

A Humanized Mouse Model of Idiopathic Nephrotic Syndrome Suggests a Pathogenic Role for Immature Cells

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ABSTRACT

Idiopathic nephrotic syndrome is characterized by glomerular proteinuria in the absence of infiltrating cells or immunoglobulin deposits. Although it is suspected that T cells secrete a circulating factor that leads to proteinuria by altering the permeability of the glomerular filtration barrier, the precise etiology of this syndrome is unknown. Because an animal model that mimics human idiopathic nephrotic syndrome does not exist, we developed a humanized mouse model of the disease by injecting CD34⁺ stem cells or CD34⁻ peripheral blood mononuclear cells from afflicted patients into immunocompromised mice. Even though both CD34⁺ and CD34⁻ cells induced the engraftment of human CD45⁺ leukocytes in mice, only the injection of CD34⁺ stem cells induced albuminuria. Ultrastructural analysis of glomeruli from the resulting proteinuric mice revealed effacement of podocyte foot processes, similar to the pathology observed in the human disease. Therefore, our data suggest that the cells responsible for the pathogenesis of idiopathic nephrotic syndrome are more likely to be immature differentiating cells rather than mature peripheral T cells.

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Idiopathic nephrotic syndrome (INS) is a disease of unknown cause that can be associated with two different histologic varieties: Minimal-change nephrotic syndrome (MCNS) with minimal histologic changes in the glomeruli and FSGS, characterized by glomerular scarring.¹ MCNS is usually steroid sensitive, whereas FSGS is often steroid resistant and progresses to end-stage renal failure in approximately 20% of patients. After transplantation, up to 40% of patients rapidly relapse to their initial disease.^{2,3} Although neither immune cell infiltration nor immune complex deposits can be identified, accumulating data suggest that steroid-sensitive MCNS and a subset of FSGS (particularly those recurring after transplantation) have an immunologic basis. Indeed, many findings (*e.g.*

sensitivity to immunosuppressive drugs; relapse initiated by immune challenge with infectious or allergic stimuli; the occurrence of MCNS and FSGS in patients with Hodgkin's disease, non-Hodgkin's lymphoma, or thymoma) have led Shalhoub⁴ to propose that MCNS and FSGS are caused by an im-

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munologic disorder. An immunologic basis for MCNS and FSGS is a widely accepted hypothesis.^{1,5} The most likely pathologic process is that T cells promote the production of a circulating factor that alters the glomerular permeability of the renal filtration barrier.^{5,6} This concept came from the observation that proteinuria recurred in the very first samples of urine from patients who received a transplant⁷ and was supported by studies demonstrating increased protein excretion in rats after the injection of serum from patients who relapsed after transplantation for FSGS.⁸ Nevertheless, both the nature of pathogenic circulating factor and the mechanisms by which the immune system is involved in the pathologic process of FSGS and MCNS remain unknown. Several experimental models of glomerular lesions resembling human FSGS or MCNS have been described in rodents,^{9–12} but these models are of limited value in the characterization of the circulating factor and the underlying immunologic abnormalities. It was shown that the Buffalo/Mna rat model was similar to human FSGS¹³; however, it has not aided in determining the mechanisms of FSGS. In the work presented here, we developed a new animal model of FSGS and MCNS by using the technology of humanizing NOD/SCID mice. The concept relies on the fact that very immunocompromised mice can be engrafted with human cells, either peripheral blood mononuclear cells (PBMC)^{14–18} or stem cells (CD34⁺ cells).^{19–22} When PBMC are injected, mature cells, mostly mature activated human T cells, proliferate in mice, whereas when CD34⁺ stem cells are injected, immature stem cells proliferate and may differentiate into immature T cell progenitors that migrate to thymus and differentiate into more mature T cells.^{20,22} We hypothesized that if such mice were engrafted with either PBMC or CD34⁺ stem cells from patients with FSGS or MCNS, they could reproduce the disease, providing an animal model in which the underlying mechanisms are similar to those observed in humans.

RESULTS

To engraft human cells into mice, we used two strategies: injection of PBMC and injection of CD34⁺ stem cells. Twelve

blood samples from six patients (Table 1) and 12 samples from 12 control subjects were separated into CD34⁺ cells and CD34⁻ PBMC. We obtained $1.28 \pm 0.21 \times 10^4$ CD34⁺ cells and $22 \pm 4.8 \times 10^6$ CD34⁻ PBMC from patients' blood samples and $1.5 \pm 0.25 \times 10^4$ CD34⁺ cells and $20 \pm 4 \times 10^6$ CD34⁻ PBMC from control blood samples.

Assessment of Engraftment

Intraperitoneal injection of CD34⁻ PBMC from control subjects or patients into sublethally irradiated NOD/SCID mice that were treated with anti-NK antibody (12 mice in each group) induced the development of human CD45⁺ cells in the peripheral blood compartment in all cases. The percentage of human CD45⁺ cells increased to approximately 13% and then decreased to <0.1% without any significant differences between groups (Figure 1A). We showed that human CD45⁺ cells were CD3⁺ and CD19⁻ (Figure 2A). These data demonstrate that intraperitoneal injection of PBMC induces a transient peripheral engraftment of human peripheral mature T lymphocytes (CD3⁺ cells) into mice in a reproducible manner.

Because we obtained $<2 \times 10^4$ CD34⁺ cells and it has been shown that intraosseous (IO) injection induces better engraftment of human CD34⁺ cells,²³ we delivered CD34⁺ cells by IO injection. We observed an engraftment of human cells into all 12 mice that were administered an injection of CD34⁺ cells from control subjects and in 11 of 12 mice that were administered an injection of CD34⁺ cells from patients. The only mouse in which engraftment did not occur was administered an injection of CD34⁺ cells from patient 6, from whom it was possible to harvest CD34⁺ cells only once. In mice with engraftment, we detected >0.2% human CD45⁺ cells in peripheral blood 3 wk after injection. This percentage increased to approximately 0.8% when mice were killed (Figure 1B), without any significant differences between groups. None of the human CD45⁺ cells detected in mice were CD3⁺ or CD19⁺ (Figure 2B). We tested seven mice that were administered an injection of CD34⁺ engraftment and detected in six of them human CD34⁺ cells in both the injected femur and the contralateral femur, sug-

Table 1. Patient characteristics^a

Patient	Age (yr)	Gender	Diagnosis	Status at Blood Harvesting
1	14	F	Steroid-resistant FSGS	Relapse of NS after renal transplantation, ESRF under chronic hemodialysis
2	13	M	Steroid-resistant FSGS	Relapse of NS after renal transplantation, ESRF under chronic hemodialysis
3	25	F	Steroid-resistant FSGS	Relapse of NS after renal transplantation, ESRF under chronic hemodialysis
4	35	M	Steroid-resistant FSGS	Relapse of NS after renal transplantation, ESRF under chronic hemodialysis
5	3	F	Steroid-sensitive MCNS	First episode of NS
6	5	F	Steroid-sensitive MCNS	First episode of NS

^aTwelve blood samples were obtained from patients: Three from patient 1; four from patient 2; two from patient 3; and one each from patients 4, 5, and 6. ESRF, end-stage renal failure; NS, nephrotic syndrome.

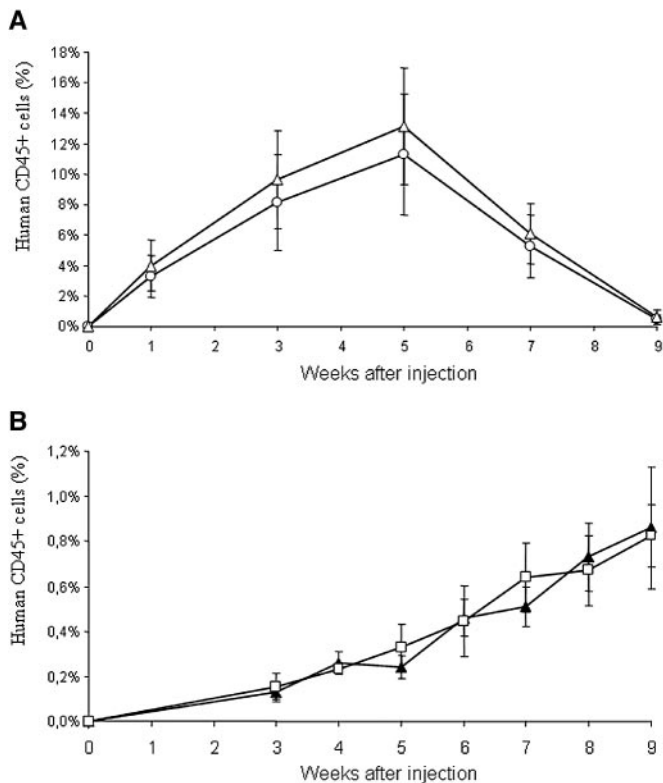


Figure 1. Kinetics of engraftment of human cells in NOD/SCID mice. Engraftment of human cells was determined by the percentage of human CD45⁺ cells in blood of mice that were administered an injection of CD34⁻ PBMC (A) of patients (O) or control subjects (Δ) and with CD34⁺ cells (B) of patients (▲) and control subjects (□). Percentages of human CD45⁺ cells were calculated as % human CD45⁺ = human CD45⁺ cells/(human CD45⁺ cells + mouse CD45⁺ cells) × 100. Data are means ± SEM of 12 mice except for those that were administered an injection of CD34⁺ cells from patients in which only the 11 mice with engraftment have been considered. CD45 is a marker of all hematopoietic and lymphoid cells except erythrocytes and platelets.

gesting an engraftment of human stem cells and a migration of these human CD34⁺ stem cells to other osseous localizations (Figure 3A). Moreover, 10 mice that were administered an injection of CD34⁺ cells from patients ($n = 9$) or from control subjects ($n = 1$) were tested for the presence of human cells in the thymus when the mice were killed. In one mouse that was administered an injection of CD34⁺ from patient 3, no human CD45⁺ cells were detected; however, in the remaining nine mice, $1.61 \pm 1\%$ CD45⁺ cells were identified, suggesting that migration of human cells, likely thymic progenitors, had occurred into the thymus. Human cells identified in thymus were CD45RA⁺, CD45RO⁺, or CD45RA⁺RO⁺ (Figure 3B), but none was CD3⁺ neither CD4⁺ nor CD8⁺, suggesting that human cells were in a differentiation process in the thymus. Also, no human engrafted cells could be detected in the secondary lymph nodes (spleen and lymph nodes), suggesting that until 9 wk after

transplantation, no mature T cells had developed and emigrated from thymus and no mature B cells had developed and emigrated from bone marrow. Our results demonstrate that approximately 10^4 CD34⁺ cells are sufficient to establish an engraftment of human cells into immunocompromised mice when injected by the IO route.

Assessment of Albuminuria

We first analyzed albumin and creatinine of 20 NOD/SCID mice, 15 times for each mouse, and determined a mean ± SD of albumin-to-creatinine ratio (ACR) value of $11.88 \pm 4.69 \mu\text{g}$ albumin/mg creatinine. We established the limit of normal value of ACR as being mean + $2 \times$ SD (*i.e.*, $21.26 \mu\text{g}$ albumin/mg creatinine).

In mice that were administered an injection of CD34⁺ cells or CD34⁻ PBMC from controls, ACR level did not change. In mice that were administered an injection of cells from patients, we showed that CD34⁻ PBMC engraftment did not change ACR levels, whereas all 11 mice with CD34⁺ cell engraftment showed a significant increase in ACR (Table 2). In these 11 mice, ACR increased significantly during the fourth week after injection and then remained significantly increased until mice were killed (Figure 4). Therefore, the increase in ACR levels paralleled the detection of CD45⁺ human cells. That the one mouse that did not display albuminuria, although administered an injection of CD34⁺ cells from a patient, was the one in which no engraftment was observed suggests that engraftment of patient cells was needed to induce albuminuria. To confirm this finding, we administered an injection of CD34⁺ cells from patients 1 and 2 to two other mice but without treating mice with anti-NK antibody. No human CD45⁺ cells were detected, and no increase of ACR occurred.

In mice that were administered an injection of CD34⁻ PBMC from patients, ACR did not increase (Figure 4) despite the engraftment of human CD3⁺ cells that increased up to approximately 13%, suggesting that mature T cells from patients are not capable of inducing ACR increase in mice. One could hypothesize that proliferation of human T cells in mice was inhibited and that this inhibition prevented an increase of ACR. This inhibition could be due to the presence of mature CD25⁺ regulatory T cells (Treg) within the injected CD34⁻ PBMC or to the residual immune system of mice that reject human T cells. To rule out these hypotheses, we purified CD25⁻ cells from CD34⁻ PBMC from two patients (patients 1 and 2) and two control subjects and injected them into sublethally irradiated NOD/SCID γc^- mice in which the γc chain was mutated, thereby preventing NK cell development. These NOD/SCID γc^- mice are more immunocompromised than NOD/SCID mice that are administered an injection of anti-NK antibody and are known to allow a higher engraftment of human cells.²⁴ Human CD45⁺ cells, made of $93 \pm 5\%$ mature CD3⁺ T cells, raised up to $82.26 \pm 9\%$, and mice died from xenograft-*versus*-host disease 4 to 8 wk after injection with-

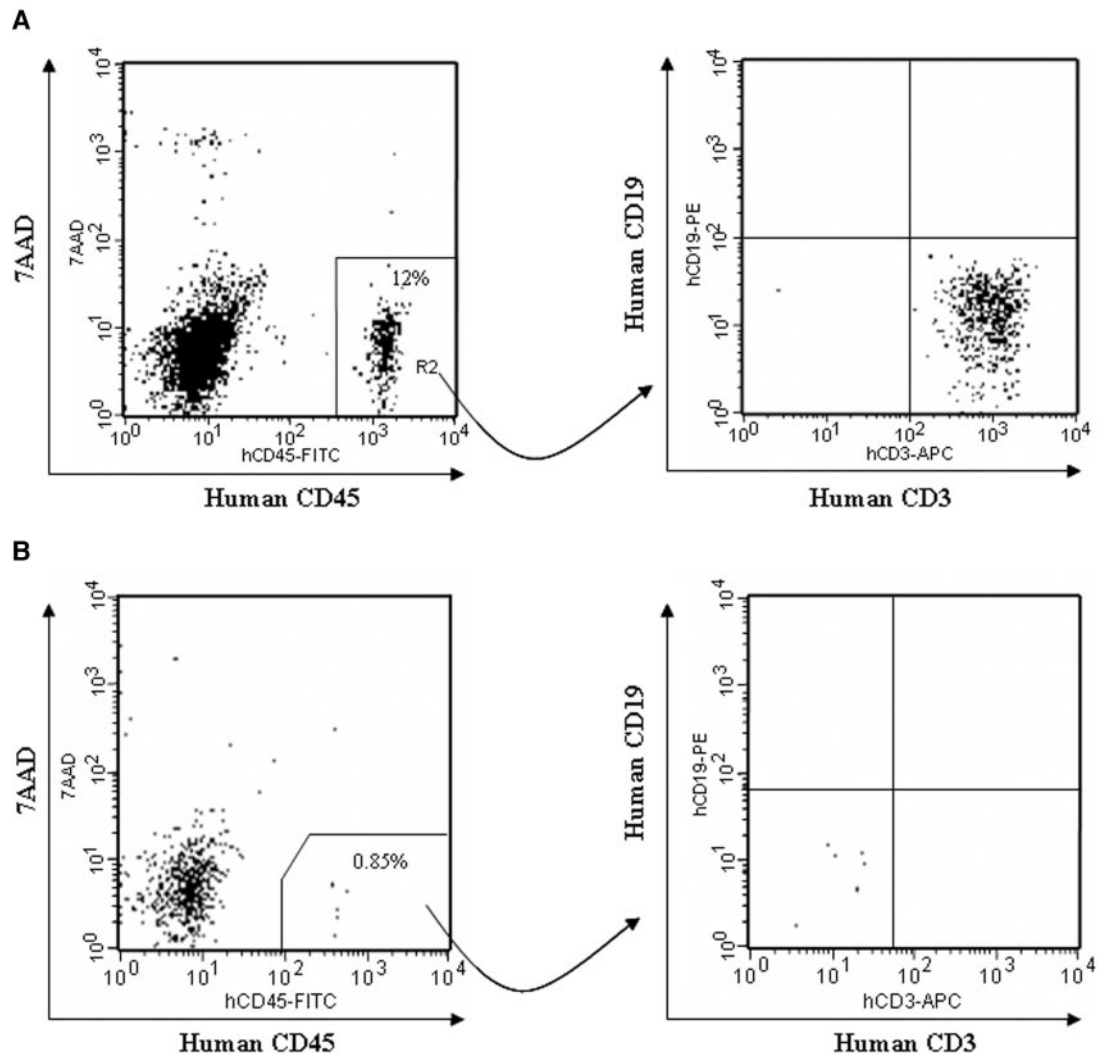


Figure 2. Phenotype of engrafted CD45⁺ human cells in NOD/SCID mice. Representative analysis by flow cytometry of peripheral blood from a mouse that received an intraperitoneal injection of CD34⁺ PBMC from patient 3 (A) and from a mouse injected by IO route with CD34⁺ cells from patient 2 (B). 7AAD⁻ (living) human CD45⁺ cells were gated (left) and analyzed for CD3 (marker of T lymphocytes) and CD19 (marker of B lymphocytes) expression (right).

out any ACR increase (monitored every 3 d). Therefore, despite a huge engraftment of mature activated T cells from patients, albuminuria could not be transferred into mice.

Assessment of Renal Pathology

To test whether renal pathology induced by the injection of CD34⁺ cells from patients was similar to that observed in human disease, we analyzed kidneys of mice with increase of ACR using light microscopy and immunofluorescence ($n = 3$) and electron microscopy ($n = 6$). As shown in Figure 5, light microscopy was normal as revealed by nonexpanded mesangium and cells per glomerular cross-section at 36 ± 7 (normal) in mice that were administered an injection of CD34⁺ cells from patients and 35 ± 4 (normal) in mice that were administered an injection of CD34⁺ cells from control subjects. Also, no Ig or complement component deposits were observed with immunofluorescence microscopy (data

not shown). However, electron microscopy revealed a partial widening and effacement of epithelial cell foot processes, as is also observed in humans with INS, whereas epithelial cell foot processes of mice that were administered an injection of cells from control subjects were normally distributed along the basal membrane (Figure 6). Glomerular basement membrane thickness was 175.8 ± 23.8 nm in mice that were administered an injection of CD34⁺ cells from control subjects ($n = 3$) and 198.4 ± 19.7 nm in mice that were administered an injection of CD34⁺ cells from patients ($n = 6$; $P = 0.16$, NS). Also, no electron-dense deposits were present. These data suggest that the increase in ACR level observed in mice was strong enough to induce effacement of epithelial cell foot processes and that the mechanism of albuminuria in mice is likely similar to the etiology hypothesized in humans; that is, a secretion of a vascular permeability factor by cells of the immune system.

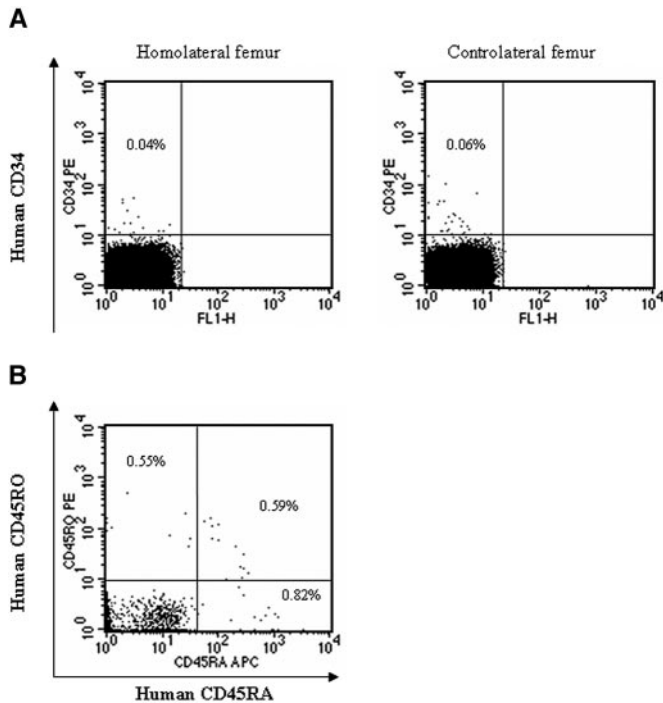


Figure 3. Detection of human cells in femur and thymus of NOD/SCID mice that were engrafted with human CD34⁺ cells. (A) Representative analysis by flow cytometry of bone marrow cells harvested in both femurs from a mouse that was administered an IO injection of CD34⁺ cells from patient 1 and killed 9 wk after injection. (B) Representative analysis of cells in thymus from one mouse that was administered an IO injection of CD34⁺ cells from patient 4 and killed 9 wk after injection. CD45RA and CD45RO both are isoforms of CD45, markers of thymocytes at different stages of differentiation.

DISCUSSION

We show that CD34⁺ cells that are from patients and injected into mice may engraft and induce albuminuria. However, ACR did not increase in mice that were administered an injection of CD34⁻ PBMC from patients despite both a higher number of cells injected and a higher number of human cells detected in mouse blood. It has been shown that injection of PBMC into immunocompromised mice induces the expansion of mature T cells,^{14–17} whereas injection of CD34⁺ cells induces the development of a human immune and hematopoietic system,^{19,21,22} with immature T cells migrating to the thymus and undergoing differentiation. Therefore, it is likely that, in our model, the cells responsible for albuminuria are immature cells undergoing differentiation. Also, ACR increase occurred 3 to 4 wk after injection of CD34⁺ cells and paralleled the detection of human CD45⁺ cells in peripheral blood, suggesting that the cells that are responsible for albuminuria were likely to originate from stem cells and their subsequent proliferation and differentiation rather than from the CD34⁺ cells themselves. Data from the literature suggest that cells that are responsible for MCNS are from the immune system, more specifically

from the T cell subset. A possible role for T cells developed from the observation that infusion of supernatants of cultured PBMC from patients with MCNS relapses induced proteinuria in rats^{25–28} and that T cell hybridomas obtained from a patient with NS secreted a factor that caused proteinuria in rats.²⁹ Studies of T cell compartments in MCNS demonstrated expansion of CD4⁺ and CD8⁺ T cell populations^{30,31} and an increased synthesis of several cytokines, such as TNF- α ,^{31,32} soluble IL-2 receptor,³² and IL-13.³³ In our model, we showed that peripheral CD3⁺ cells of patients were not capable of inducing albuminuria in mice, even in cases in which CD3⁺ cells could proliferate in mice, after depletion of Treg and injection in more immunocompromised mice. Given all of the data in the literature suggesting the involvement of T cells and considering that, in our model, albuminuria occurs after engraftment of CD34⁺ cells but not after engraftment of CD3⁺ cells, it is tempting to propose that cells that are responsible for albuminuria are undifferentiated cells undergoing differentiation into T cells. Our results showing that engrafted human cells could migrate to the mouse's thymus in mice that were administered an injection of CD34⁺ stem cells and that no mature CD3⁺ T cells could be found in mice until they were killed are consistent with a role for immature rather than mature T cells. However, one cannot rule out the hypothesis that immature cells of other lineages may be responsible for albuminuria.

Other experimental animal models resembling MCNS or FSGS have been described, such as age-associated nephropathy,¹¹ nephron reduction,¹² or puromycin or aminonucleoside toxic-induced nephrosis.^{9,10} However, despite some similarities with human FSGS, the outcome of proteinuria in these models does not involve the immune system and does not involve a circulating factor. Therefore, these models are of limited value in terms of understanding the pathologic process of human primary FSGS. The Buffalo/Mna rat model in which rats spontaneously develop lesions mimicking human idiopathic FSGS³⁴ is more interesting. Indeed, proteinuria relapsed after transplantation of normal kidneys from healthy LEW.1W rats into Buffalo/Mna recipients, suggesting that this model resembles human primary FSGS.¹³ Moreover, Buffalo/Mna rats exhibit renal macrophage activation and TH2 polarization that precedes the development of NS.³⁵ It is noteworthy that Buffalo/Mna rats were first reported because of the spontaneous formation of a thymoma associated with muscular weakness. Also in humans, FSGS may be associated with thymoma, and it was recently suggested, by T cell transcriptome analysis, that INS was likely a thymic disorder.³⁶ Given this experimental data and our results suggesting that immature T cells may be responsible for proteinuria, it is tempting to propose that immune cells passing through the thymus to undergo differentiation and selection may be involved in the pathogenesis of FSGS or MCNS. However, neonatal³⁷ and adult thymectomy³⁵ had no effect on proteinuria in the Buffalo/Mna rat model, making it an unlikely hypothesis for this model.

The outcome of albuminuria and the structural abnormalities observed using the electron microscope strongly

Table 2. Outcome of albuminuria in humanized mice^a

Injected Cells	No. of Mice Administered Injection	Engraftment of Human Cells	ACR > Mean + 2 × SD
CD34 ⁻ PBMC from control subjects	12	12	None
CD34 ⁺ from control subjects	12	12	None
CD34 ⁻ PBMC from patients	12	12	None
CD34 ⁺ from patients	12	11	11 ^b

^aEngraftment of human cells was considered positive when the percentage of human CD45⁺ cells detected in blood was >0.1% at any time after injection.

^bThe only mouse in which ACR remained under mean + 2 × SD was the mouse in which no engraftment occurred.

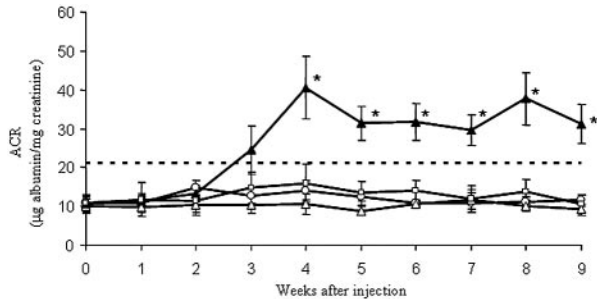


Figure 4. Evolution of ACR in mice that were administered an injection of CD34⁻ PBMC from patients (○) or control subjects (△) or of CD34⁺ cells from patients (▲) or control subjects (□). Data are means ± SEM of 12 mice in each group except for mice that were administered an injection of CD34⁺ cells from patients in which only the 11 mice with engraftments have been considered. *Significance at $P < 0.001$ versus all other mice or each group of mice. Dashed line represents the superior limit of ACR as determined as mean + 2 × SD in 20 control NOD/SCID mice.

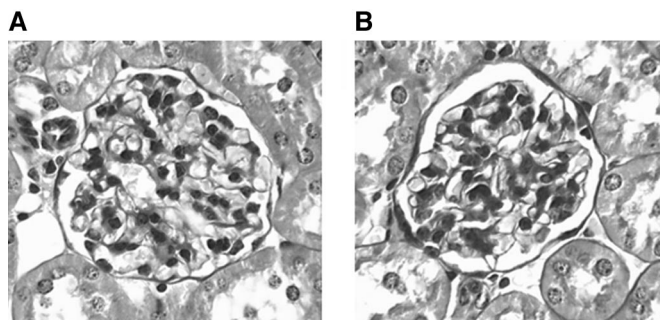


Figure 5. Normal aspect of glomeruli on light microscopy. Representative light microscopy of glomeruli from control mice (A) and albuminuric mice (B). Formalin-fixed, paraffin-embedded section stained with periodic acid-Schiff. Magnification, ×1000.

suggest that we can reproduce the pathologic process underlying albuminuria in FSGS and MCNS. Indeed, these structural abnormalities together with the significant ACR increase observed in all mice that were engrafted with CD34⁺ cells from patients but not in mice that were engrafted with CD34⁺ from control subjects suggest that the observed changes in mice are related to a pathologic mechanism similar to one involved in FSGS in human, despite the low magnitude of ACR increase in comparison with what is observed in INS in human. Also, it is likely that the modest

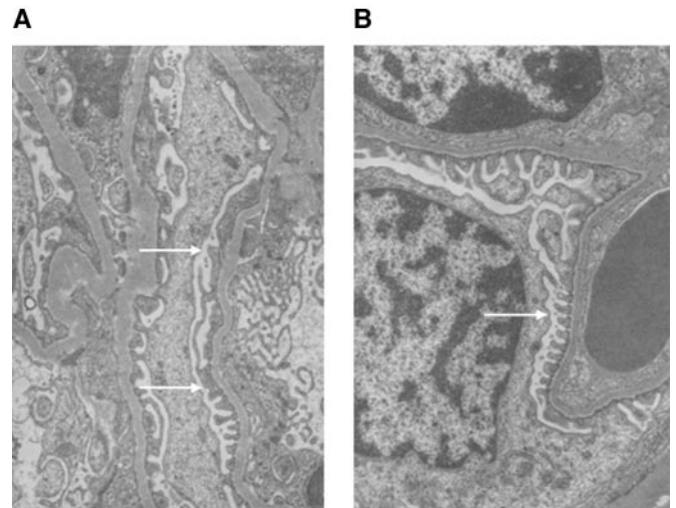


Figure 6. Epithelial cell foot fusion in mice receiving CD34⁺ cells. Representative electron microscope images of a glomerulus from albuminuric (A) and control (B) mice. The albuminuric mouse was administered an injection of CD34⁺ cells from patient 1 and shows partial widening and effacement of epithelial cell foot processes (arrows, A), whereas the mouse that was administered an injection of CD34⁺ cells from a control subject has normal and regular epithelial cell foot processes (arrow, B).

increase of ACR is because engraftment of patients' cells was very low. One could hypothesize that a higher engraftment could induce a higher ACR increase, and this would need to be demonstrated in models allowing a higher engraftment of human stem cells. That we did not observe adhesions in glomeruli is likely due to the short period of time during which mice displayed albuminuria before being killed. Also, that albuminuria was observed with both similar kinetics and ranges after injection of CD34⁺ cells from four patients with FSGS and from one patient with MCNS suggests that MCNS and FSGS share the same pathologic process and likely represent a different spectrum of the same disease.

Our data demonstrate that NOD/SCID humanized mice may be a useful tool to study human disease. In the context of FSGS and MCNS, we could reproduce albuminuria in mice together with renal lesions, seen using electron microscopy. Our model suggests that the cells that are responsible for albuminuria are likely to be immature cells rather than mature CD3⁺ peripheral T cells. These results can also be seen as a proof of concept for using the technology of

humanized mice to study human diseases that are linked to a pathologic process involving the hematopoietic or the immune system.

CONCISE METHODS

For more details, see supplemental Concise Methods online.

Patients

Blood samples (10 ml) were obtained from six patients and 12 control subjects. Among control subjects, 10 were normal volunteers and two were adults under chronic hemodialysis for end-stage renal failure not related to INS. This work was approved by the local ethics committee. Informed consent was obtained from the parents of pediatric patients (and whenever possible from the pediatric patients themselves), as well as from adult patients and normal volunteers.

Purification of Cells

Blood samples were separated into CD34⁺ and CD34⁻ PBMC using magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34⁺ fraction (purity >85%) and CD34⁻ fraction (purity = 100%) were centrifuged and resuspended in PBS before being injected to mice. In two experiments, the CD25⁻ fraction of CD34⁻ PBMC was also sorted, and its purity was 100%.

Injection of Human Cells into NOD/SCID Mice

NOD/SCID mice were sublethally irradiated (2.5 Gy) and treated with TM- β 1 (gift from Dr. T. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), an antibody blocking the mouse NK cell function, shown to induce better engraftment of human cells.²⁰

Engraftment and Albuminuria

Peripheral blood was aspirated weekly from the retro-orbital sinus under anesthesia, and bone marrow, thymus, and spleen were harvested when mice were killed. The presence of human cells was analyzed by flow cytometry. At the indicated time points, urine samples (minimum of 50 μ l) were collected. Urine creatinine and albumin concentrations were determined in the same samples with an Olympus analyzer AU400 (Olympus Instruments, Japan). Urine albumin excretion was expressed as the ACR.

Microscopic Examination

Mouse kidney samples were obtained at the time of killing, 9 wk after injection. Kidney specimen were analyzed by optic microscopy, direct immunofluorescence, and electronic microscopy. All analyses were performed in a masked manner. Kidney pathology was assessed according to a slightly modified procedure previously described.³⁸

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DISCLOSURES

None.

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