Podocyte Injury Damages Other Podocytes

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ABSTRACT
Loss of podocytes promotes glomerulosclerosis, but whether this results from a continued primary insult or a secondary mechanism triggered by the initial loss of podocytes is unknown. We generated chimeric mice in which only a subpopulation of podocytes expressed hCD25, which is the receptor for the immunotoxin LMB2. In addition, genetic labeling of hCD25-negative cells with human placental alkaline phosphatase allowed the study of these two distinct podocyte populations. Administration of LMB2 did not cause podocyte injury in hCD25-negative control mice. In contrast, LMB2 severely damaged or sloughed off the subpopulation of hCD25-positive podocytes within the chimeric glomeruli. Moreover, hCD25-negative podocytes, which were immune to the initial toxin injury, developed injury as early as 4 d after LMB2 injection, evidenced by foot process effacement, upregulation of desmin, and downregulation of nephrin, podocin, and podocalyxin. Furthermore, the magnitude of secondary injury correlated with the magnitude of primary injury, supporting the concept of an amplified cascade of podocyte injury. In conclusion, podocyte damage can propagate injury by triggering secondary damage of “remnant” intact podocytes, even when the primary insult is short-lived. This transmission of podocyte injury may form a vicious cycle leading to accelerated podocyte deterioration and glomerulosclerosis.


Loss of podocytes leads to glomerular sclerosis, the morphologic hallmark of chronic kidney disease.1–5 A number of factors, including genetic, mechanical, and immunological stresses, as well as toxins, can cause podocyte injury.6–9 As podocyte deterioration is often relentless, the question as to whether the ongoing injury is a result of a continued primary insult or a secondary mechanism triggered by the initial loss of podocytes per se has remained unresolved.

Previously, we established a transgenic mouse line (NEP25), which expresses human (h) CD25 selectively on podocytes.10 By injecting the hCD25-targeted recombinant immunotoxin, anti-Tac(Fv)-PE38 (LMB2),11 podocyte-selective injury can be induced in NEP25 mice in a dose-dependent manner. With high-dose LMB2 (≥1.25 ng/g BW), NEP25 mice develop massive nonselective proteinuria, severe glomerulosclerosis, and renal failure, and die within 14 d. With low-dose LMB2 (0.625 ng/g BW), NEP25 mice develop moderate proteinuria, which peaks 1 to 2 wk after injection and gradually decreases. Although LMB2 is rapidly cleared from the circulation with half life of 35 min,11 podocyte injury progresses over weeks. Thus, 3 wk after injection, mice develop focal segmental glomerulosclerosis. With either dose, the injury is initially confined to podocytes, but later other cells
within and outside the glomerulus become affected. These progressive injury phenotypes prompted us to hypothesize that injury of some podocytes secondarily injures other podocytes that escaped the initial injury.12

In this regard, numerous studies in subtotally nephrectomized animals have shown that loss of a large number of nephrons imposes stresses, e.g., glomerular hypertension or secondary glomerular hypertrophy with decrease in podocyte density in the remnant glomeruli, thereby secondarily damaging podocytes.13–18 Thus, this mechanism appears to become critically important at the late phase of renal injury. We were intrigued by the possibility that podocyte damage in and of itself could damage other podocytes within the glomerulus at an early phase, i.e., before maladaptive responses to nephron loss occur.

In both the experimental animal models and clinical nephropathies studied thus far, the entire podocyte population is exposed to the primary insult. These settings, including our NEP25 model, preclude determination of whether continued podocyte damage is caused by a lingering effect of primary insult or by a mechanism secondary to the initial loss of some podocytes. To overcome this problem, we generated chimeric mice in which only a subpopulation of the podocytes carries the hCD25 transgene and, therefore, are selectively targeted by the immunotoxin LMB2. We demonstrate that hCD25-negative podocytes, which are not targeted by LMB2, are damaged by a secondary mechanism triggered by initial damage of hCD25-positive cells. These studies provide evidence of podocyte-to-podocyte transmission of damage that may cause a vicious cycle leading to sclerosis.

RESULTS

Identification of hCD25-Positive versus hCD25-Negative Podocytes

hCD25 is regulated by the nephrin promoter and is, therefore, rapidly downregulated after toxin injury when podocyte nephrin expression is reduced. To identify the genotype of each podocyte in paraffin and electron microscopy tissue sections, we created chimeric mice using R26-hPAP transgenic mice as the source of non-hCD25-carrying cells. The R26-hPAP line is insensitive to LMB2 and ubiquitously expresses human placental alkaline phosphatase (PLAP), which is readily identifiable by histochemical or immunological staining.19 We stained PLAP histochemically and immunologically in paraffin sections of the kidney of heterozygous R26-hPAP transgenic mice. As reported previously, both methods intensely and highly specifically stained all types of cells including podocytes in R26-hPAP mice (Figure 1 A through D and S1).

To test whether injured podocytes are stably labeled with PLAP, we generated NEP25/R26-hPAP double transgenic mice, in which all cells express both hCD25 (susceptible to toxin) and PLAP. After LMB2 injection (0.625, 1.25, 2.5, and 25 ng/g BW), NEP25/R26-hPAP mice were analyzed at various time points (4 to 42 d). Periodic acid—Schiff (PAS) staining and immunostaining for nephrin and podocalyxin confirmed that glomeruli in these kidneys have various degrees of podocyte injury. All cells clearly stained for PLAP, even glomeruli with severe downregulation of nephrin, podocalyxin, and the transgene product hCD25 (Figure 1 E through H). Thus, PLAP can serve as an hCD25(-) marker, even in injured podocytes.

Chimeric Mice: Assessment of Podocytes at Baseline

We next generated NEP25↔R26-hPAP chimeric mice, in which only a fraction of podocytes expresses hCD25. We then performed renal biopsy in NEP25↔R26-hPAP chimeric mice before LMB2 injection. Although the percentage of hCD25-positive (+) podocytes was variable among glomeruli and among mice, the average percentage of PLAP(+) or hCD25(+) cells within a given chimeric kidney was similar from section to section, as these sections sampled a large number, >50 glomeruli. In addition, in wild-type↔R26-hPAP chimeric mice, we confirmed that distribution of PLAP-positive cells was similar in left and right kidneys within a given mouse. In the subsequent studies, therefore, we assessed hCD25 (+)
staining in a single section with >55 glomeruli for each chimeric mouse and calculated the hCD25 index to represent the average percentage of hCD25-positive podocytes (Table 1).

PLAP staining was seen primarily on the cell membranes. It was therefore difficult to determine PLAP positivity for the interior glomerular cells. In contrast, PLAP positivity versus negativity was clearly evident for most surface podocytes (Figure 2 I and J). Immunofluorescent staining (Figure 2 A through C) and immunoelectronmicroscopy (Figure 3A) confirmed that PLAP protein was distributed in a chimeric pattern.

Analysis of serial sections confirmed that each surface podocyte was positive for either PLAP or hCD25 (Figure 2 G and H) with the exception that some areas of foot processes appeared positive for both, possibly reflecting interdigitation of foot processes of PLAP (--) podocytes and hCD25 (+) podocytes. Therefore, in subsequent analyses, we identified the genotype of individual surface podocytes by PLAP positivity on the apical membrane in close proximity to podocyte nuclei. The term “hCD25(--) podocytes” is used to indicate podocytes not carrying hCD25 as determined and validated by PLAP-positive staining.

**Early Response of Chimeric Mouse Podocytes**

We first performed analyses at a very early phase after high-dose LMB2 (25 ng/g BW). Four days after toxin injection, non-chimeric NEP25 mice with ICR genetic background showed massive proteinuria. Urinary albumin/creatinine ratio (ACR) increased, from baseline 0.10 ± 0.02 to 58.36 ± 4.41 (mg/mg). In most podocytes, nephrin staining was diminished, with nephrin index averaging 3.24 ± 0.32 (scale: 0 to 8). Of note, neither proteinuria nor podocyte injury was observed at any time points in wild-type mice or R26-hPAP transgenic mice not carrying hCD25 after toxin injection (data not shown).

In chimeric NEP25→R26-hPAP mice, there was variable chimerism of podocytes at baseline, with an index of hCD25 ranging from 0.11 to 0.78, where 0.00 is no hCD25 and 1.00 is 100% of podocytes carrying hCD25. Four days after LMB2 injection, urinary ACR ranged from 1.08 to 67.26 (Table S2), and nephrin index ranged from 3.67 to 7.93 (Table 1). The nephrin index was negatively correlated with the degree of chimerism (Table 1). Most hCD25 staining was lost in all chimeric mice except for mouse C2, in which a few podocytes with faint hCD25 staining were observed in several glomeruli.

In all chimeric mice, most hCD25 (+) podocytes showed downregulation of nephrin after LMB2, confirming that LMB2 effectively injured hCD25 (+) podocytes (Figures 2 D through F, K through N, and S1). In addition, 9.7 to 58.5% of hCD25(--) surface podocytes (with definitive PLAP positivity), although not targeted by LMB2, also showed downregulation of nephrin (Figures 2 D through F, K through N, and S1). Downregulation of podocin and vascular endothelial growth factor (VEGF) and upregulation of desmin were also observed in PLAP (+) surface podocytes (Figure S3).

The percentage of hCD25 podocytes with downregulated nephrin was variable among mice (Figure S2), but correlated with the percentage of hCD25 (+) cells at baseline (Spearman’s rank correlation coefficient (rs), 0.83, P < 0.01; Figure 4). Thus, more podocytes injured by the primary toxin insult resulted in more podocytes injured by secondary, non-toxin-mediated mechanisms.

**Table 1. Profiles of the chimeric mice used in the present study**

<table>
<thead>
<tr>
<th>Dose of LMB2 (ng/g BW)</th>
<th>Time Interval between LMB2 Study (d)</th>
<th>Code of Each Chimeric Mouse</th>
<th>hCD25 Index before LMB2 (range 0 to 1)*</th>
<th>Nephrin Index before LMB2 (range 0 to 8)</th>
<th>Podocin Index before LMB2 (range 0 to 8)</th>
<th>rs (P)*</th>
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<tr>
<td>25</td>
<td>4</td>
<td>C1</td>
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<tr>
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<td>0.19</td>
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<td>0</td>
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A total of 15 chimeric mice (C1 through C15) were used. Each chimeric mouse showed a unique percentage of hCD25-positive podocytes on biopsy specimens before LMB2 injection, represented as the hCD25 index. After LMB2 injection (dose and time interval indicated), these mice showed variable podocyte injury, represented as the nephrin and podocin indices. Nephrin and podocin indices closely correlated with rs = 0.99 (P < 0.001).

* 0: no hCD25 staining, 1: 100% hCD25 staining.

* 0: complete loss of nephrin/podocin staining, 8: intact nephrin/podocin staining.

* Spearman’s rank correlation coefficient (rs) between hCD25 and nephrin indices with P value for each experiment.
Late Secondary Responses of Chimeric Mouse Podocytes

We next studied nonchimeric and chimeric mice at a later stage, 9 d after LMB2 toxin injection. Given that most nonchimeric NEP25 mice died within 7 d after 25 ng/g BW of LMB2,10 the dosage of LMB2 was decreased to 2.5 ng/g BW. Nonchimeric NEP25 mice (n = 3) on ICR genetic background showed massive proteinuria, with ACR averaging 73.69 ± 5.10, and developed severe glomerular damage with nearly complete downregulation of nephrin, podocalyxin, and hCD25. Nephrin indices in these mice were 0.25, 0.32, and 0.75, respectively (scale: 0 to 8).

We next studied the extent of injury in chimeric mice (n = 5). The hCD25 index at baseline ranged from 0.26 to 0.99, reiterating variable chimerism. After LMB2 injection, all chimeric mice showed massive proteinuria. Morphologically, injury assessed by nephrin downregulation was less than the nonchimeric NEP25 transgenic mice, ranging from 0.89 to 5.29, and the nephrin index was inversely correlated with the hCD25 index assessed before LMB2 injection (Table 1).

In two mice with low hCD25 index (mice C7, C8), glomerular structure was remarkably preserved, and most surface podocytes could be identified. In these mice, the majority of surface podocytes were positive for PLAP, indicating that most hCD25(+) podocytes had been lost. Nephrin and podocin were downregulated, and desmin was

Figure 2. NEP25→R26-hPAP chimeric mice at baseline and 4 d after 25 ng/g BW of LMB2. (A through F) Double immunofluorescent staining for nephrin (green) and PLAP (red) in a chimeric mouse before LMB2 (A through C) demonstrates global staining of nephrin and patchy (i.e., chimeric-pattern) staining of PLAP. Asterisks show RBCs with autofluorescence. Arrows depict DAPI nuclear staining (blue) with nephrin and PLAP, i.e., hCD25 (-) podocytes, and arrow heads depict DAPI with nephrin alone, i.e., hCD25 (+) podocytes. After LMB2 (D through F), nephrin staining is diminished globally, both in PLAP (+) podocytes (arrows) and in PLAP (-) podocytes (arrow heads), demonstrating nephrin is downregulated in hCD25 (-) podocytes. These panels and those in Figure S1 were stained in an identical fashion at the same time and photographed under the same conditions. (G and H) Serial sections from a NEP25→R26-hPAP chimeric mouse at baseline were stained for PLAP and hCD25. PLAP (+) cells are negative for hCD25, and PLAP (-) surface cells are positive for hCD25. (I through N) Serial section analysis for PLAP and nephrin. I and J, K and L, M and N are from adjacent sections. Before LMB2 (I and J), both PLAP (+) (arrows) and PLAP(-)(arrow heads) surface podocytes show normal nephrin staining pattern. After LMB2 (K and L), nephrin staining is downregulated in some PLAP (+) surface podocytes (arrows) in both glomeruli with (M and N) and without (K and L) adhesion. Magnification, ×400.
upregulated even in the remaining hCD25(-) podocytes (Figures 5 C through H and S1). Double immunofluorescent staining for desmin and PLAP revealed that the majority of desmin-positive podocytes was also positive for PLAP (Figure 5 I through K). Immuno EM for PLAP demonstrated that PLAP(+), i.e., hCD25(-)-negative podocytes were injured with extensive foot process effacement (short arrows) and microvillous transformation (asterisks). Bars represent 1 μm.

We next investigated the extent of injured podocytes versus extent of podocytes susceptible to toxin. As shown in Figure S4, the intact podocyte area after toxin decreased below the hCD25-negative podocyte area measured before toxin in these chimeric mice, confirming that damage propagated beyond toxin-susceptible cells into hCD25(-) podocytes.

Long-Term Effects of Minimal Initial Injury in Chimeric Mice

We next studied the long-term effects of injury in chimeric mice with low percentage of hCD25(+) podocytes (n = 4). Assessment was made 42 d after injection of high-dose (25 ng/g BW) LMB2. Unlike nonchimeric NEP25 mice, all four chimeric mice survived until sacrifice at day 42. Urinary ACR peaked on day 7 with 5.25 to 51.77, and gradually decreased to 0.65 to 3.81 by day 42 (Table S2). These chimeric mice showed focal segmental glomerular sclerosis (Figure 6). Glomeruli had neither hCD25(+) podocytes nor PLAP(-) surface podocytes, indicating that all hCD25 podocytes were eliminated.

Notably, in nonsclerotic portions in sclerotic glomeruli, nephrin was preferentially downregulated in podocytes adjacent to sclerotic lesion, although those podocytes were PLAP(+), i.e., insensitive to LMB2 (Figure 6). Glomeruli without sclerosis showed normal immunostaining for nephrin (Figure 6), podocin, and desmin (data not shown). In addition, there was no abnormal reabsorption of albumin in proximal tubular cells, confirming that the filtration barrier remained intact in these glomeruli. Thus, a substantial number of glomeruli maintained normal structure after losing a fraction of podocytes (Table 2).

Figure 7 shows the frequency distribution of hCD25 positivity (left columns) among chimeric glomeruli studied at baseline in four chimeric mice compared with the percentage of sclerotic glomeruli (right columns) at 42 d after LMB2 in the same mice. Within a given kidney, glomeruli with a greater population of podocytes with LMB2 susceptibility more readily developed sclerosis. The threshold of hCD25 positivity for a given glomerulus to develop sclerosis was between 0.25 and 0.5. However, scarcity of chimeric mice within this range limited further analysis.
failure, typically in the subtotal nephrectomy model. The loss or establishment of glomerulosclerosis. This observation podocyte injury before development of any sign of nephron served at the very early stages, as early as 4 d after the primary damage. Notably, this secondary podocyte injury can be ob-
injury from one glomerulus to another in late stages, glomeruli. This classic vicious cycle can explain the spread of remnant glomeruli, which lead to sclerosis in these remnant causes hemodynamic derangements and hypertrophy in the remnant glomeruli. Thus, substantial nephron loss or establishment of glomerulosclerosis. This observation contrasts the pattern documented in the late phases of renal failure, typically in the subtotal nephrectomy model. The glomerulus-to-glomerulus expansion of sclerosis after subtotal nephrectomy is thought to reflect excessive stress imposed on the remnant glomeruli. Thus, substantial nephron loss causes hemodynamic derangements and hypertrophy in the remnant glomeruli, which lead to sclerosis in these remnant glomeruli. This classic vicious cycle can explain the spread of injury from one glomerulus to another in late stages, i.e., after loss of a large population of nephrons.

More recently, Sato et al., using a diphtheria toxin receptor transgenic rat model, a model similar to the nonchime-
rnic NEP25 model, demonstrated persistent podocyte loss over 14 wk, duplicating the pattern seen in PAN model. Of note, in all these models of glomerulosclerosis using podocyte toxins, all podocytes were exposed to the primary insult; hence, progressive podocyte damage and loss may reflect heterogeneity among podocytes in the rate of re-
sponse to the toxins. In this regard, the present study clearly demonstrates that expansion of segmental sclerosis involves propagation of damage into podocytes that are not exposed to a primary insult (Figure 6).

Focal segmental glomerulosclerosis may be primary, caused by toxins, gene mutation, or idiopathic, or sec-
ondary, i.e., after loss of nephron number. Theses two forms of focal segmental glomerulosclerosis may have two separate mechanistic paradigms for expansion of podocyte injury. The present study indicates that, in either or both forms, the initial primary insult per se, if it occurs in large scale, can be brief in duration for triggering subsequent pro-
gressive loss of podocytes through the autonomous propa-
gation of podocyte-to-podocyte damage. A phenomenon similar to this appears to occur in inducible podocin knock-
out mice, which have mosaic glomeruli.

Kriz and others proposed earlier that synechiae formation and misdirected filtration are key morphologic events leading to glomerulosclerosis. The synechiae formation may, in turn, create a hostile environment for neighboring podocytes to preserve their structural integrity. In the pres-
ent study, spread of podocyte damage started at early stages, i.e., the allotted time was too short for synechiae to form.

Occasionally, in the NEP25 model, cells carrying parietal epithelial cell characteristics cover the surface of severely in-
jured glomerular capillaries (Figure S5). Among the chimeric mice analyzed 4 d after LMB2 injection, in which the genotype of individual podocytes was determined, only mouse C6 rarely contained such lesions. These cells were positive for claudin-1 and negative for nephrin and podocalyxin (Figure S5). Of note, throughout the analyses of individual podocytes (Figures S2 and 4), when surface cells were completely negative for nep-
hrin, we referred to the adjacent section stained for podocalyxin. We excluded the cells that were negative for podocalyxin from the analysis. This was to avoid the possibility that rare vescerally residing parietal epithelial cells were counted as damaged podocytes.

For initially intact podocytes to be damaged as a result of damage in other podocytes, in all likelihood, the threat to intact podocytes must come from injury in adjacent podo-
cytes. Thus, podocyte injury may cause release of toxic sub-
stances by damaged podocytes, including transforming growth factor β, endothelin-1, chemokines, and wingless-
related MMTV integration site (Wnt) family members. These factors are increased in injured glomeruli and excarate podocyte injury in other models. Their expres-
sions are increased in glomeruli of NEP25 mice after LMB2 injection (unpublished observation). Conversely, podocyte injury, causing reduction in intact podocyte population, may cause decrease in the ambient concentration of protective cell survival factors, such as VEGF. Secondary podocyte injury could also result from loss of proper cell-to-cell in-
teractions that are essential for podocyte survival. Another potential mechanism is transmission of death signal through the gap junction. Although LMB2 (Mw 63,000 Dal-
tons) itself, or its toxin moiety (Mw 38,000 Daltons), un-
likely passes through gap junctions, small molecules that transmit death signal may pass. Lastly, inflammatory cell infiltration is absent from glomeruli in this model.

In an attempt to identify the specific substances involved in the podocyte-to-podocyte transmission of damage, we sought an in
paired and from adjacent sections. Before LMB2 (A and B), both PLAP (2.5 ng/g BW of LMB2. (A through H) Serial section analysis. Upper and lower panels are observations made over the past 30 yr.46

Kriz presented an elegant overview of the potential toxic role of serum proteins in the context of various experimental observations.4 0–4 4is in favor of this notion. Although the notion of proteinuria for the subsequent progression of chronic kidney disease47 is determined by the magnitudes of increase in toxic substances and/or decrease in survival factors, depending on the extent of initial podocyte injury.

Figure 5. Injury in hCD25 (-) podocytes of NEP25→R26-hPAP chimeric mice 9 d after 2.5 ng/g BW of LMB2. (A through H) Serial section analysis. Upper and lower panels are paired and from adjacent sections. Before LMB2 (A and B), both PLAP (+) (arrows) and PLAP(-) (arrow heads) surface podocytes show normal nephrin staining pattern. After LMB2, nephrin (A and D) and podocin (E and F) are downregulated and desmin (G and H) is upregulated in some PLAP (+) surface podocytes (arrows). (I through K) Double immunofluorescent staining for desmin and PLAP. Desmin is upregulated both in PLAP (+) podocytes (arrows) and in PLAP (-) podocytes (arrow heads), demonstrating damage of hCD25 (-) podocytes. Of note, PLAP (+) cells do not carry the hCD25 transgene and therefore are not targeted by LMB2. Magnification, ×400.

vitro protocol that simulates the in vivo phenomenon. As described in the supplement and Table S1, however, we could not establish an in vitro protocol that allows podocyte-to-podocyte transmission of damage to occur. In this regard, a recent study in mice undergoing experimental unilateral ureteral obstruction39 showed that podocyte injury is drastically curtailed in the absence of physical forces for filtration, suggesting a certain limitation of an in vitro system to dissect the nature of cell-to-cell interactions occurring in progressive podocyte damage.

If the podocyte-to-podocyte damage transmission is, indeed, uniquely an in vivo phenomenon, the substances or factors involved appear to operate only in the presence of filtration. For example, podocyte injury causes leakage of macromolecules into Bowman’s space through filtration, which may not only be a result but possibly a cause of podocyte injury. The well-known predictive value of nonselective proteinuria for the subsequent progression of chronic kidney diseases40–44 is in favor of this notion. Although the notion of toxic effect of leaked serum proteins on podocytes is not new,45 Kriz presented an elegant overview of the potential toxic role of serum proteins in the context of various experimental observations made over the past 30 yr.46

We observed infrequent detached PLAP (+) podocyte debris in Bowman’s capsule 4 d after LMB2 injection (Figure S6).
females at 2.5 dpc; 574 pairs of embryos were aggregated in 14 experiments, and 112 mice were born. However, the efficiency of integration of both types of embryos was low. Only 18 NEP25R26-hPAP chimeric mice were obtained; 14 of these and one NEP25 wild-type mouse were used in the present study.

Chimeric mice carrying both NEP25 and R26-hPAP transgene were identified by PCR performed on tail DNA as previously reported. Renal biopsy was performed before LMB2 injection, and the ratio of hCD25-positive podocytes was assessed by immunostaining. The ratio of hCD25 positivity was semiquantified as described later.

Table 2. Substantial number of glomeruli maintained normal structure after losing a minor population of podocytes

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<tr>
<th>Code of Each Chimeric Mouse</th>
<th>Glomeruli Containing One or More hCD25-Positive Podocytes at Baseline (%)</th>
<th>Sclerotic Glomeruli 42 d after 25 ng/g BW of LMB2 (%)</th>
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Percentage of glomeruli containing one or more hCD25-positive podocytes was determined on biopsy specimens obtained before LMB2 injection. Percentage of sclerotic glomeruli was determined on autopsy specimens obtained 42 d after 25 ng/g BW of LMB2. Both values were obtained by three-dimensional analysis using serial sections.

**Induction of Podocyte Injury**

Recombinant immunotoxin, LMB2 (Anti-Tac (Fv)-PE38), was generated in *E. Coli* and purified as previously. More than two weeks after baseline biopsy, 2.5 or 25 ng/g BW of LMB2 was intravenously injected. Four, 9, or 42 d after LMB2 injection, mice were euthanized. Kidneys were perfusion-fixed with 4% buffered paraformaldehyde at mean arterial pressure for some assessments. In addition, nonchimeric NEP25 mice on ICR genetic background were treated and analyzed in an identical fashion as comparisons. Kidney samples were further fixed in 4% buffered paraformaldehyde overnight and embedded in paraffin. Parts of kidney samples were incubated in graded (15 to 30%) sucrose/PBS solution and frozen for immunoelectron microscopy.

**Histologic Methods**

PAS staining, various immunostaining, and histochemical staining for PLAP were performed in 2-μm thick serial paraffin sections.

For alkaline phosphatase histochemistry, deparaffinized and rehydrated sections were heated in a substrate buffer (0.1M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM MgCl₂) at 65 °C for 2 h, incubated in BCIP-NBT solution (Sigma) at 30 °C for 16 h, and then counterstained lightly by PAS staining and covered with aqueous mounting medium, Crystal Mount (Biomedica Corp., Foster City, CA).

The following primary antibodies were used at indicated dilutions: mouse monoclonal anti-synaptopodin antibody (1:1, Progen, clone G1D4), guinea pig polyclonal anti-mouse nephrin antibody (1:200, Progen, GP-N2), rabbit anti-podocalyxin antibody (1:2000; a generous gift from Dr. Kurihara), mouse monoclonal anti-human desmin antibody (1:50, DAKO, D33), rabbit polyclonal anti-human podocin antibody (1:1000, Sigma), mouse monoclonal anti-human CD25 antibody (1:20, NeoMarkers, 25C04), rabbit monoclonal anti-human PLAP antibody (1:50, NeoMarkers, SP15), and rabbit polyclonal anti-claudin-1 antibody (1:200, abcam). For synaptopodin, nephrin, PLAP, hCD25, and claudin-1, slides were heated in citrate buffer (pH 6.0). For podocalyxin and podocin, slides were digested with 0.1% trypsin for 10 min before staining.

For immunoelectron microscopy, 8-μm thick frozen sections were cut, air dried, rinsed in PBS, incubated in 0.114% periodic acid for 10 min, and then in 0.114 mg/ml NaBH₄ for 30 min. After blocking in diluted normal goat serum, the sections were incu-
Sclerosis

podocytes. sclerosis than mice C14 and C15 with widespread hCD25(H11001)/H11001 podocytes and showed much less
H11001/H11001 area greater than 50% before LMB2, indicating
sclerosis, threshold hCD25 positivity for sclerosis is variable among
showed at the whole kidney level. According to this limited anal-
grams of normal and sclerotic glomeruli after LMB2 (right columns). Each panel represents res-
men positive patient). The left columns represent frequency distribution of baseline hCD25 positivity and the right columns represent the percentage of normal and sclerotic glomeruli after LMB2. The former was determined on the basis of >55 glomeruli in a single section, and the latter was determined by serial section analysis on >70 glomeruli. hCD25 scores 0.00, 0.25, and 0.5 represent hCD25 areas of 0, 1 to 25, and 26 to 50%, respectively. None of these mice had even a single glomerulus with hCD25(+) area greater than 50% before LMB2, indicating that chimeric contribution of LMB2-susceptible podocytes was small in these mice. Mice C14 and C15 had greater number of glomeruli with LMB2-susceptible podocytes than mice C12 and C13, and later showed greater number (25.8 to 41.8%) of sclerotic glomeruli than C12 and C13 did (1 to 8.5%). To determine the threshold percentage of LMB2-susceptible podocytes that is sufficient to develop sclerosis, the two columns obtained from independent analyses are compared based on the notions (1) that the percentage of hCD25 (+) podocytes is similar between biopsied and autopsied kidneys of the same chimeric mouse, as demonstrated earlier, and (2) that glomeruli with more hCD25 (+) podocytes are more prone to develop glomerulosclerosis, which was shown at the whole kidney level. According to this limited analysis, threshold hCD25 positivity for sclerosis is variable among mice and is affected by percentage of glomeruli containing hCD25 (+) podocytes. Thus, mice C12 and C13 had the fewest glomeruli containing hCD25(+) podocytes and showed much less sclerosis than mice C14 and C15 with widespread hCD25(+) podocytes.

Semiquantification of Immunostaining

In biopsy samples stained for hCD25, the area of hCD25 staining was semiquantified in all glomeruli (n > 55/sample) using scores 0 (hCD25 area = 0), 0.25 (1% ≤ hCD25 area ≤ 25%), 0.5 (26 to 50%), 0.75 (51 to 75%), and 1.0 (≥ 76%). For each chimeric mouse, the average hCD25 score was calculated and designated as hCD25 index.

For evaluating podocyte injury after LMB2, nephrin and podocin immunostaining was semiquantified. For this, each quadrant of each glomerulus was scored as 0 (no staining), 1 (diminished), or 2 (normal), with total glomerular score ranging from 0 to 8. Scores from all glomeruli on a section for each mouse (>70) were averaged and defined as the nephrin or podocin index. These indices were used to represent the extent of overall podocyte injury in each mouse and are shown in Table 1.

PLAP positivity and nephrin staining were evaluated in individual surface podocytes of all glomeruli in a section. For this, serial sections after LMB2 were stained for PLAP, nephrin, and podocalyxin, and each glomerulus was photographed by BZ9000 (Keyence). In each surface podocyte of which the nucleus was observed, nephrin staining was evaluated as either normal (continuous) or diminished (discontinuous). If nephrin staining was completely negative, podocalyxin staining on the adjacent sections was assessed to confirm that the surface cell was a podocyte. Rare podocalyxin-negative surface cells (Figure S5) were not included in the analysis. Podocytes involved in adhesion were also excluded from the analysis. The genotype of hCD25 was determined on the other adjacent section stained for PLAP. In each glomerulus, surface podocytes were categorized into four types: hCD25(+) with normal nephrin, hCD25(+) with diminished nephrin, hCD25(-) with normal nephrin, and hCD25(-) with diminished nephrin. Mean numbers for these types of surface podocytes are shown in Figure S2. To represent the extent of secondary podocyte injury in each chimeric mouse, we calculated and designated the following percentage as the average percentage of injured surface hCD25(-) podocytes: [mean number of hCD25(-) podocytes with diminished nephrin/mean hCD25(-) podocytes with normal nephrin + mean hCD25(-) with diminished nephrin] × 100. Correlation between this percentage versus hCD25 index is shown in Figure 4.

For analysis of the chimeric mice 9 d after LMB2 injection, immunohistochemistry was assessed in kidney sections processed and embedded in single blocks to avoid variable conditions. Baseline hCD25 and nephrin in each glomerulus were photographed by BZ9000. The ratios of hCD25 and nephrin-stained areas to total tuft area were quantified using image analysis software, WinROOF (Mitani Co.). Serial sections of kidneys at sacrifice after toxin were stained for nephrin and podocalyxin. The ratios of nephrin- and podocalyxin-stained areas to total tuft area were quantified in a similar manner. These data are shown in Figure S4.
The hCD25 positivity at baseline and the ratio of glomerulosclerosis 42 d after LMB2 were evaluated by three-dimensional analyses. Serial sections (>70 sections) at baseline were stained for hCD25, and the ratio of glomeruli containing more than one hCD25 (+) podocyte was determined. The data are presented in Figure 7 and Table 2. Serial sections (>70 sections) at sacrifice after LMB2 were stained for PAS and the percentage of glomeruli with sclerosis assessed. The data are presented in Table 2.

**Statistical Analysis**
Comparison of nephrin index between glomeruli with and without adhesion in chimeric mouse was performed using Mann–Whitney U test. Association between extent of hCD25(−) podocytes at baseline and injury of hCD25(+) podocytes after LMB2 (Figure 4) and that between hCD25 index and nephrin indices (Table 1) were evaluated by calculating the Spearman rank correlation coefficient. \( P < 0.05 \) was regarded as statistically significant.

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**DISCLOSURES**
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**REFERENCES**


Online supplement
Supplementary methods and results

*Morphometrical analysis for late (9 days after toxin) secondary responses*

All chimeric mice were embedded in the same paraffin block, and thus sectioned at the same thickness, and underwent an immunostaining process. Serial sections before LMB2 were stained for hCD25 and nephrin, and each glomerulus was photographed by Keyence Biorevo BZ9000. The ratios of hCD25-positive and nephrin-positive areas to total tuft area were quantified using WinRoof. Serial sections of kidneys after LMB2 were stained for nephrin and podocalyxin. The ratios of nephrin and podocalyxin-stained areas to total tuft area were similarly quantified.

In all chimeric mice except for one (mouse C11), nephrin-positive area (N) was greater than hCD25-positive area (C) when analyzed in serial sections (Figure S4). Since all podocytes are stained for nephrin before LMB2, the difference between the two staining-positive areas (N-C) columns in Figure S4 reflects hCD25 (-) podocyte areas. If all hCD25 (-) podocytes remained free of injury after LMB2 injection, nephrin-positive areas would be similar to hCD25 (-) podocyte areas. However, measurements revealed that the nephrin-positive area after LMB2 was always smaller than the hCD25 (-) podocyte area. The area for podocalyxin, a more stable podocyte marker than nephrin, was also smaller than hCD25 (-) podocyte area. For example, in a median glomerulus (horizontal columns) of a chimeric mouse (mouse C7), 32.4% was stained positive for nephrin, representing podocyte area, and 7.1% was stained positive for hCD25. The difference, 24.5%, reflects hCD25 (-) podocytes. After LMB2, only 8.26 and 14.1% stained positive for nephrin and podocalyxin, respectively. These data indicate that a substantial population of hCD25 (-) podocytes were injured and lost nephrin and podocalyxin staining.

In another chimeric mouse (mouse C11), most podocytes were hCD25 (+). Before LMB2, nephrin-positive and hCD25-positive areas were similar, and the median difference was essentially 0, thus confirming the validity of the analysis. After LMB2, nephrin and podocalyxin areas were near 0.

*Propagation of injury to hCD25-negative podocytes did not occur in vitro.*

In an attempt to identify the mechanism involved in the
Podocyte-to-podocyte transmission of damage, we examined an *in vitro* co-culture protocol. For this purpose, a rat podocyte cell line was stably transfected with hCD25 expression vector, and a subline, designated as hCD25(+), was established and characterized. We first observed that LMB2 added to the culture media dose-dependently suppressed leucine incorporation (IC50 9.8 pM) by hCD25(+), but not by hCD25(-) cells. hCD25(+) and hCD25(-) cells were then separately cultured in DMEM/F12 containing ITS-A supplement (0.5%) and FCS (5%) on laminin-coated dishes at 33°C. A fraction of cells was stained with DiO (Invitrogen) at 37°C for 15 minutes. Subsequently, DiO-labeled hCD25(-) cells were mixed with unlabelled hCD25(+) cells at various ratios and plated on 3 cm laminin-coated dishes (dishes 2-4)(4.4 X 10⁴/dish) and these were compared with dishes containing DiO-labeled hCD25(-) cells alone (dish 1) or DiO-labeled hCD25(+) cells alone (dish 5)(Table S1). To induce differentiation, cells were incubated at 37°C for 4 days, and on the 4th day, FCS concentration was decreased to 0.5%. At this time, all cells on dishes 1-5 were confluent and all cells on dishes 1 and 5 were confirmed to be positive for DiO. On the next day, LMB2 (500 pM) was added to the medium. Three days later, many hCD25 (+) cells in dish 1 were damaged. Detached cells were harvested, fixed in 2% paraformaldehyde/PBS, and cytopsins were prepared. The glass slides were stained with DAPI and photographed with AxioCam (Zeiss) or BZ-8100 (Keyence). Total numbers of DAPI signal and that of DiO (+) DAPI signal were counted using image analysis software, WinROOF (Mitani Co.). In a separate set of experiments, we confirmed that most detached cells were dead as they had incorporated propidium iodide.

If propagation of injury to hCD25(-) podocytes occurs *in vitro* as observed in chimeric mice, substantial number of hCD25 (-) cells would be dead and detached when they were co-cultured with hCD25 (+) cells. However, as a result, most detached cells from the above dish 2-4 were DiO (-) hCD25(+) cells, and the number of DiO (+) hCD25(-) cells did not appreciably exceed the expected number of spontaneously detached cells (Table S1).

These data indicate that propagation of injury to hCD25-negative podocytes that was observed *in vivo* does not occur *in vitro*.
Table S1

Propagation of injury to hCD25 negative podocytes did not occur in vitro

<table>
<thead>
<tr>
<th>Dish</th>
<th>Ratio of cultured cells (1)</th>
<th>% of dead cells to plated cells (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hCD25 (-)</td>
<td>hCD25 (+)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

hCD25 (-) and/or hCD25 (+) podocytes were co-cultured at the indicated ratio and LMB2 was added to the medium. Three days later, dead detached cells were harvested and hCD25 (+) cells and hCD25 (-) cells were separately counted.

In dishes 2-4, only 0.8 to 1.2% of hCD25 (-) cells were dead, contrasting to hCD25 (+) cells. The percentage of dead hCD25 (-) cells in these dishes was comparable to that of spontaneous dead cells in dish 1. Thus, the presence of dead hCD25 (+) cells did not increase the death of hCD25 (-) cells.

(1) Represented as % of the total number of cells plated on each dish, 44000.

(2) Represented as % of the number of plated cells of each hCD25 type.
Table S2

Urinary albumin/creatinine ratio (ACR) of chimeric mice before and after LMB2 injection

<table>
<thead>
<tr>
<th>Mouse code</th>
<th>ACR at baseline</th>
<th>ACR after LMB2 injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 days</td>
</tr>
<tr>
<td>C1</td>
<td>0.06</td>
<td>1.08</td>
</tr>
<tr>
<td>C2</td>
<td>0.37</td>
<td>5.65</td>
</tr>
<tr>
<td>C3</td>
<td>0.52</td>
<td>51.27</td>
</tr>
<tr>
<td>C4</td>
<td>0.52</td>
<td>30.10</td>
</tr>
<tr>
<td>C5</td>
<td>0.52</td>
<td>67.26</td>
</tr>
<tr>
<td>C6</td>
<td>0.22</td>
<td>64.76</td>
</tr>
<tr>
<td>C12</td>
<td>0.06</td>
<td>23.62</td>
</tr>
<tr>
<td>C13</td>
<td>0.17</td>
<td>5.25</td>
</tr>
<tr>
<td>C14</td>
<td>0.11</td>
<td>50.45</td>
</tr>
<tr>
<td>C15</td>
<td>0.03</td>
<td>51.77</td>
</tr>
</tbody>
</table>

For chimeric mice, C7-C11, which were analyzed 9 days after LMB2 injection, we confirmed massive proteinuria by SDS-PAGE analysis, but did not quantify albumin/creatinine ratio.

Figure legends

Figure S1
Double immunofluorescent staining for nephrin and PLAP.

In wild-type mice without R26·hPAP and hCD25 (top panels), nephrin (green) is globally and continuously stained, whereas PLAP (red) is negative. In R26·hPAP mice without hCD25 (panels in the second row), all nephrin staining overlaps with PLAP staining. In NEP25→R26·hPAP chimeric mice 4 days after 25 ng/g BW of LMB2 (panels in the third row), nephrin staining is globally downregulated showing discontinuous pattern both in PLAP (+) and PLAP (−) podocytes. In chimeric mice 9 days after 2.5 ng/g BW of LMB2 (bottom panels), nephrin staining almost disappears, while most of the surface of the glomerulus appears PLAP (+). Although cells that completely lose nephrin staining cannot be identified as podocytes, a small number of
surface podocytes (arrows) with diminished nephrin are positive for PLAP, thus convincingly demonstrating damage in hCD25 (-) podocytes. Asterisks show RBCs with autofluorescence.

Figure S2
Quantitative analysis of surface podocytes in \textit{NEP25\texttt{↔}R26-hPAP} chimeric mice

Before and after LMB2, nephrin staining positivity per podocyte was examined, and in the adjacent section, hCD25 genotype was determined for the same podocyte by PLAP staining. The number of podocytes (Y-axis) with each staining category (X-axis) per glomerulus is shown (Mean±SD).

(A) Before LMB2, all surface podocytes showed intact nephrin as there was no hCD25 (+) or hCD25(-) podocytes with diminished nephrin, i.e., no closed bar is shown in the top panel.

Glomeruli of two chimeric mice contained only rare hCD25(+) surface podocytes (mice C1, C2), shown by the red open bars, compared to hCD25(+) podocytes shown by the blue open bars, while in another chimeric mouse (mouse C6), the majority of surface podocytes were hCD25(+), and there were only rare hCD25(-) podocytes. The mean percentage of hCD25(+) surface podocytes ranged from 11\% (mouse C1) to 78\% (mouse C6).

(B) After LMB2, only few hCD25(+) surface podocytes remained, i.e., diminished red bars, open or closed. Most of those hCD25(+) podocytes had diminished nephrin (indicated by the closed red bars higher than the red open bars). The majority of the remaining surface podocytes were hCD25(-) podocytes (blue bars). Some of those hCD25(-) podocytes had diminished nephrin, i.e., in transition from (A) to (B), blue closed bars appeared along with diminution of the height of blue open bars. In a chimeric mouse with the most severe injury (C5), more than half (52\%) of hCD25(-) surface podocyte were injured (the highest blue closed bar), while a lower percent of hCD25(-) surface podocytes was injured in chimeric mice with low hCD25(+) podocyte percent population (mice C1, C2).

Figure S3
Injury in hCD25 (-) podocytes of \textit{NEP25\texttt{↔}R26-hPAP} chimeric mice 4 days after 25 ng/g BW of LMB2

A through D, as well as E and F, respectively, are from adjacent sections.
A-D: Podocin (A), VEGF (C) and nephrin (D) staining was downregulated in hCD25 (-) podocytes which are indentified by positive PLAP staining (B) (arrows).

E and F: Some hCD25 (-), i.e. PLAP (+)(E) podocytes express desmin (F)(arrows). (x400).

Figure S4
Propagation of injury into hCD25 (-) podocytes in NEP25→R26-hPAP chimeric mice 9 days after 2.5 ng/g BW of LMB2

Before LMB2 (abbreviated as “-LMB2”), serial sections from biopsy specimens were stained for nephrin and hCD25. In each glomerulus, nephrin and hCD25 staining areas were assessed. Nephrin positive staining areas (N) are larger than hCD25(+) areas (C) in these chimeric mice (mice C7, C8, C9 and C10). For each glomerulus, the difference between the two areas of nephrin-positive and hCD25-positive staining (N-C) was calculated, which theoretically reflects the hCD25(-) podocyte area. Therefore, in one chimeric mouse shown (mouse C11), most podocytes were hCD25 (+). Although the calculated hCD25(-) podocyte areas are variable, the median is essentially zero in this mouse, thus validating the method of this analysis.

After LMB2 (abbreviated as “+LMB2”), serial sections were stained for nephrin and podocalyxin. In the chimeric mice (mice C7, C8, C9, C10), the areas of both nephrin (N) and podocalyxin (P) are smaller than hCD25(-) podocyte area (N-C) assessed before LMB2, suggesting that substantial hCD25 (-) podocytes have lost nephrin and podocalyxin staining. Each data point taken from a single glomerulus is shown by an open circle. Values are presented as % areas relative to the whole tuft area.

Horizontal bars represent median. * represents significant difference (p<0.05) compared with N-C by non-parametric multiple comparison (Steel Dwass).

Figure S5
Parietal epithelial cell (PEC) lesions depicted by claudin-1 staining in chimeric mice 4 days after LMB2
A-C and D-F are adjacent sections, respectively. A-C: adhesion lesion occasionally observed in C5 and C6 mice. D-F: PECs covering the surface of glomerulus were rarely observed only in mouse C6. Cells in these PEC lesions are positive for claudin-1 and negative for nephrin and podocalyxin. In determining the genotype of individual podocytes, cells with negative podocalyxin staining were excluded from the analysis.

Figure S6
PLAP (+) podocyte debris in chimeric mice

Two panels are from adjacent sections of C3 chimeric mouse 4 days after LMB2. Rare cell debris with nephrin staining is observed in Bowman’s space which are all positive for PLAP (arrows).
Figure S2

(A) before LMB2

number of podocytes /glomerulus

hCD25(+), normal nephrin
hCD25(+), diminished nephrin
hCD25(-), normal nephrin
hCD25(-), diminished nephrin

(B) after LMB2

number of podocytes /glomerulus

C1 C2 C3 C4 C5 C6
Figure S4

Area (%)

C7  C8  C9  C10  C11

-N  C  N-C  N  P  -LMB2  +LMB2  -LMB2  +LMB2  -LMB2  +LMB2  -LMB2  +LMB2  -LMB2  +LMB2
Figure S5

A  claudin-1
B  nephrin
C  podocalyxin

D
E
F
Figure S6

PLAP  nephrin