Research Article

Analysis of T Cell Subsets in Adult Primary/Idiopathic Minimal Change Disease: A Pilot Study

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Aim. To characterise infiltrating T cells in kidneys and circulating lymphocyte subsets of adult patients with primary/idiopathic minimal change disease. Methods. In a cohort of 9 adult patients with primary/idiopathic minimal change recruited consecutively at disease onset, we characterized (1) infiltrating immune cells in the kidneys using immunohistochemistry and (2) circulating lymphocyte subsets using flow cytometry. As an exploratory analysis, association of the numbers and percentages of both kidney-infiltrating immune cells and the circulating lymphocyte subsets with kidney outcomes including deterioration of kidney function and proteinuria, as well as time to complete clinical remission up to 48 months of follow-up, was investigated. Results. In the recruited patients with primary/idiopathic minimal change disease, we observed (a) a dominance of infiltrating T helper 17 cells and cytotoxic cells, comprising cytotoxic T cells and natural killer cells, over Foxp3+ Treg cells in the renal interstitium; (b) an increase in the circulating total CD8+ T cells in peripheral blood; and (c) an association of some of these parameters with kidney function and proteinuria. Conclusions. In primary/idiopathic minimal change disease, a relative numerical dominance of effector over regulatory T cells can be observed in kidney tissue and peripheral blood. However, larger confirmatory studies are necessary.

1. Introduction

Primary minimal change disease (MCD) is a common cause of idiopathic nephrotic syndrome. Histopathologically, it is characterised by the apparent lack of abnormalities on light or immunofluorescence microscopy but effacement of foot processes on electron microscopy. It is believed that podocyte injury underlies the fundamental dysfunction in this disease. MCD is, therefore, also classified as a type of primary podocytopathy [1]. In this condition, disruption of the glomerular filtration barrier leads to increased glomerular permeability to proteins, causing severe urinary loss of proteins including albumin, immunoglobulins, complement, lipid-binding proteins, and clotting factors. The ensuing complications include hypoalbuminemia, generalised oedema, and malnutrition, as well as predisposition of patients to risk of infection, thrombotic events, and dyslipidemia.

Although the mechanisms underlying podocyte injury may include both nonimmunological factors and immunological factors [2, 3], they remain largely speculative and
Thymic Foxp3 molecules and self-antigens present in the thymus. The role of their immunoregulatory capacities in the thymus while two main subsets, thymic and peripheral Foxp3, are known to have different origins as well as distinct and complementary functions in both physiological and pathological immune situations [10–12]. Thymic Foxp3+ Treg cells acquire their immunoregulatory capacities in the thymus while being selected by the self-major histocompatibility complex molecules and self-antigens present in the thymus. The role of thymic Foxp3+ Treg cells in the periphery, therefore, appears to be maintenance of immune tolerance through modulation of activation, differentiation, and proliferation of potential autoreactive T cell clones [13, 14].

Another study performed on 21 adults with primary MCD demonstrated decreased suppressive capacity of Foxp3+ Treg cells, although patients had similar numbers of circulating Foxp3+ Treg cells in comparison with controls [19]. In another report, 21 children with steroid-resistant nephrotic syndrome had increased ratios of circulating interferon-γ (IFN-γ)-gamma-secretin Th1 cells and IL-4-secreting Th2 cells over Foxp3+ Treg cells when compared to 22 children with steroid-sensitive nephrotic syndrome in clinical remission and 14 healthy controls [20]. The interplay between Th17 cells and Foxp3+ Treg cells has also been reported in two studies. One study showed an increase in circulating Th17 cells over Treg cells in 25 adult patients with primary MCD when compared to healthy controls, which also correlated with severity of proteinuria [21]. The other study performed on 36 children with idiopathic nephrotic syndrome demonstrated an increase in circulating Th17 cells over Treg cells when compared to healthy controls, which was associated with an increase in intrarenal IL-17 expression [22].

Our primary aim was to confirm whether a similar immune imbalance of T cell subsets is a feature of primary MCD in our population. We focused primarily on the numerical balance of Th17 and cytotoxic cells [comprising CTL and natural killer (NK) cells] over Foxp3+ Treg subset in kidney tissue by immunohistochemistry, using their relative ratio as an arbitrary measure of such balance. We have described our tissue results as a case series due to the lack of normal kidney tissue for comparison. We also detected and quantified different Treg cells in the peripheral blood of patients using flow cytometry but focused our main analysis on Th17 cells, CD8+ T cells (a surrogate phenotype for CTL), and Foxp3+ Treg cells. We compared our findings in the peripheral blood of these patients to our observations in healthy controls. Finally, we explored correlations between our chosen immune parameters and clinical outcomes, including deterioration of kidney function and proteinuria, as well as time to complete clinical remission. Therefore, this is a pilot descriptive study reporting the observed frequencies of different immune cell subsets in kidney tissue and peripheral blood of MCD patients and their association with clinical outcomes.

2. Material and Methods

2.1. Study Design and Patients. This was a single-centre prospective study in a cohort of 9 consecutive patients aged 21–80 years with new-onset biopsy proven MCD thought to be idiopathic in nature. Patients were recruited at the Department of Renal Medicine of the Singapore General Hospital (SGH) between 1 January 2012 and 31 March 2014 at the time when native kidney biopsy was performed for the investigation of new-onset nephrotic syndrome and followed for up to 48 months for outcomes analysis. Healthy individuals were recruited to serve as a control group for many of the comparisons in the peripheral blood studies. Signed informed consent was taken from all participants and our protocol was approved by our Centralised Institutional Review Board (Approval number 2009/672/E).
2.2. Exclusion Criteria. HIV infection, history of haematological malignancies, children, pregnant women, poor cognitive capacity, prisoners, and inability to understand the research protocol and give consent were the exclusion criteria.

2.3. Clinical Data. Baseline demographic and clinical characteristics were retrieved from patient hard-copy case notes and electronic medical records. Use and type of immunosuppressants prescribed were also recorded.

2.4. Routine Laboratory Investigations. Serum creatinine was measured and proteinuria was quantified by means of either the urine protein to creatinine ratio or a 24-hour collection of total urinary protein. Calculated estimated glomerular filtration rate (GFR) was obtained through the modified diet in renal disease (MDRD) equation. All laboratory parameters were retrieved prospectively from electronic medical records at 6-month intervals from the time of native kidney biopsy up to 48 months of follow-up. All laboratory investigations were conducted at the SGH's clinical laboratory, which is accredited by the College of American Pathologists.

2.5. T Cell Subset and B Cell Detection in Native Kidney Biopsies by Immunohistochemistry. Immunohistochemistry for detection of T cell subsets and B cells in native kidney tissue biopsies was performed at the laboratories in both the Renal Department and Department of Pathology of the SGH. In brief, sections prepared from formalin-fixed and paraffin-embedded kidney tissue specimens were stained with monoclonal antibodies conjugated with either horseradish peroxidase or alkaline phosphatase and directed against different phenotypic markers, including CD4 (used as a surrogate marker of CD4+ T cells), CD8 (used as marker of CD8+ T cells), CD19 (used as marker of B cells), IL-17 (used as marker of IL17-secreting Th17 cells), granzyme B (a marker of cytotoxic cells, comprising both CTL and NK cells), and Foxp3 (used as a surrogate marker of Treg cells) using an automatic stainer machine. The binding of the different antibodies onto the kidney tissue samples was revealed using the respective chromogenic substrates for those enzymes. Isotype-matched antibodies were used as negative controls. Tonsil tissue served as positive control. Staining was visualized and measured by light microscopy semiquantitatively as percentage of infiltration for CD4+, CD8+, and CD49+ cells and expressed as number of cells per mm² of kidney biopsy (cell density) by adjusting to biopsy surface area measured by Olympus CellSens software for Foxp3+, IL-17+, or granzyme B+ cells.

2.6. Phenotypic Analysis of T Cell Subsets by Flow Cytometry. The quantification of total CD3+ T cells, CD4+ T cells, CD8+ T cells, and CD19+ B cells in peripheral whole blood of patients and healthy controls was performed by flow cytometry using a BD LSR II machine available at the haematology laboratory of the SGH, using fluorochrome-labelled monoclonal antibodies against CD45, CD3, CD4, CD8, and CD19. The quantification of CD4+CD25+Foxp3+ Treg cells, CD4+CD161+ Th17 cells, CD4+CD45RA memory T cells, CD4+CD45RA naïve T cells, CD8+CD45RA memory T cells, CD8+CD45RA naïve T cells, CD3+CD56+ natural killer T (NK)T cells, CD3+TCRd1+ gamma-delta T cells, CD3+TCRd2+ gamma-delta T cells, CD4+CD161+TCRa7.2+ mucosal-associated invariant T (MAIT) cells, and CD5+CD56+ natural killer (NK) cells in stored peripheral blood mononuclear cells of patients and healthy controls was performed by flow cytometry using a BD LSR II machine available at the Singapore Immunology Network, using fluorochrome-labelled monoclonal antibodies against different phenotypic markers including CCR6, CD3, CD4, CD5, CD8, CD14, CD19, CD25, CD27, CD28, CD39, CD45RA, CD45RO, CD49d, CD56, CD127, CD152, CD161, GARP, TCRa7, TCRd1, and TCRd2. All the stained cells were acquired in a 20-colour flow cytometer, and the results were analysed using FlowJo v9.5 software. For compensation and calibration purposes, commercially available fluorochrome-labelled beads were used as controls. Percentages of marker-expressing cells were reported and adjusted as percentage of the lymphocyte counts. Absolute numbers of these T cell subsets were obtained by adjusting the percentages to the lymphocyte counts obtained through measuring the full blood count in the SGH's clinical laboratory.

2.7. Statistical Analysis. Being an exploratory, descriptive study in consecutively recruited patients during the allowed recruitment period, sample size was not calculated. Descriptive statistics were used to display our results obtained from our immunohistochemical investigations. No comparison with healthy control kidney tissue was possible. The different lymphocyte and T cell subset percentages and ratios in peripheral blood were compared between the patients with primary MCD and the healthy controls using the Mann–Whitney U test. Spearman correlation was used to assess strength of association of lymphocyte and T cell subset percentages and numbers in kidney tissue and peripheral blood with different clinical outcomes, including changes in serum creatinine, MDRD GFR, and proteinuria, as well as time to complete clinical remission, doubling of creatinine, and need for dialysis. All analyses were performed using SAS V9.3 software (SAS Inc., Cary, NC, USA).

3. Results

Nine patients with primary MCD were included in this study. All had their native kidney biopsy specimens tested by immunohistochemistry for detection of different T cell subsets and B cells. The obtaining of normal kidney tissue (or a surrogate tissue for normality) as control for our immunohistochemical studies was not possible. Eight patients provided blood sample for measurement of different T cell subsets in peripheral blood by flow cytometry. As many as twelve healthy individuals served as controls for our immune cell detection in peripheral blood by flow cytometry. Baseline demographic and clinical characteristics of recruited patients are shown in Table 1. Most patients were young
Chinese males with full-blown nephrotic syndrome and not taking steroids or immunosuppression at the time of biopsy. Detailed clinical parameters for each patient are presented in Table 2.

Figure 1 shows a phenotypic panel (by immunohistochemistry) of several T cell subsets and B cells in a patient with primary MCD. The pictures were taken at an area of moderate infiltration of both T and B cells for demonstration of our detection capability as the infiltrate was scarce and the overall staining was discrete in most patients, but sufficient for subset identification and quantification. The cell infiltrate in MCD patients was composed mainly of CD4+ and CD8+ T cells with a small population of B cells. The median percentage of infiltration of CD4+ T cells, CD8+ T cells, and CD19+ B cells, and the median cell density (cells per square millimeter of kidney biopsy) of the IL-17+ cells (Th17 cells), granzyme B+ cytotoxic cells (comprising both CTL and NK cells), and Foxp3+ cells (Treg cells) is plotted in Figure 2. We observed higher cell densities of Th17 cells and cytotoxic cells when compared to Foxp3+ Treg cells. As a consequence, the ratios of Th17 cells over Foxp3+ Treg cells and cytotoxic cells over Foxp3+ Treg cells, an arbitrary measurement of the numerical balance between these subsets, were increased. We had no access to normal kidney tissue for comparison of our findings and to investigate whether our observed frequencies and ratios were different to the ones observed in healthy individuals.

In peripheral blood we measured the percentage (from the lymphocyte count) and the absolute numbers per microlitre of blood of circulating CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, and monocytes, as well as CD4+CD25+ Foxp3+ Treg cells, CD4+CD161+ Th1 cells,
Table 1: Baseline clinical and demographic characteristics of the patients with primary MCD.

|                        | n   |
|------------------------|-----|
| Age (years) *          | 31.4|
| Male sex (#)           | 6/9 |
| Race, Chinese (#)      | 8/9 |
| Diabetes mellitus (#)  | 1/9 |
| GFR at biopsy (ml/min/1.73 m2)* | 83.6 |
| Proteinuria at biopsy (g/day)* | 7.46 |
| Albumin (g/L)*         | 15  |
| Steroids use at biopsy (#) | 2/9 |
| MMF or AZA use at biopsy (#) | 0/9 |
| Cytotoxics use at biopsy (#) | 0/9 |

AZA: azathioprine; GFR: glomerular filtration rate; MMF: mycophenolate mofetil. *Results reported as median values.

Although the immunopathogenesis of primary MCD is complex and remains largely nondeciphered, it has been proposed that disturbances in the immune balance between CD4+CD45RA⁻ memory T cells, CD4+CD45RA⁺ naïve T cells, CD8⁺CD45RA⁺ memory T cells, CD8⁺CD45RA⁻ naïve T cells, CD3⁺CD56⁺ NKT cells, CD3⁺TCRd1⁺ gamma-delta T cells, CD3⁺TCRd2⁺ gamma-delta T cells, CD4⁺CD161⁺ TCRαβ7.2⁺ MAIT cells, and CD3⁻CD56⁺ NK cells. Figure 3 illustrates some of the T cell subsets detected using multicolour flow cytometry in a representative patient with primary MCD. In peripheral blood, we found significantly higher percentages of total CD3⁺ T cells (p = 0.0092) and of total CD8⁺ T cells (p = 0.0358) in primary MCD patients when compared to healthy controls (Figures 4(b) and 4(f)), but there was no significant difference in the percentages or numbers of naïve and memory CD8⁺ T cells between the two groups. Foxp3⁺ Treg cells were not significantly reduced in primary MCD patients in comparison with controls (p = 0.0333; Figure 4(q)). There were no statistically significant differences in the other T cell subsets or immune cells studied between primary MCD and the healthy controls (Figure 5), except for the finding of lower circulating NK cells in patients with primary MCD. Finally, there were no differences in the calculated ratios of circulating Th17 cells over Foxp3⁺ Treg cells or circulating memory CD8⁺ T cells over Foxp3⁺ Treg cells (Figure 6).

We correlated selected immune cellular parameters with clinical outcomes to address any potential clinical meaning, as an exploratory analysis. Table 3 shows the statistically significant correlations found between the circulating and infiltrating immune subsets with serum creatinine, MDRD GFR, and proteinuria (at different time points during follow-up), as well as time to complete clinical remission in our exploratory analyses. Complete clinical remission was achieved in these patients with a median time of 28 days (Figure 7) with subsequent stabilisation of serum creatinine. No patient had a doubling of creatinine or underwent dialysis.

In relationship with our original hypothesis, at disease onset, the number of circulating CD8⁺ T cells correlated positively with serum creatinine (Figure 8(a)) and negatively with MDRD GFR (Figure 8(b)), the percentage of kidney tissue infiltrating CD4⁺ T cells (Figure 8(c)) and the percentage of circulating naïve CD8⁺ T cells (Figure 8(d)) correlated positively with proteinuria, and the ratio of tissue cytotoxic over Foxp3⁺ Treg cells correlated negatively with MDRD GFR (Figure 8(e)).

4. Discussion

Although the immunopathogenesis of primary MCD is complex and remains largely nondeciphered, it has been proposed that disturbances in the immune balance between...
Table 2: Summary of the clinical characteristics and outcomes of the patients with primary MCD.

| Patient | Age/sex | Race      | Diabetes mellitus | GFR at onset | GFR last follow-up | Proteinuria at onset | Proteinuria last follow-up | Albumin at onset | Steroids at biopsy | Days to complete remission | Total days of follow-up |
|---------|---------|-----------|-------------------|--------------|-------------------|----------------------|--------------------------|------------------|------------------|--------------------------|-------------------------|
| 1       | 25/F    | Chinese   | No                | 167.8        | 132.2             | 7.9                  | 0.16                     | 17               | No               | 356                      | 1385                    |
| 2       | 52/M    | Chinese   | No                | 73.9         | 93.4              | 5.6                  | 0.1                      | 9.0              | No               | 25.0                     | 1320                    |
| 3       | 31/M    | Indian    | No                | 48.7         | 96.7              | 10.3                 | 0.05                     | 13               | No               | 20                       | 1364                    |
| 4       | 21/M    | Chinese   | No                | 17.7         | 77.9              | 7.5                  | 0.1                      | 15.0             | No               | 97                       | 1256                    |
| 5       | 47/F    | Chinese   | No                | 132.9        | 132.9             | 13.7                 | 13.7                     | 14.0             | No               | UNK                      | 1                      |
| 6       | 21/F    | Chinese   | No                | 83.6         | 84.4              | 5.0                  | 0.1                      | 15.0             | Yes              | 6                        | 1197                    |
| 7       | 25/F    | Chinese   | No                | 146.4        | 115.4             | 6.0                  | 0.0                      | 17.0             | Yes              | 20.0                     | 298                     |
| 8       | 62/M    | Chinese   | No                | 84.8         | 57.3              | 3.4                  | 0.3                      | 30.0             | No               | 31                       | 1102                    |
| 9       | 65/M    | Chinese   | Yes               | 14.0         | 67.6              | 14.0                 | 14.4                     | 20.0             | No               | 343                      | 925                     |

F: female; GFR: glomerular filtration rate; M: male; MCD: minimal change disease; UNK: unknown due to loss of follow-up.
Table 3: Correlation between lymphocyte and T cell subsets and clinical outcomes in primary MCD.

| Immune cell parameter versus | Clinical outcome | $R$   | $p$ value |
|-----------------------------|-----------------|-------|-----------|
| Circulating total CD8+ T cells (#) | Serum creatinine t0 | 1     | 0.0004    |
| Circulating memory CD8+ T cells (%) | Serum creatinine t6 | -0.9429 | 0.0167    |
| Circulating total CD8+ T cells (#) | Serum creatinine t12 | 0.9276 | 0.0167    |
| Circulating monocytes (#) | Serum creatinine t12 | 0.7545 | 0.0377    |
| Circulating memory CD4+ T cells (#) | Serum creatinine t24 | -1     | 0.0167    |
| Circulating memory CD4+ T cells (#) | Serum creatinine t36 | -0.9747 | 0.0333    |
| Circulating total CD8+ T cells (#) | GFR t0 | -0.9286 | 0.0067    |
| Tissue cytotoxic/Treg ratio | GFR t0 | -0.7197 | 0.0342    |
| Circulating Th17 cells (%) | GFR t6 | 0.8986 | 0.0278    |
| Blood Th17/Treg ratio | GFR t6 | 0.8986 | 0.0278    |
| Circulating monocytes (#) | GFR t6 | -0.7306 | 0.0476    |
| Circulating monocytes (#) | GFR t36 | 1.0000 | 0.0167    |
| Circulating naïve CD4+ T cells (%) | Proteinuria t0 | 0.7857 | 0.048     |
| Circulating naïve CD4+ T cells (%) | Proteinuria t12 | 0.7619 | 0.0368    |
| Circulating naïve CD3+ T cells (%) | Proteinuria t24 | -0.9747 | 0.0333    |
| Circulating Th17 cells (%) | Proteinuria t36 | -0.9747 | 0.0333    |
| Blood Th17/Treg ratio | Proteinuria t36 | -0.9747 | 0.0333    |
| Tissue cytotoxic/Treg ratio | Proteinuria t36 | -0.8407 | 0.0444    |
| Circulating TCRgd2+ T cells (%) | Time to complete remission | 0.8469 | 0.0238    |
| Circulating TCRgd2+ T cells (%) | Time to complete remission | 0.7748 | 0.0492    |
| Circulating monocytes (%) | Time to complete remission | 0.8368 | 0.0072    |
| Circulating monocytes (%) | Time to complete remission | 0.8201 | 0.0095    |

Teff cells and Foxp3$^+$ Treg cells play a potential pathogenic role [23]. This pilot study was conducted to describe the relative frequencies of different Teff cells and Foxp3$^+$ Treg cells, in both tissue and peripheral blood, as an arbitrary measurement of their immune balance in primary MCD. Despite the small sample size, its main strength is the combining of tissue data with detailed phenotypic analysis of different immune cell subsets in peripheral blood as well as with clinical parameters. To the best of our knowledge, this has not been attempted in previous studies.

In tissue, we found the overall T cell infiltration into the kidney interstitium of patients with primary MCD to be minimal (Figure 2). Our finding that kidney interstitial infiltrates of these primary MCD patients were composed mainly of T cells rather than B cells is in keeping with the notion that primary MCD is fundamentally a T cell disorder. In addition, there appeared to be a relative deficiency of Foxp3$^+$ Treg cells over Th17 cells and cytotoxic cells. Our study showed that the ratios of Th17 cells over Foxp3$^+$ Treg cells, as well as cytotoxic cells over Foxp3$^+$ Treg cells, were elevated (arbitrarily deciding a ratio above 1). Although ideally infiltrates in normal healthy subjects would be used for comparison, it was ethically unachievable in our descriptive study. Other studies have compared their observations with those from living donor kidney tissue or patients with thin-membrane disease [18, 24]. At the SGH, no biopsy is obtained preimplantation in living donors, and none of the patients recruited in our parallel studies had thin-membrane disease as diagnosis. In those studies, healthy or “normal” controls were found also to have a scarce cellular infiltrate, which could be or not comparable to that of our patients with MCD. As a consequence, in this paper, we are mainly describing and partially dissecting phenotypically the observed infiltrate and reporting their relative frequencies, which is a commonly encountered way to assess immune balance. But we do accept that this is an arbitrary and imperfect way to assess immune balance, especially when in vivo a complex functional and numerical interplay of the different immune arms activated or dysfunctional in several human disorders, rather than just a numerical balance, is taking place. However, that interplay can only be assessed with a multidimensional interrogation of the functional phenotypes present in disease states, as well as a detailed assessment of the hierarchy of involved cellular and molecular interactions, but this was beyond the scope and reach of our study design and aims. In addition, our findings are consistent with published data that demonstrates...
Figure 3: Representative surrogate T cell subsets in peripheral blood of a patient with primary MCD. CD4⁺CD25⁺Foxp3⁺ Treg cells (a), CD4⁺CD161⁺Th17 cells (b), CD4⁺CD45RA⁻ memory T cells (c), CD4⁺CD45RA⁺ naïve T cells (d), CD8⁺CD45RA⁻ memory T cells (e), CD8⁺CD45RA⁺ naïve T cells (f), CD3⁺CD56⁻NKT cells (g), CD3⁺CD56⁺NK cells (h), CD3⁺TCRd1⁺gamma-delta T cells (i), CD3⁺TCRd2⁺gamma-delta T cells (j), and CD4⁺CD161⁺TCRa7.2⁺ MAIT cells (k) were detected by 20-colour flow cytometry, using the appropriate antibodies as described in the methods section.

A possible deficiency of tissue-infiltrating Foxp3⁺ Treg cells in primary MCD [18]. In addition, in a study reported in 2002, gene expression for granzyme B was detected intrarenally in 2 of 7 children with minimal change disease [25].

Concordant with published observations [19], our primary MCD patients displayed similar numbers of circulating Foxp3⁺ Treg cells in comparison with healthy controls. However, counter to our hypothesis and the two reports previously mentioned [21, 22], we did not observe an increased ratio of circulating Th17 cells over Foxp3⁺ Treg cells in patients with primary MCD in comparison to healthy controls. It is possible that these discrepant observations were due to...
Figure 4: Comparative circulating cell numbers per microlitre of blood and comparative percentages from total lymphocytes of circulating total CD3⁺ T cells (a, b), total CD4⁺ T cells (c, d), naïve CD4⁺CD45RA⁺ T cells (g, h), memory CD4⁺CD45RA⁻ T cells (k, l), total CD8⁺ T cells (e, f), naïve CD8⁺CD45RA⁺ T cells (i, j), memory CD8⁺CD45RA⁻ T cells (m, n), CD4⁺CD25⁺Foxp3⁺ Treg cells (o, p), and CD4⁺CD161⁺ Th17 cells (q, r) between 7 primary MCD patients and up to 12 healthy controls. Cells were detected by 20-colour fluorescence-activated flow cytometry. The median cell numbers are represented by the horizontal lines. The asterisks indicate that the comparisons were statistically significant and the respective p value is given.
the different technological approaches to detect Th17 cells. We used CD161 as a surrogate marker for Th17 cells (on CD3+CD4+ T cells) and we observed a decreased number of circulating Th17 cells in the patients with primary MCD in comparison to healthy controls. On the contrary, those authors based their detection on intracytoplasmic IL-17 expression, which indeed could be a more reliable marker. However, in those studies, the detection of IL-17-expressing cells required in vitro stimulation of T cells, which in our opinion (based on our unpublished observations with CyTOF mass cytometry in a different project in kidney transplantation) would likely overrepresent the actual frequencies of this subpopulation in vivo. Irrespective of this possibility, our tissue and peripheral blood results mirror themselves as we observed an apparent reduction of both infiltrating and circulating Th17 cells, implying that our observation not necessarily reflects technological difference but possibly a characteristic of this condition. Once again this is not conclusive as we lack a tissue control for comparison.

A technical limitation worth mentioning in our study, inherent of single-staining immunohistochemistry using surrogate cell markers, is the assumption that all the CD4+ expressing cells were CD4+ T cells, all the Foxp3+ cells were Treg cells, and all the granzyme B+ cells were CTL and NK cells. Immunofluorescence could have circumvented this limitation, which can be considered in subsequent grant applications.

It was interesting to observe some degree of association of our immune parameters with clinical features at presentation and with disease outcomes. Most of our patients achieved...
Figure 6: Comparative ratios of circulating Th17 cells over Treg cells (a) and memory CD8$^+$ T cells over Treg cells (b) between 7 primary MCD patients and 3 healthy controls. Cells were detected by 20-colour fluorescence-activated flow cytometry. The median ratios are represented by the horizontal lines. The comparisons did not reach statistical significance.

Overall, our results show that a numerical imbalance of different immune subsets, in particular a deficiency of Foxp3$^+$ Treg cells or a dominance of effector Th17 cells and cytotoxic cells, could be a characteristic of primary MCD. However, larger confirmatory studies looking into physiopathogenic mechanisms are necessary.

5. Conclusion

Primary MCD is an important cause of idiopathic nephrotic syndrome in adults. Different immune subsets can be detected in the renal interstitium of MCD patients, where a relative numerical abundance of Th17 cells and cytotoxic cells over Foxp3$^+$ Treg cells appears to be a characteristic of this condition. Although our observations are preliminary, they enhance the current limited knowledge on this topic, and although our paper does not provide functional data, our findings could incite further detailed studies to assess more mechanistically the so-called immune imbalance between Foxp3$^+$ Treg and Teff cells and its contribution in the pathogenesis of podocyte injury in primary MCD. A better understanding of the immunopathogenesis of primary MCD is necessary for the improvement of our current diagnostic armamentarium and the development of more targeted immunotherapy, which might potentially involve T cell subset modulation.

Abbreviations

| Abbreviation | Description                          |
|--------------|--------------------------------------|
| CTL          | Cytotoxic T lymphocyte               |
| GFR          | Glomerular filtration rate           |
| IFN          | Interferon                           |
| MCD          | Minimal change disease               |
| MDRD         | Modified diet in renal disease       |
| MAIT         | Mucosal-associated invariant T        |
| NK           | Natural killer                        |
| NKT          | Natural killer T                     |
| SGH          | Singapore General Hospital           |
| Teff         | Effector T                           |

Complete clinical remission promptly after initiation of steroid therapy and none doubled serum creatinine levels or required dialysis. Therefore, we focused our clinical outcomes analysis on levels of proteinuria and kidney function at disease onset and on time to clinical remission. In tune with our overall hypothesis, we found associations of infiltrating CD4$^+$ T cells and circulating CD8$^+$ T cells with higher proteinuria at onset and also correlation of circulating CD8$^+$ T cells and the infiltration ratio of cytotoxic cells over Foxp3$^+$ Treg cells with worse kidney function at presentation. Overall, these observations appear to support the long-standing notion that primary MCD could be a T cell-mediated disorder or that T cell dysfunction could play a significant role in its pathogenesis. However, these observations arose from exploratory analyses in a limited number of cases and should be taken with reserve. Nevertheless, we are reporting them for potential hypothesis-generation purposes rather than to suggest conclusions from them.
Additional Points

Summary. Primary minimal change disease is a common cause of idiopathic nephrotic syndrome in adults. Infections, certain environmental factors, and T cell dysfunction with increased levels of a soluble glomerular permeability factor have been proposed to play a central role in the pathogenesis of this condition. Therefore, a dysfunction, or a relative deficiency of Foxp3+ regulatory T cells over effector T cells, could be implicated in its immunopathogenesis. We present here a pilot phenotypic analysis of the major T cell subsets infiltrating the kidney and in the peripheral blood of adult patients suffering from primary minimal change disease. An apparent numerical dominance of Th17 cells and cytotoxic cells over Foxp3 Treg cells was detected in the renal interstitium in primary/idiopathic minimal change disease, but we had no control normal kidney tissue for comparison. In peripheral blood, on the other hand, we observed an increase in the circulating total CD8+ T cells in patients with minimal change disease. These results suggest that in primary/idiopathic minimal change disease, a relative numerical dominance of effector over regulatory T cells can be observed in kidney tissue and peripheral blood. However, larger confirmatory studies are necessary.

Ethical Approval

The study protocol was approved by the Centralised Institutional Review Board of SingHealth, Singapore (Approval no. 2009/672/E).

Consent

Signed informed consent was taken from all participants before being subjected to a native kidney biopsy, which was clinically indicated and not an experimental procedure in our protocol.

Disclosure

Deidentified data was shared among few of the authors for the purpose of data analysis. No data was shared to third parties.

Conflicts of Interest

There are no conflicts of interest among the authors or the participating institutions, and the authors do not have any financial relationships to disclose.

Authors’ Contributions

Francisco Salcido-Ochoa designed the study, revised all the collected data, analysed and plotted the data, and wrote
and revised the paper; Susan Swee-Shan Hue analysed histopathological data and revised the paper; Doreen Haase designed the study and analysed the flow cytometry data; Jason Chon Jun Choo provided clinical advice and revised the paper; Nurhashikin Yusof performed the flow cytometry experiments and collected clinical data; Reiko Lixiang Li performed the immunohistochemistry experiments and collected clinical data; John Carson Allen Jr. performed and supervised statistical analysis and revised the paper; Jabeed Iqbal analysed histopathological data and revised the paper; Alwin Hwai Lian Loh analysed histopathological data and revised the paper; Olaf Rotzscheke designed the study, provided basic science advice, and revised the paper.

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References

[1] L. Barisoni, H. W. Schnaper, and J. B. Kopp, "A proposed taxonomy for the podocytopathies: a reassessment of the primary nephrotic diseases," Clinical Journal of the American Society of Nephrology, vol. 2, no. 3, pp. 529–542, 2007.
[2] G. Cara-Fuentes, W. L. Clapp, R. J. Johnson, and E. H. Garin, "Pathogenesis of proteinuria in idiopathic minimal change disease: molecular mechanisms," Pediatric Nephrology, vol. 31, no. 12, pp. 2179–2189, 2016.
[3] C. Dossier, A. Jamin, and G. Deschênes, "Idiopathic nephrotic syndrome: the EBV hypothesis," Pediatric Research, vol. 81, pp. 233–239, 2016.
[4] L. C. Clement, C. Avila-Casado, C. Macé et al., "Podocyte-secreted angiopoietin-like-4 mediates proteinuria in glucocorticoid-sensitive nephrotic syndrome," Nature Medicine, vol. 17, no. 1, pp. 117–122, 2011.
[5] R. J. Shalhub, "Pathogenesis of lipid nephrosis: a disorder of T cell function," The Lancet, vol. 2, no. 7880, pp. 556–560, 1974.
[6] E. Schnenberger, J. H. Ehrich, H. Haller, and M. Schiffer, "The podocyte as a direct target of immunosuppressive agents," Nephrology Dialysis Transplantation, vol. 26, no. 1, pp. 18–24, 2011.
[7] J. G. Van Den Berg, J. Aten, M. A. Chand et al., "Interleukin-4 and interleukin-13 act on glomerular visceral epithelial cells," Journal of the American Society of Nephrology, vol. 11, no. 3, pp. 413–422, 2000.
[8] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases," Journal of Immunology, vol. 155, no. 3, pp. 1151–1164, 1995.
[9] S. Sakaguchi, T. Yamaguchi, T. Nomura, and M. Ono, "Regulatory T cells and immune tolerance," Cell, vol. 133, no. 5, pp. 775–787, 2008.
[10] M. Miyara, Y. Yoshioka, A. Kitoh et al., "Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor," Immunity, vol. 30, no. 6, pp. 899–911, 2009.
[11] A. D. Campbell and M. A. Koch, "Phenotypical and functional specialization of FOXP3+ regulatory T cells," Nature Reviews Immunology, vol. 11, no. 2, pp. 119–130, 2011.
[12] Y. Ding, J. Xu, and J. S. Bromberg, "Regulatory T cell migration during an immune response," Trends in Immunology, vol. 33, no. 4, pp. 174–180, 2012.
[13] C. Benoist and D. Mathis, "Treg cells, life history, and diversity," Cold Spring Harbor Perspectives in Biology, vol. 4, no. 9, article a007021, 2012.
[14] J. A. Bluestone and H. Bour-Jordan, "Current and future immunomodulation strategies to restore tolerance in autoimmune diseases," Cold Spring Harbor Perspectives in Biology, vol. 4, no. 11, 2012.
[15] W. Chen, W. Jin, N. Hardegen et al., "Conversion of peripheral CD4+CD25− naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3," The Journal of Experimental Medicine, vol. 198, no. 12, pp. 1875–1886, 2003.
[16] I. Apostolou and H. Von Boehmer, "In vivo instruction of suppressor commitment in naive T cells," Journal of Experimental Medicine, vol. 199, no. 10, pp. 1401–1408, 2004.
[17] M. A. Curotto de Lafaille, N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J. J. Lafaille, "Adaptive Foxp3+ Regulatory T Cell-Dependent and -Independent Control of Allergic Inflammation," Immunity, vol. 29, no. 1, pp. 114–126, 2008.
[18] K. Benz, M. Böttner, K. Dittrich, V. Campean, J. Dötsch, and K. Amann, "Characterisation of renal immune cell infiltrates in children with nephrotic syndrome," Pediatric Nephrology, vol. 25, no. 7, pp. 1291–1298, 2010.
[19] A. Araya, L. Diaz, C. Wasserfall et al., "T regulatory cell function in idiopathic minimal lesion nephrotic syndrome," Pediatric Nephrology, vol. 24, no. 9, pp. 1691–1698, 2009.
[20] A. Jaiswal, N. Prasad, and V. Agarwal, "Regulatory and effector T cells changes in remission and resistant state of childhood nephrotic syndrome," Indian Journal of Nephrology, vol. 24, no. 6, pp. 349–355, 2014.
[21] L.-L. Liu, Y. Qin, J.-F. Cai et al., "Th17/Treg imbalance in adult patients with minimal change nephrotic syndrome," Clinical Immunology, vol. 139, no. 3, pp. 314–320, 2011.
[22] S. X. Shao, X. Q. Yang, X. D. Zhao et al., "The prevalence of Th17 cells and FOXP3 regulate T cells (Treg) in children with primary nephrotic syndrome," Pediatric Nephrology, vol. 24, no. 9, pp. 1683–1690, 2009.
[23] S. S. Hue, S. M. Suhail, J. C. J. Choo et al., "Do Foxp3+ regulatory T cells (treg cells) play a role in the immunopathogenesis of primary/idiopathic minimal change disease?" ISRN Pathology, vol. 2014, 8 pages, 2014.
[24] D. B. Ascon, M. Ascon, S. Satpute et al., "Normal mouse kidneys contain activated and CD3+CD4-CD8- double-negative T lymphocytes with a distinct TCR repertoire," Journal of Leukocyte Biology, vol. 84, no. 6, pp. 1400–1409, 2008.
[25] J. Strehlau, A. D. Schachter, M. Pavlakis, A. Singh, A. Tejani, and T. B. Strom, "Activated intrarenal transcription of CTL-effectors and TGF-β1 in children with focal segmental glomerulosclerosis," Kidney International, vol. 61, no. 1, pp. 90–95, 2002.