudinal follow-up study, increased frequency of Tregs and its cytokines (IL-10 and TGF-β) and decreased frequency of Th1 and Th2 lymphocytes and their cytokines (IFN-γ and IL4) along with decreased expression of P-gp on PBLs was observed during remission and vice versa at the time of relapse in NS patients.

Tregs are a specialized subpopulation of T cells that actively suppress activation of the immune system [22]. Tregs act by consumption of IL-2, inhibition of mRNA synthesis in non Treg cells, secretion of anti-inflammatory cytokines (TGF-β, IL-10, IL-35), blockade of co-stimulatory signals in effector T cells, and by blocking the granzyme dependent direct killing by pathogenic effector T cells [23,24]. Increased frequency of Tregs and its cytokines during remission in our study suggest a possible role of Tregs in controlling the disease activity in INS patients following treatment with glucocorticoids. Simultaneous decrease in frequency of Th1 and Th2 cells and their proinflammatory cytokines and decreased expression of P-gp favors the active suppressive role of Tregs in our study.

Table 2
Frequency of Tregs, Th1 and Th2 cells and percentage of P-gp expressing Tregs, Th1 and Th2 cells at baseline, during remission and at relapse.

Characteristics	Baseline NS (A)	Remission (B)	Relapse (C)	*P*-values
Tregs (CD4+CD25+FoxP3+)	1.83 ± 0.84	6.82 ± 4.12	3.03 ± 1.18	A vs. B, 0.001; B vs. C, 0.016
CD4+CD25+FoxP3+P-gp+	1.27 ± 0.87	2.79 ± 1.94	1.64 ± 1.27	A vs. B, 0.002; B vs. C 0.10
% of P-gp expressing Tregs	(69.39%)	(40.90%)	(54.12%)	
Th1(CD4+IFN-γ+)	16.18 ± 7.19	9.9 ± 4.65	19.83 ± 3.47	A vs. B, 0.018; B vs. C, 0.001
CD4+IFN-γ+P-gp+	8.55 ± 6.73	3.77 ± 2.29	12.23 ± 4.66	A vs. B, 0.004; B vs C, 0.001
% of P-gp expressing Th1 cells	(52.84%)	(38.08%)	(61.67%)	
Th2(CD4+IL-4+)	10.50 ± 4.66	4.81 ± 1.42	9.89 ± 5.18	A vs. B, 0.001; B vs. C, 0.008
CD4+IL-4+P-gp+	5.9 ± 4.14	2.14 ± 0.96	6.09 ± 3.90	A vs. B, 0.001; B vs. C, 0.001
% of P-gp expressing Th2 cells	(56.19%)	(44.49%)	(61.57%)	

Cell mediated immunity and lymphocytes are the putative therapeutic targets of steroids in treatment of INS [25]. Glucocorticoids are substrate for P-gp which in a state of overexpression may lead to resistance to the action of glucocorticoids on target cells. It has been reported that overexpression of P-gp is associated with poor response to steroid or steroid dependence in INS [26]. Funaki et al reported decrease in the MDR1 gene expression after complete remission and advocated that P-gp may play a role in the tapering of corticosteroids after remission in steroid sensitive Nephrotic Syndrome [27]. The poor treatment response due to increased P-gp expression has also been observed in other non-nephrological conditions like patients with malignancies [28], systemic lupus erythematosus [29], inflammatory bowel disease [30], rheumatoid arthritis, and other autoimmune diseases [31]. It has been demonstrated that P-gp expression and function can be suppressed by different compounds such as cyclosporine-A, and tacrolimus [32,33]. This could be the reason why many relapsing INS patients respond to calcineurin inhibitors. We have successfully demonstrated that P-gp expression was negatively correlated with immunosuppressive cytokines and positively correlated with proinflammatory cytokines. The negative correlation of P-gp expression on PBLs with IL-10 and TGF-β and positive correlation with cytokines IFN-γ and IL-4 in our study suggest P-gp expression is upregulated on PBLs in pro-inflammatory state. We postulate that during remission, increased Tregs may have mopped up IL2, a cytokine that affects the mRNA synthesis by MDR-1 gene and lead to decreased expression of P-gp on PBLs (34). Natural Treg cells constitutively express CD25, the high affinity receptor for IL-2. Treg cells by sopping up IL-2 suppress Teff cells proliferation and differentiation and Teff cells undergo apoptosis [34]. Increased Tregs during remission in the present study may have counterbalanced the effect of Teff cells (Th1 and Th2), however, we have not studied the levels of IL-2. A recent study has clearly demonstrated that P-gp positive Th17 cells may be important mediators particularly in setting of steroid resistant inflammatory disease [35]. P-gp expression increases on pathogenic T cells in diseased condition. P-gp has been recently targeted as the molecule responsible for poor response to many chemotherapeutic agents. Increased P-gp expression could be one of the possible factors responsible for relapse besides other immunological and non immunological factors.

4.1. Strength and limitations of study

The major strength of our study is that it is the longitudinal follow up study of the same cohort of NS patients; therefore the possible effect of the other confounding factors affecting the response and relapse of NS is least. Moreover, we have demonstrated that the cytokine profile of different phenotypes of stimulated T cells was matching their respective T cell phenotype. Thereby, strengthening our observation that Tregs were functionally dominant during remission in NS patients. The major limitations of our study were that expression of P-gp on podocytes was not studied and sample size is small.

5. Conclusion

Glucocorticoid therapy in INS induces decreased P-gp expression on PBLs along with increased frequency and cytokine response of T-regulatory cells and reduced frequency and cytokine response of Th1 and Th2 cells during remission. However, the frequency and cytokine response of Tregs, Th1 and Th2 and P-gp expression on PBLs was reversed during relapse. P-gp expression on lymphocytes remains significantly high during remission in relapsing patients as compared to non-relapsing patients. Monitoring of P-gp expression may help in management of INS patients.

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