Lutheran/basal cell adhesion molecule accelerates progression of crescentic glomerulonephritis in mice

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Migration of circulating leukocytes from the vasculature into the surrounding tissue is an important component of the inflammatory response. Among the cell surface molecules identified as contributing to leukocyte extravasation is VCAM-1, expressed on activated vascular endothelium, which participates in all stages of leukocyte-endothelial interaction by binding to leukocyte surface expressed integrin VLA-4. However, not all VLA-4-mediated events can be linked to VCAM-1. A novel interaction between VLA-4 and endothelial Lutheran (Lu) blood group antigens and basal cell adhesion molecule (BCAM) proteins has been recently shown, suggesting that Lu/BCAM may have a role in leukocyte recruitments in inflamed tissues. Here, we assessed the participation of Lu/BCAM in the immunopathogenesis of crescentic glomerulonephritis. High expression of Lu/BCAM in glomeruli of mice with rapidly progressive glomerulonephritis suggests a potential role for the local expression of Lu/BCAM in nephritogenic recruitment of leukocytes. Genetic deficiency of Lu/BCAM attenuated glomerular accumulation of T cells and macrophages, crescent formation, and proteinuria, correlating with reduced fibrin and platelet deposition in glomeruli. Furthermore, we found a pro-adhesive interaction between human monocyte α4β1 integrin and Lu/BCAM proteins. Thus, Lu/BCAM may have a critical role in facilitating the accumulation of monocytes and macrophages, thereby exacerbating renal injury.

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Necrotizing crescentic rapidly progressive glomerulonephritis (RPGN) is a class of acquired renal disease that remains one of few human autoimmune diseases that represent an acute threat to survival.¹ This has stimulated investigation into the immunobiology of the condition in the hope of understanding the pathogenesis not only of anti-glomerular basement membrane disease, but also of other forms of glomerulonephritis (GN) in which the aggravating antigen(s) is as yet unknown. Focal necrotizing crescentic GN is the renal lesion typically associated with the clinical syndrome of RPGN and is a medical emergency that requires side-effect prone immunosuppressive therapies. Untreated RPGN progresses rapidly to renal insufficiency. This process is almost always associated with severe interstitial and periglomerular inflammation. The inflammatory infiltrate gives way to a progressive fibrotic process involving the crescents and the periglomerular and peritubular interstitium, accompanied by tubular atrophy and progressive renal failure. Although the pathogenesis of crescentic RPGN is incompletely understood and likely involves several convergent pathways, there is general agreement that circulating mononuclear phagocytes have a central role. Administration of nephrotoxic serum to rodents or rabbits results in a severe proliferative and necrotizing GN that is characterized by glomerular crescent formation and accumulation of leukocytes.²⁻⁴ These infiltrating cells may then release inflammatory mediators that influence the behavior of glomerular, tubular, and interstitial cells. This interaction between infiltrating and resident cells leads to cellular proliferation, matrix expansion, and may ultimately lead to glomerular sclerosis and interstitial fibrosis. Monocytes and macrophages have a critical role as shown by ablation of macrophages in murine crescentic GN that reduced renal injury and improved renal function.⁵ Part of the deleterious action of glomerular macrophages could be directly linked to the augmented glomerular procoagulant activity as a result of their expression of surface membrane procoagulant activity.
and by their potential to indirectly augment glomerular procoagulant activity by the production of cytokines capable of enhancing endothelial cell procoagulant activity. In addition, infiltrating glomerular macrophages are the major source of IL-1 and tumor necrosis factor (TNF). TF-a proteins has been recently shown to mediate sickle cell integrin in sickle red cells with endothelial cellNice deficiency significantly prevented both the incidence and severity of crescentic glomerulonephritis. In mice, albuminuria (Figure 1a) and blood urea nitrogen and serum creatinine concentrations (Figure 1b and c), which were normal in Lu−/− mice.

As an index of early renal microvascular damage, we measured renal blood flow velocity in the renal artery before and on day 4 of NTS-induced RPGN. Whereas renal blood flow velocity remained normal in NTS-challenged Lu−/− animals, Lu+/+ animals displayed a significantly more profound diminution of mean renal blood flow on day 4 than that measured in Lu−/− counterparts (Figure 2). More severe alteration of renal blood flow in Lu+/+ animals was concomitant to equal, and later significantly higher, systolic blood pressure levels compared with those measured in Lu−/− mice (Figure 2). This suggests that Lu/BCAM deficiency limited the early rise in renal vascular resistance.

We histologically examined WT mice injected with NTS, and found severe GN by day 21 (Figures 3a–f), whereas Lu−/− littersmates had significantly less renal damage (Figure 3g–i). Overall, Lu−/− mice displayed significantly fewer (3.5-fold less) crescentic glomeruli (Figure 3m). Lu−/− mice also exhibited fewer fibrocellular crescents (Figure 3n), no increases in glomerular diameter, and virtually no rupture of Bowman’s capsule (Supplementary Figure S1A and B online).

Reduced ultrastructural alterations in Lu−/− glomeruli

Because glomerular expression of Lu/BCAM is constitutive and differential loss of glomerular permselectivity with heavy albuminuria preceded the development of the crescents already on day 7, we evaluated the morphological features of podocytes in Lu+/+ and Lu−/− mice on day 4 after an injection of NTS. Notably, podocyte ultrastructure was normal and identical in Lu+/+ and Lu−/− mice under control conditions with focal thickening of the external lamina of the GBM in Lu−/− condition as previously described. In response to NTS, WT Lu+/+ mice displayed mild-to-severe effacement of the foot processes of podocytes (Figure 3o). These ultrastructural alterations were markedly attenuated in Lu−/− animals (Figure 3p). Consistently Lu/BCAM deficiency was associated with fewer loss of differentiated podocytes than in Lu+/+ mice after NTS challenge, as assessed by WT-1 immunostaining on day 21 (Figure 3q and r).

Role of Lu/BCAM in the immuno-inflammatory response associated with RPGN

Although T cells and macrophages are central players both in our mouse model of NTS-induced GN and in human crescentic RPGN, antibody deposition may also have a pathophysiological role during the early stages of the disease, promoting activation of complement. Therefore, we assessed the humoral response of Lu−/− and Lu+/+ mice to sheep IgG. Sheep IgG deposition in glomerular basement membranes in the kidneys of both groups after NTS injection displayed similar intensity and pattern (Figure 4a). Glomerular deposition of mouse IgG was also similar in both NTS-injected groups (Figure 4b). Serial

RESULTS

Attenuated RPGN and no renal failure in Lu−/− mice

A total of 12 Lu−/− and 14 wild-type (WT) male littersmates of mixed 129/Ola-C57BL/6J genetic background had similar renal histology and functional parameters (albuminuria to creatinine ratio, serum creatinine, and blood urea nitrogen) at baseline (Figure 1). Injection of anti-glomerular basement membrane (anti-GBM) nephrotoxic serum (NTS) induced nephrotic syndrome in WT animals. Nephrotic syndrome is caused by hypoproteinemia due to massive urinary loss of large proteins, particularly albumin, leading to hypoalbuminemia and ascites. Lu/BCAM deficiency significantly prevented both the incidence and severity of ascites (not shown) as well as the renal dysfunction reflected by albuminuria (Figure 1a) and blood urea nitrogen and serum creatinine concentrations (Figure 1b and c), which were normal in Lu−/− mice.
Lu/BCAM expression favors glomerular fibrin and platelet deposition

At sites of glomerular damage, the primary hemostatic reaction involves platelet and fibrin deposition. At these sites, circulating leukocytes marginate and become activated. Furthermore, glomerular fibrin deposition is important in the pathogenesis of renal failure and crescent formation in GN.18 In addition, adhered platelets can support leukocyte localization.19,20 Remarkably, although Lu+/+ nephritic kidney displayed prominent fibrin immunoreactivity in 80% of glomeruli after 3 weeks (Figure 6), Lu/BCAM-deficient mice exhibited fewer fibrin and platelet deposition in glomeruli than their WT counterparts 21 days after NTS challenge (Figures 6 and 7). In contrast, glomeruli from Lu−/− mice exhibited better preservation of podocin expression than that observed in Lu+/+ mice (Figures 6c and 7c).

Lu/BCAM in the immune-inflammation associated with RPGN.

Lu/BCAM deficiency prevents macrophage and T-lymphocyte infiltrates in the kidney

Macrophages and T cells are central players both in our mouse model of NTS-induced GN and in human crescentic RPGN.4,5,21,22 Therefore, to determine whether the differences in immune cell infiltration may explain the augmented glomerular and interstitial damage of the WT group to NTS, the kidneys were stained for the immune cell markers CD3 for T lymphocytes (Figure 8a and b) and F4/80 for resident macrophages (Figure 8c and d). CD3-positive lymphocytes were present around the glomeruli and throughout the renal interstitium in the NTS-infused WT group, but not in the Lu−/− group (P<0.05; Figure 8b). Lu/BCAM-deficient mice displayed fewer F4/80+ macrophages in the kidney cortex and around glomeruli than their WT counterparts on day 21 after NTS injection (P<0.01; Figure 8d). A similar picture was observed when using other macrophage markers such as CD11b, CD68, and Mac-3 (Figure 9 and Figure 10), although at least clearly two distinct populations of myeloid cells were recruited in Lu+/+ nephritic kidneys but not in Lu−/− kidneys. F4/80- and CD11b-positive cells surrounded parietal basal membrane of diseased glomeruli and infiltrated the kidney interstitium. By contrast, staining for CD68 and Mac-3 recognized a population of not only interstitial but also intraglomerular cells (Figures 9 and 10). Mac-3-positive cells were prominently a part of crescentic structures (Figure 9c, detail). Again, Lu/BCAM-deficient animals displayed significantly fewer infiltrates of Mac-3-expressing cells in glomeruli (Figure 10c). Although not fully co-localized, Mac-3 expression and fibrin deposition in glomeruli were highly associated, irrespective of genotype (Supplementary Figure S2). Thus, Lu/BCAM deficiency on renal endothelial
cells leads to marked diminution of the accumulation of macrophages and T lymphocytes in the kidney despite severe stimulus for experimental RPGN. In blood, Lu⁺/⁺ animals displayed lymphopenia and granulopenia after 2 weeks of experimental RPGN, whereas no such alterations were observed in Lu⁻/⁻ animals (Table 1).

Glomerular protection conferred by Lu/BCAM deficiency is confirmed in congenic C57Bl6/J mice

To exclude genetic differences beside the Lu gene as an explanation for the observed differences between Lu⁻/⁻ and WT Lu⁺/⁺ littermates of mixed 129/Ola-C57Bl6/J background, we applied the same passive anti-GBM nephritis protocol to induce RPGN in Lu⁻/⁻ and Lu⁺/⁺ of congenic C57Bl6/J background. Again, Lu/BCAM deficiency blunted part of the increase in albumin urinary excretion and prevented renal failure as assessed by blood urea nitrogen level (Supplementary Figure S3 online). Accordingly, Lu⁻/⁻ animals displayed fewer crescentic glomeruli and lower fibrin and platelet deposition and glomerular infiltrates with Mac-3-expressing cells (Supplementary Figure S4 online).

Lu/BCAM deficiency prevents increase in circulating classical monocyte Ly6Chi subset and kidney entry of both the classical and nonclassical monocyte subsets

Mouse monocytes comprise at least two phenotypically distinct subsets: Ly6Chi 7/4hi CCR2⁺ CX3CR1lo and Ly6Clo 7/4lo CCR2⁻ CX3CR1hi monocytes. 'Inflammatory'

Figure 3 Deletion of Lu gene prevents fatal glomerular destruction. Photomicrographs illustrating Masson trichrome staining of kidney sections on different days after NTS in Lu⁺/⁺ (a–f) and Lu⁻/⁻ (g–l) mice. In unchallenged animals (controls, CTR) and on day 4 no significant damage was observed in both groups, but on day 21 Lu⁻/⁻ NTS-treated mice displayed fewer and less severe glomerular and tubulo-interstitial lesions than their Lu⁺/⁺ counterparts that displayed 3.5 times as many crescents in glomeruli and more tubules with proteinaceous casts (a–c and g–l; bar = 80 μm); (d–f and j–l; bar = 20 μm). (m and n) Glomerular damage assessed by histopathological evaluation of total crescent formation (m) and fibrocellular crescent formation (n) on day 21 after NTS infusion in NTS-injected Lu⁺/⁺ and Lu⁻/⁻ mice. **P < 0.01 and ***P < 0.001 versus Lu⁺/⁺ NTS-treated mice. (o and p) Ultrastructural analysis of podocytes and the glomerular basement membrane by transmission electron microscopy (TEM) in NTS-treated Lu⁺/⁺ glomeruli (o) with representative effacement of the foot processes (thick arrows) and focal thickening of the GBM (thin arrow). Such features were limited in Lu⁻/⁻ glomeruli (p). Bars = 0.05 μm. (q) Representative photomicrographs illustrating the progressive loss of WT-1 expression in Lu⁻/⁻ glomeruli (p). Bars = 0.05 μm. (r) Average number of WT-1-positive cells per glomerular section on day 21. *P < 0.05 versus Lu⁻/⁻ on day 21, **P < 0.01 vs. respective group on day 0. Data are means ± sem. NTS, nephrotoxic serum.
Ly6Chi monocytes rapidly enter sites of inflammation, whereas ‘Resident’ Ly6Clo monocytes enter lymphoid and non-lymphoid organs under homeostatic conditions and patrol across the vascular endothelium.\textsuperscript{23} The specific role of each lymphoid organs under homeostatic conditions and patrol across the vascular endothelium.\textsuperscript{23} The specific role of each subset of monocytes in blood and kidneys. In kidneys, \(Lu^-/-\) displayed fewer monocyte infiltrates of both subsets (\(36.5 \pm 7.2\%\) and \(29.6 \pm 5.8\%\) for \(Ly6Chi\) and \(Ly6Clo\), respectively, \(P<0.05\) vs. \(Lu^+/+\) group), with no significant change in subsets ratio. \(Lu^+/+\) mice displayed higher \(Ly6Chi\) and \(Ly6Clo\) populations ratio in blood on day 8 as compared with \(Lu^-/-\) animals \(P<0.05\) (Supplementary Figure S5 online).

**Adhesion of peripheral human leukocytes to Lu/BCAM-Fc under flow conditions**

To determine whether Lu/BCAM glycoprotein could interact with integrin \(\alpha 4\beta 1\), we performed flow adhesion assays of human PBMCs using Lu/BCAM-Fc protein coated on plastic microchannels. PBMCs, primarily monocytes, adhered to coated Lu/BCAM-Fc in the presence of \(Ca^2+\) and \(Mg^2+\) even in high shear stress conditions (4 dynes/cm\(^2\)) (Figure 11a). Preincubation of freshly isolated PBMCs with recombinant Lu-Fc protein significantly reduced leukocyte adhesion as compared with cells incubated with control Fc fragment. As a positive control, leukocytes adhered to VCAM-1-Fc (Vascular Cell Adhesion Molecule-1), a known ligand for integrin \(\alpha 4\beta 1\). Under flow conditions, human monocytes expressing integrin \(\alpha 4\beta 1\) adhered specifically to immobilized Lu/BCAM-Fc proteins. Cell adhesion to Lu/BCAM-Fc was inhibited by blocking monoclonal antibodies anti-\(\alpha 4\) integrin (Figure 11b) through a large range of physiological shear stress (analysis of variance, ANOVA: \(P<0.001\) vs. adhesion after incubation with isotype-matched control antibody).

**DISCUSSION**

Necrotizing crescentic RPGN is an inflammatory process directed by cognate immune responses, which results in severe glomerular injury and renal failure.\textsuperscript{1} In the past, the participation of antibody and complement in human RPGN was emphasized, leading to the view that humoral immune effectors, principally immune complexes or anti-glomerular basement membrane (GBM) antibody, were the predominant pathogenic mediators in this severe form of GN. In fact, B cell-deficient mice that have normal cell-mediated immunity but cannot produce Ig develop crescentic anti-GBM GN to the same extent as their normal littermates, confirming that crescent formation can occur completely independent of humoral immune responses.\textsuperscript{24} Thus, the involvement of T cells and macrophages has been recognized, suggesting an additional contribution of cell-mediated immunity.\textsuperscript{1,11,21,25–31} Although numerous studies have examined the role of...
soluble factors in mediating recruitment of lymphocytes and macrophages in the kidney, the mechanisms whereby myeloid cells interact with the renal endothelium in the progression of RPGN are still not fully understood. Considering the constitutive expression of Lu/BCAM on the surface of endothelial cells in the kidney, we postulated that they could be involved in the binding of integrins on myeloid cell, contributing to their reinforced adhesiveness to vascular endothelium. Our hypothesis was supported by the presence of $\alpha_4\beta_1$ consensus-binding motifs in the extracellular domain of Lu/BCAM. Furthermore, endothelial Lu/BCAM was recently shown to be a receptor for the $\alpha_4\beta_1$ integrin in red blood cells (RBCs), thereby promoting adhesion of sickle RBCs to the endothelium.

Regulation of $\alpha_4\beta_1$ activation in vivo may promote leukocyte adhesion. Likewise, a pathological role for the integrin receptor Lu/BCAM is being realized in SS RBC aggregation to monocytes in sickle cell disease. Erythroid Lu/BCAM proteins are implicated in these aggregates through their interaction with $\alpha_4\beta_1$ integrin on peripheral blood mononuclear cells. In the context of RPGN, monocyte adhesion to the $\alpha_4\beta_1$ ligands thrombospondin, VCAM-1, and fibronectin or other factors in the vasculature may markedly impact glomerular damage. Because mice erythrocytes do not express Lu/BCAM, we hypothesize that Lu/BCAM promotes glomerular inflammation and demotion through its endothelial expression. Therefore, to directly examine the actions of Lu/BCAM, a VLA-4/ $\alpha_4\beta_1$ integrin receptor on the endothelium in the pathogenesis of RPGN, we used $\text{Lu}^+/-$ and $\text{Lu}^+/+$ littermate mice and then infused these mice with anti-GBM NTS. We observed that genetic deficiency in Lu/BCAM prevents renal injury (albuminuria, glomerular fibrinoid necrosis, and crescent formation) along with reduced kidney infiltrates of T lymphocytes and macrophages. In many kidney diseases, the density of mononuclear cell accumulation correlates with the degree of renal dysfunction and is predictive of disease progression. Previous examination of the expression of VCMA-1 in experimental RPGN has been performed during the very early stages of nephritis. Infusion of antibody to the

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**Figure 5** Immunostaining for Lu/BCAM in normal and RPGN mouse kidneys, showing strong expression in the glomerular endothelium. (a) Representative photomicrographs after immunofluorescence staining for Lu/BCAM and nidogen in healthy control condition and on day 4 and 21 of NTS-induced RPGN. Bar = 20 μm. Note that prominent Lu/BCAM expression is endocapillary. Some fainter mesangial expression is also observed. (b) Immunochemistry confirmed prominent endothelial pattern of Lu/BCAM expression that is consistent in healthy and diseased glomeruli. Representative photomicrograph after immunofluorescence staining (c) and immunohistochemistry (d) for Lu/BCAM in $\text{Lu}^+/-$ kidney cortex. (e) Western blot analysis of Lu/BCAM and tubulin ratio in renal cortex from wild-type $\text{Lu}^+/-$ mice treated or not by NTS ($n = 5$ per group) and $\text{Lu}^+/-$ control cortex ($n = 1$). Data are means ± s.e.m. NTS, nephrotoxic serum; RPGN, rapidly progressive glomerulonephritis.
Figure 6 | Deletion of Lu gene prevents glomerular deposition of fibrin and loss of podocin expression. (a) Immunohistochemical detection of fibrin formation in kidney cryosections from Lu+/+ and Lu−/− mice without (day 0) or with NTS-induced RPGN (on day 4 and day 21). Bar = 50 μm. (b) Comparative evolution of immunoreactive fibrin deposition in glomeruli from NTS-challenged Lu+/+ and Lu−/− animals. Semiquantitative scoring showed significantly increased fibrin deposition score in nephritic cortex from Lu+/+ mice as compared with Lu−/− mice on day 21. *P<0.05; **P<0.01. (c) Representative immunofluorescence photomicrographs after double staining for podocin (red) and fibrin (green) in kidney cryosections from Lu+/+ and Lu−/− mice without (day 0) or with NTS-induced RPGN (on day 4 and day 21). Note that podocin expression was markedly more blunted in Lu+/+ than in Lu−/− on day 21. Bar = 80 μm. NTS, nephrotoxic serum; RPGN, rapidly progressive glomerulonephritis.

Figure 7 | Deletion of Lu gene prevents glomerular deposition of platelets and loss of podocin expression. (a) Comparative evolution of thrombocyte deposition in glomeruli from NTS-challenged Lu+/+ and Lu−/− animals. Bar = 50 μm. (b) Image analysis showed significantly increased fibrin deposition score in nephritic cortex from Lu+/+ mice as compared with Lu−/− mice on day 21. **P<0.01 vs. Lu−/− condition on day 21. (c) Double immunofluorescence staining for podocin (red) and platelets (green) in kidney cryosections from Lu+/+ and Lu−/− mice without (day 0) or with NTS-induced RPGN (on day 4 and day 21). Note that podocin expression was markedly more blunted in Lu+/+ than in Lu−/− on day 21. Bar = 80 μm. NTS, nephrotoxic serum; RPGN, rapidly progressive glomerulonephritis.
glomerular basement membrane in rats induced glomerular
upregulation of ICAM-1, ELAM-1, and VCAM-1 in a TNF-
alpha-dependent manner. Anti-VLA-4 mAb modestly
inhibited PMN migration and had no significant effect on
proteinuria. We did not observe any change in neutrophil
infiltrates in Lu/BCAM-deficient nephritic animals. However,
our study may have missed transient action on neutrophils as
studies of similar models of acute GN have revealed that
neutrophil recruitment to the glomerulus follows a rapid, yet
restricted, time course. Neutrophils accumulate in glomeruli
within hours of induction of inflammation, but are not
present 4-8 h later. More efficient than VLA-4 antagonism,
inhibition of Mac-1 was found to alleviate the GBM
antibody– associated increase in glomerular injury, demon-
strating an essential role for the elevation in neutrophil dwell
time in mediating glomerular injury, demonstrat-
ing an essential role for the elevation in neutrophil dwell
time in mediatiing glomerular injury. Thus, this latter step
is supposed to be independent of Lu/BCAM–VLA-4
interaction. Interestingly, a subsequent work demonstrated
that mAbs to VCAM-1 had no significant effect on
glomerular damage in a rat model of NTS-induced RPGN,
whereas mAbs to VLA-4 significantly attenuated renal
injury. This study concluded that α4β1 integrin/VLA-4 is
an important mediator of glomerular injury, operating after
transendothelial leukocyte migration, and that kidney
endothelial VCAM-1 has little part in early leukocyte influx
to glomeruli. In fact, the marked protection conferred by
Lu/BCAM deficiency suggests that this endothelial
glycoprotein could be an alternative ligand promoting
inflammation of the kidney vasculature.

To examine the type of macrophage recruited within
glomeruli, we performed immunohistochemistry for several
markers of macrophage, including CD68, Mac-3, CD11b,
and F4/80. Macrosialin/CD68 may not be a specific
marker for macrophages but rather an antigen indicative
of phagocytosis. It is expressed on tissue macrophages,
Langerhans cells, and at low levels on dendritic cells.
Nevertheless, in crescentic RPGN, this marker was
markedly found to be expressed by virtually all CD45
monocytes/macrophages, whereas F4/80-expressing cells
represented a minority of CD45+ monocytes. Although
immunostaining techniques failed to provide evidence of
monocyte intraglomerular influx on day 4 when recruitment
of the innate immunity is intense, significant alleviation
of platelet and fibrin deposition at a later time point in
glomeruli from NTS-challenged Lu/BCAM-deficient mice
suggests that the Lu/BCAM molecule has a major
pathophysiological role in the process of microvascular
injury leading to RPGN. Accordingly, early concomitant
increase in renal vascular resistance index measured in
WT animals was alleviated in Lu/BCAM-deficient mice.
Macrophages have been shown to be responsible for
glomerular fibrin deposition in anti-GBM antibody–
induced GN. Likewise, significant fibrin deposition was
observed to be associated with glomerular macrophage
accumulation in our passive model of RPGN. Glomerular
tissue factor gene and protein expression were strikingly
increased in human crescentic RPGN, in particular within
the crescents and in the mesangial area in the proximity of

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Figure 8 | Lu/BCAM deficiency prevents T lymphocyte- and F4/80-positive macrophage infiltrates. (a) Immunostaining for CD3+
lymphocytes and (b) quantification by image analysis of CD3-positive infiltrates in renal cortex from NTS-injected mice (on day 0, 4, and 21).
(c) Immunostaining for F4/80+ macrophages and (d) quantification by image analysis of F4/80-positive infiltrates in renal cortex
from NTS-injected mice. Data are means ± s.e.m. (n = 12 per group). *P < 0.05 and **P < 0.01 versus baseline on day 0 and versus Lu−/−
on day 21. Bar = 80 μm. NTS, nephrotoxic serum.
monocytes. Furthermore, fibrin deposition also translates thrombin activity that has been shown to aggravate RPGN per se. Interestingly, $Lu^{+/−}$ animals displayed significantly fewer intraglomerular CD68+ and Mac-3+ cells and much fewer periglomerular and interstitial CD11b+ and F4/80+...
Erythrocytes (x10⁶/ml) 8.85

Further studies will be required to determine whether Lu/BCAM expression may promote preferential recruitment of specific monocyte subsets at different time points. Deciphering whether Lu/BCAM had an impact on the later F4/80 high population that originates from the resident population derived from the yolk sac and is not recruited from the circulation as reported recently by Geissmann and coll.⁴⁶ will also require further studies. As F4/80+ macrophages develop and stay in close association with epithelial structures and are associated with capillaries within the microcirculation,⁴⁷ they may be secondarily responsive to Lu/BCAM-dependent capillary and epithelial injury. Similarly, alleviation of endothelial and tissue injury in Lu/BCAM-deficient kidneys may have had an impact on the VLA-4 independent recruitment of T lymphocytes in the interstitium. Overall, Lu/BCAM deficiency was sufficient to prevent severe glomerular damage and renal failure in mice. Furthermore, we describe a novel pathophysiological interaction between leukocyte integrin α4β1 and endothelial Lu/BCAM proteins. Integrin α4β1 is known to have high affinity for VCAM-1, which is only expressed on the surface of activated endothelial cells. As Lu/BCAM proteins are expressed on both resting and activated cells, we hypothesize that they could contribute to abnormal adhesion of myeloid cells to resting endothelium either by tethering myeloid cells or by reinforcing their adhesion along with other described interactions to the glomerular endothelium. These results indicate for the first time that Lu/BCAM exhibits a broad pro-nephritic and pro-inflammatory action in the course of experimental RPGN and may represent a future therapeutic target in inflammatory diseases.

### MATERIALS AND METHODS

#### Experimental animal models

Homozygous Lu−/− and their littermates WT mice Lu+/+ of hybrid 129/Ola-C57BL/6j genetic background were generated by heterozygous mating at the Institut Clinique de la Souris (ICS, Strasbourg, France) as previously described.¹⁴ We used male mice between 12 and 16 weeks of age. After eight generations of backcrossing, by using marker-assisted technologies on a C57Bl6/J background (N8), 10 homozygous Lu−/− and 10 congenic littermate Lu+/+ mice were used in another set of experiments.

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**Table 1** Hematological parameters indicate that Lu+/+ animals displayed lymphopenia and granulopenia after 2 weeks of experimental RPGN; such alterations were not observed in Lu−/− animals (P<0.05 vs. Lu+/+ at baseline).

| Hematological parameters | Baseline | Day 14 post NTS |
|--------------------------|----------|----------------|
| Erythrocytes (x10⁶/ml)   | 8.85 ± 0.34 | 5.91 ± 0.77   |
| White blood cells (x10⁶/ml) | 8.35 ± 0.66 | 5.04 ± 0.76* |
| Monocyte (x10⁶/ml)       | 0.62 ± 0.05 | 0.59 ± 0.15   |
| Granulocytes (x10⁶/ml)   | 0.46 ± 0.06 | 0.25 ± 0.07* |
| Lymphocytes (x10⁶/ml)    | 7.14 ± 0.43 | 3.83 ± 0.58* |
| Platelets (x10⁶/ml)      | 962.3 ± 65.6 | 745.80 ± 82.99 |

**Abbreviations:** Lu, Lutheran; NTS, nephrotoxic serum.

*P<0.05 vs. Lu+/+ at baseline.

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**Figure 11 | Lu/BCAM interaction with β1 integrin mediates monocyte adhesion in shear stress conditions.**

(a) Human monocytes adhered to coated Lu/BCAM-Fc in the presence of Ca²⁺ and Mg²⁺ even in high shear stress conditions. As a positive control, leukocytes adhere to VCAM-1-Fc protein, a known ligand for integrin α4β1. White bars: adhesion to immobilized Fc proteins; black bars: adhesion to immobilized Lu/BCAM-Fc proteins; gray bars: adhesion to immobilized VCAM-1-Fc proteins. (b) Part of adhesion to Lu/BCAM-Fc but not to Fc alone was blunted when leukocytes were preincubated with an anti-β1 antibody, suggesting specific interaction (analysis of variance, ANOVA: P<0.001 versus adhesion after incubation with isotype-matched control antibody).
Passive anti-GBM nephritis protocol in mice has been popularized and induced as described previously. Anti-GBM nephrotoxic serum was injected through the retro-orbital venous sinus at 6 μg/g body weight for 3 days continuously as previously described. We collected urine and blood on day 4, 7, 10, 14, and 21. On day 14 (C57BL/6j mice) or on day 21 (129/Ola-C57BL/6j mice), animals were euthanized. Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Institut National de la Santé et de la Recherche Médicale (INSERM).

Physiological parameter measurements
Cardiac and renal hemodynamic studies were assessed by echocardiography (Vivid 7, GE Medical Systems ultrasound, Velizy-Villacoublay, France) equipped with a 12-MHz linear transducer (121). The measurement procedure was described previously.

Biochemical analysis
Urinary concentrations of albumin were normalized by creatinine concentrations in individual samples. Albumin excretion was expressed as grams per mol of creatinine. Serum creatinine and urea concentrations were expressed as μmol/l and mmol/l, respectively. Analyses were performed by the IBISA core facility at Institut Claude Bernard, Paris. Assessment of creatinine levels was performed using a mouse creatinine ELISA kit (Cusabio Biotech, Wuhan, P.R. China).

Renal histology and morphological evaluation
The kidneys were fixed in formalin 10% v/v (Labonord, Templemars, France) for at least 24h, dehydrated, and sections of 3 μm were realized and stained with Masson’s trichrome. Glomerular crescents were defined as glomeruli containing two or more layers of cells in Bowman’s space and scored as severe glomerular involvement when present. The crescent glomerulus ratio was calculated by counting injury and normal glomeruli (60–70 glomeruli for each section). The proportion of pathological glomeruli was calculated by crescents were defined as glomeruli containing two or more layers of cells in Bowman’s space. The crescent glomerulus ratio was calculated by counting injury and normal glomeruli (60–70 glomeruli for each section). The proportion of pathological glomeruli was calculated by crescents were defined as glomeruli containing two or more layers of cells in Bowman’s space. The crescent glomerulus ratio was calculated by counting injury and normal glomeruli (60–70 glomeruli for each section).

Transmission electron microscopy
The preparation of the kidneys for transmission electron microscopy was performed according to procedures as previously described.

Western blotting
After 6 days of culture, the cell outgrowths from decapsulated glomeruli were carefully separated and placed in 200 μl Phospho-SafeExtraction Reagent (Merck Biochemistry, Fontenay-sous-Bois, France) on ice, and lyzed with a sonicator. Total protein concentration was measured with Novagen BCA Protein Assay Kit (Merck Biochemistry). Protein amounting to 20 μg was subjected to electrophoresis in Criterion XT precast gel (4–12% Bis-tris, Bio-Rad). The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Marnes-la-Croquette, France). After washing three times with phosphate-buffered saline Tween-20 (PBST), the membrane was blocked for 1 hour at room temperature with 5% milk (PBST) and then incubated with rabbit polyclonal anti-murine (m) Lu antibody 455 (1:15,000, kindly provided by Yves Colin-Aronovicz, Inserm, INTS), rabbit polyclonal to NPHS2 antibody (podocin) (1:2000, Abcam, Paris, France), rat monoclonal anti-tubulin antibody (1:5000, Abcam) in PBST overnight at 4°C. After washing three times with PBST, the membrane was incubated with ECL horseradish peroxidase-linked whole antibody (1:5000, Amersham Biosciences, Glattbrugg, Switzerland) for 60 min at room temperature. The detection of specific signals was performed using the Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) with an LAS-4000 imaging system (Fuji, Stamford, CT) used to reveal the bands; densitometric analysis was used for quantification.

Immunofluorescence detection
For indirect immunofluorescence staining, kidney sections were embedded in OCT (Sakura, Alphen aan den Rijn, The Netherlands) compound frozen in isopentane cooled in liquid nitrogen. The frozen sections (5 μm) were fixed with cold acetone for 10 min and then washed three times in PBS, blocked in 5% BSA in PBS for 1 h at room temperature, and then incubated with primary antibodies diluted in 1% BSA-PBS overnight at 4°C: rabbit polyclonal anti-murine (m) Lu antibody 455 (1:1500, kindly provided by Yves Colin-Aronovicz), rabbit polyclonal to NPHS2 (podocin) antibody (1:2000, Abcam). After washing the unbound antibodies three times in PBS, Cy3-conjugated secondary antibodies (Chemicon International, Millipore SAS, Molsheim, France) were used at a 1:1000 dilution for 1 h at room temperature. After three washes, the cells were treated with the second primary antibody FITC-conjugated polyclonal goat antiserum to mouse fibrinogen (Nordic Immunological Laboratories, Susteren, The Netherlands, 1:10) or anti-thrombocyte-FITC Ab (Cedarlane, TEBU-BIO, Le Perray en Yvelines, France, 1:200) diluted in 1% BSA-PBS and incubated for 30 min at room temperature for double immunofluorescence staining. The slides were mounted with Fluoprep mounting medium, and analyzed using the Zeiss 2 fluorescent microscope, AxioCam HRC camera, and the Axiosview 4.3 software (Carl Zeiss S.A.S., Marly le Roi, France).

To detect Lu expression in podocyte, cells were cultured on μ-slides (Ibidi). After a 15 min fixation with 2% paraformaldehyde, cells were washed twice with PBS and incubated for 5 min with 0.2% triton (Sigma-Aldrich Chimie S.À.R.L., Saint-Quentin Fallavier, France). BSA-PBS. The same protocol for frozen kidney sections was used for cultured cells.

To assess podocyte differentiation and leukocyte infiltration in the kidneys, 4 μm paraffin-embedded kidney sections were cut, dehydrated, and treated with target retrieval solution (Dako, Dako France S.A.S., Les Ulis, France) for 20 min at 98°C in a water bath to unmask specific antigens. After cooling, the sections were incubated with peroxidase blocking reagent (Dako) for 20 min and then in 10% goat serum-1% BSA in PBS for 1 h. The sections were incubated overnight at 4°C with anti-Wilms tumor-1 (WT-1) (1:500, Santa Cruz Biotechnology, Heidelberg, Germany), anti-CD3 (1:100, Dako), anti-F4/80 (1:500, Serotec, Oxford, UK), anti-tubulin antibody (1:5000, Abcam) in PBST overnight at 4°C. After washing three times with PBST, the membrane was incubated with ECL horseradish peroxidase-linked whole antibody (1:5000, Amersham Biosciences, Glattbrugg, Switzerland) for 60 min at room temperature. The detection of specific signals was performed using the Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) with an LAS-4000 imaging system (Fuji, Stamford, CT) used to reveal the bands; densitometric analysis was used for quantification.

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MAC-3 (1:50, BD Pharmingen, Le Pont de Clai, France), CD11b (1:100, Abcam), CD68 (1:100, Sorotec), or fibrinogen (1:500, Dako) antibody. Histofine reagents (Nichirei Biosciences, Tokyo, Japan) were used and signal was detected in the presence of AEC substrate chromogen (Dako). Sections were counterstained with hematoxylin (Vector Laboratories, Eurobio, Courtaboeuf, France). The number of WT-1-positive cells was counted in 30 glomeruli for each kidney sample. The leukocyte infiltration quantification was accessed using Image J software (NIH). Positively stained Mac-3 cells per 50 glomeruli of each mouse were counted and expressed per glomerular cross-section. Glomerular fibrin deposition was assessed by studying at least 50 glomeruli per mouse with the help of polyclonal rabbit antihuman fibrinogen Ab (1:500, Dako) according to a previously published protocol. The degree of glomerular fibrin deposition was scored semiquantitatively (on a scale of 0–3) as follows: 0, no fibrin deposition; 1, fibrin occupying up to one-third of the glomerular cross-sectional area; 2, fibrin occupying one-third to two-thirds of the glomerulus; and 3, greater than two-thirds of the glomerular cross-section covered by fibrin.

For sheep IgG and mouse IgG staining, 5 μm kidney cryosections were fixed in cold acetone for 10 min, then washed in PBS and blocked with 5% BSA-PBS for 1 h at room temperature. Kidney sections were then stained with Alexa Fluor 594 anti-sheep IgG, Alexa Fluor 594 anti-mouse IgG (Invitrogen, Life Technologies SAS, Saint Aubin, France, 1:500). After washing, the slides were mounted with Fluorprep (Biomérieux, Craponne, France) and analyzed using the Zeiss 2 fluorescent microscope, AxioCam HRCamera and the Axiovision 4.3 software.

For double immunofluorescent staining, frozen kidney sections (5 μm) were fixed with cold acetone for 10 min and then washed three times in PBS, blocked in 5% BSA-PBS in PBS for 1 h at room temperature, and then incubated overnight at 4°C with single or mixed primary antibodies diluted in 1% BSA-PBS: rabbit polyclonal anti-murine (m) Lu antibody 455 (1:1500, kindly provided by Yves Colin-Aronovicz, Inserm, INTS) and rat anti-nidogen antibody (1:500, Chemicon International) or rabbit polyclonal to NPHS2 antibody (podocin) (1:2000, Abcam). After washing three times, unbound antibodies—secondary FITC- and Cy3-conjugated antibodies (Chemicon International)—were added at a 1:500 and 1:1000 dilution for 1 h at room temperature.

Direct immunofluorescence microscopy was performed with goat IgG against mouse fibrinogen, conjugated with FITC (Nordic Immunology, 1:10) and FITC-conjugated anti-mouse thrombocyte rat IgM (Cedarlane, 1:200) diluted in 1% BSA-PBS.

To examine the localization of fibrin and macrophages in glomeruli, double fluorescent labeling was performed on 4 μm kidney paraffin-embedded sections. Macrophage were detected with anti-Mac-3 Ab (1:50, BD Pharmingen) and fibrin was detected with polyclonal rabbit antihuman fibrinogen Ab (1:500, Dako). The secondary antibodies used were donkey anti-rabbit conjugated with FITC and donkey anti-rat conjugated with Cy3 (both 1:500, Jackson ImmunoResearch Europe, Suffolk, UK). The slides were mounted with Fluorprep (Biomérieux), and analyzed using the Zeiss 2 fluorescent microscope, AxioCam HRCamera and the Axiovision 4.3 software.

**Measurement of the murine anti-sheep IgG immune response**

Serum mouse anti-sheep IgG levels were measured by enzyme-linked immunosorbent assay13 by using plates (Nunc Maxisisorb; Fisher Scientific, Illkirch, France) coated overnight at 4°C with 100 μg/ml of sheep IgG (Sigma). After blocking with 3% bovine serum albumin, diluted serum samples were incubated for 1 h at 37°C. For each experiment a range of serum dilutions was tested with a standard curve of a known positive sample. After washing, peroxidase-coupled anti-mouse IgG (Fc-specific) (Rockland Immunochemicals, Gilbertsville, PA) and peroxidase substrate were added.

**Flow cytometry analysis**

Kidney cortex were weighed, minced, and digested in 450 U/ml Collagenase I, 125 U/ml Collagenase XI, 60 U/ml DNaseI, and 60 U/ml hyaluronidase (Sigma-Aldrich) for 1 h at 37°C. Cells were also isolated from venous blood. All cell suspensions were layered on Histopaque 1083 (Sigma-Aldrich) for gradient density centrifugation. The mononuclear cell fraction was counted and stained using anti-mouse CD11b-PercPCy5.5, Ly6G-PE, CD115-PE (BD Biosciences, Le Pont de Clai), Ly6C-FITC or Ly6C-APC, or the corresponding isotypes, and analyzed on a LSRII Flow Cytometer (BD Biosciences) with the FacsDiVa software (BD Biosciences). CD11bhiLy6G−/NK1.1−/CD115+ cells were considered to be monocytes, and subset discrimination was made upon Ly6C expression.

**Adhesion assay under flow conditions**

All adhesion assays were performed with 5 ml freshly drawn EDTA-anticoagulated venous blood from three healthy donors after informed consent was obtained in accordance with the Declaration of Helsinki. The study has been approved by the Scientific Committee of the Institut National de la Transfusion Sanguine. Lu-Fc was also purchased with human VCAM-1-Fc from R&D Systems Europe (Lille, France).

Leukocyte adhesion to microslides coated with fusion protein was measured under flow conditions with a capillary flow chamber. Fc, Lu/BCAM-Fc, VCAM-Fc were coated into capillaries (microslides: Sigma-Aldrich; internal channel dimensions: length 50 mm, width 5 mm, height 0.2 mm) at 4°C overnight and microslides were mounted as described. The leukocytes were separated from the peripheral blood of healthy donors using Histopaque 1119 (Sigma-Aldrich). After hypotonic RBC lysis, 5×10^6 cells/ml were incubated with 2 μg/ml murine monoclonal anti-human IgG (Immunotech, Marseille, France, clone 8a4) for 30 min at 37°C in order to block Fc receptors. Washed cells were suspended in Hanks buffer (10 mM HEPES, 1 mM MgCl2, 1 mM CaCl2) with 5%BSA and then incubated with 1 μM MnCl2 for 15 min at 37°C. Stimulated cells were injected for 10 min at shear stress of 0.2 dyne/cm², and 5-minute washouts were carried out with Hanks buffer at 0.4, 1, 2, and 4 dyne/cm². After each washing step, adherent cells were quantified in five representative areas along the centerline of microslide with the help of the AxioObserver Z1 microscope and AxioVision 4 analysis software (Carl Zeiss). Adhesion was totally abolished when 1 mM EDTA was added, indicating that the interaction is dependent on divalent cations as expected with integrins. For inhibition assays, cells were preincubated for 20 min at room temperature with 10μg/ml anti-CD29/Integrin B1-blocking antibody (clone Mab13) or isotype-matched control antibody (BD Pharmingen). Each adhesion experiment was performed three times.

**Statistical analysis**

Data are expressed as means ± SEM. The two-tailed Mann–Whitney test, the Wilcoxon signed-rank test and the Student t-test were used as appropriate. For experiments with more than two subgroups,
the nonparametric Kruskal–Wallis analysis of variance (ANOVA) followed by Dunn's multiple comparison test was used. Values of \( P<0.05 \) were considered significant. All analyses were performed using Prism version 5.04 for Windows, (GraphPad Software, La Jolla, CA).

**DISCLOSURE**

All the authors declared no competing interests.

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