# Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis

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## Abstract

Pauci-immune focal necrotizing glomerulonephritis (FNGN) is a severe inflammatory disease associated with autoantibodies to neutrophil cytoplasmic antigens (ANCA). Here we characterize autoantibodies to lysosomal membrane protein-2 (LAMP-2) and show that they are a new ANCA subtype present in almost all individuals with FNGN. Consequently, its prevalence is nearly twice that of the classical ANCAs that recognize myeloperoxidase or proteinase-3. Furthermore, antibodies to LAMP-2 cause pauci-immune FNGN when injected into rats, and a monoclonal antibody to human LAMP-2 (H4B4) induces apoptosis of human microvascular endothelium *in vitro*. The autoantibodies in individuals with pauci-immune FNGN commonly recognize a human LAMP-2 epitope (designated $P_{41-49}$) with 100% homology to the bacterial adhesin FimH, with which they

cross-react. Rats immunized with FimH develop pauci-immune FNGN and also develop antibodies to rat and human LAMP-2. Finally, we show that infections with fimbriated pathogens are common before the onset of FNGN. Thus, FimH-triggered autoimmunity to LAMP-2 provides a previously undescribed clinically relevant molecular mechanism for the development of pauci-immune FNGN.

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Pauci-immune crescentic FNGN is an acute inflammatory disease that results in rapid, irreversible kidney failure, typically in the context of a systemic small vessel vasculitis, such as microscopic polyangiitis or Wegener's granulomatosis[1,2]. Injury is caused by neutrophils and macrophages that localize in glomerular capillaries without detectable immune deposits. Despite this, pauci-immune FNGN is thought to be an autoimmune disease because of its almost invariable association with ANCA. ANCA were originally described in 1982 (ref. [3]) and have proved invaluable for diagnosis and monitoring of disease activity[4].

The pathogenicity of ANCA has been examined extensively ever since the soluble lysosomal enzymes myeloperoxidase and proteinase-3 were identified as targets[2,5]. Antibodies to these enzymes activate primed neutrophils and cause neutrophil-dependent endothelial injury *in vitro*[2], and administration of antibodies to myeloperoxidase provokes FNGN in rodents[6], especially when injected together with lipopolysaccharide (LPS)[7]; antibodies to proteinase-3 are less effective[8,9]. However, individuals with FNGN have other autoantibodies as well—for example, autoantibodies to the extracellular domain of human LAMP-2 (ref. [10])—but their role in pathogenesis role is unknown.

LAMP-2 is a heavily glycosylated type 1 membrane protein[11]. In humans, three splice variants are known (LAMP-2A, LAMP-2B and LAMP-2C), all with identical extracellular domains[12]. LAMP-2 has established roles in adhesion[13] and cellular homeostasis, including autophagocytosis[14] and antigen presentation[15]. In neutrophils, LAMP-2 is integrated into the membranes of myeloperoxidase- and proteinase-3–containing intracellular vesicles, and thus autoantibodies to human LAMP-2 give positive results in fluorescence ANCA assays. In contrast to myeloperoxidase and proteinase-3, human LAMP-2 shuttles between lysosomes and the cell membrane[16], is abundant on neutrophil and endothelial cell surfaces and is thus directly accessible to circulating antibodies.

It is not known what provokes autoantibody synthesis in pauci-immune FNGN, but bacterial infections may be involved, as first postulated by Wegener in his original 1936 report of granulomatous 'septic' vasculitis[17], an idea subsequently confirmed by others[18,19]. Three explanations have been invoked[20,21]: infection-induced systemic activation of granulocytes, induction of autoantibodies by bacterial superantigens and molecular mimicry between microbial antigens and host proteins. However, there are no known sequence homologies between bacterial proteins and myeloperoxidase and proteinase-3, except for a complex homology with a complementary sequence of proteinase-3 (ref. [22]). Similar analyses have not been applied to human LAMP-2.

Here we establish that autoantibodies to human LAMP-2 are highly prevalent in pauci-immune FNGN and provide evidence of their pathogenicity by showing that they activate neutrophils and kill human blood microvascular endothelium *in vitro* and cause pauci-immune FNGN when administered to rodents. Unexpectedly, auto-antibodies to LAMP-2 in individuals with FNGN commonly recognize an epitope with considerable homology to the bacterial adhesin FimH and cross-react with it. We therefore determined whether exposure to FimH could induce antibodies to human LAMP-2 and initiate pauci-immune FNGN through molecular mimicry. The results lead us to propose a previously undescribed molecular mechanism both for the induction and development of injury in this human disease.

# RESULTS

## Autoantibodies to human LAMP-2 are common in FNGN

We established the prevalence of autoantibodies to hLAMP-2 in sera from 84 individuals with biopsy-proven active pauci-immune FNGN, either at presentation ($n = 62$) or during relapse ($n = 22$). ANCA were detectable by standard immunofluorescence assays in 80 of them (95%), and ELISA for the canonical ANCA were positive in 70 of them (83%); myeloperoxidase-specific ANCA were found in 38 people, and proteinase-3–specific ANCA were found in 39 people, including seven with antibodies to both antigens. Using a specific ELISA, we detected antibodies to human LAMP-2 in 78 of the 84 (93%) sera (Fig. 1a), and we validated the results by western blotting and indirect immunofluorescence on the *O*-glycosylation deficient CHO cell line ldlD cells stably expressing human LAMP-2 on their surface (Supplementary Fig. 1a online). Notably, the human LAMP-2 ELISA was negative in all but six of the individuals when they were in remission after immunosuppressive therapy. Assays for human LAMP-2 were also negative in 53 healthy controls and in all but 1 of 30 ANCA-negative subjects with other types of glomerulonephritis. We also found human LAMP-2–specific antibodies in eight ANCA-positive individuals with FNGN and antibodies to myeloperoxidase in other contexts, including Goodpasture's disease and systemic lupus erythematosus, whereas assays were only positive in 1 of 20 individuals with localized vasculitis without renal disease, supporting the association of autoantibodies to human LAMP-2 with active inflammation in individuals with pauci-immune FNGN.

Our standard ELISA used recombinant unglycosylated human LAMP-2 expressed in *E. coli* as substrate, so we developed additional ELISAs to test whether autoantibodies from subjects with FNGN also recognized glycosylated mammalian human LAMP-2. These ELISAs used as substrate either glycosylated human LAMP-2 purified from culture supernatants of CHO DG44 cells expressing a soluble extra-cellular domain or glycosylated human LAMP-2 that had been digested with the *N*-glycanase PGNaseF to remove *N*-glycans. The results were compared with those from the standard ELISA with unglycosylated human LAMP-2 prepared in *E. coli* (characterized in Supplementary Fig. 1b). The autoantibodies bound equally well to glycosylated and unglycosylated human LAMP-2, indicating that the epitopes they recognize are not occluded by glycosylation (Fig. 1b). The results were confirmed by indirect immunofluorescence that showed the autoantibodies bound to human LAMP-2 expressed on the surface of ldlD cells before and after removal of *O*- and *N*-glycans (data not shown). Thus, autoantibodies from subjects with FNGN bind epitopes on the protein backbone that remain accessible in fully glycosylated mammalian human LAMP-2 in the plasma membrane.

## Injection of IgG antibodies to human LAMP-2 causes FNGN

To determine whether antibodies to LAMP-2 cause injury *in vivo,* we injected 15 WKY rats intravenously with human LAMP-2–specific rabbit IgG that cross-reacts with rat LAMP-2. All rats developed sustained hematuria quantified by Combur-Test (Roche) according to the manufacturer's instructions: negative at baseline and at 2 h ($n = 2$); negative to trace at 24 h ($n = 4$); 1+ to 2+ at 48 h ($n = 5$) and 2+ to 3+ at 120 h ($n = 4$). The urine protein:creatinine ratio increased 25-fold from $0.017 \pm 0.019$ at baseline to $0.305 \pm 0.098$ and $0.416 \pm 0.14$ at 24 h and 120 h, respectively. The treated rats developed severe renal injury with leukocyte infiltration (Fig. 1c). Rats culled after 24 h had focal capillary necrosis in a mean of 22.2% of glomeruli (range 17–25%). Rats culled later had crescents resulting in a mean of 21% of glomeruli after 48 h (range 16–24%) and 18.5% after 120 h (range 6–20%; Fig. 1d). Injected antibodies were rapidly cleared from the circulation, and only minimal deposition of rabbit IgG was detectable in kidneys of rats killed 2 h after injection, whereas there was none at later time points (Fig. 1e and Supplementary Fig. 1c). Normal control rats ($n = 8$) and rats injected with normal rabbit IgG ($n = 4$) did not develop hematuria, proteinuria (data not shown) or morphological injury

(Fig. 1d, control). Thus, antibodies to LAMP-2 are pathogenic and can cause pauci-immune FNGN.

### Antibodies to LAMP-2 activate neutrophils and endothelium

Because ANCAs specific for myeloperoxidase and proteinase-3 activate primed human neutrophils *in vitro*[23,24], we tested whether antibodies to human LAMP-2 do the same. Incubation with H4B4, a monoclonal antibody to human LAMP-2, induced significantly more neutrophil 'shape change'[25] than a monoclonal antibody to CD4; H4B4 (10 μg ml$^{-1}$) resulted in 20.3% shape changed of neutrophils (range 17.0–22.3%) versus 8.5% (7.1–9.3%) with CD4 ($P < 0.05$). The results with 20 μg ml$^{-1}$ of the antibodies were 43.0% (36.9–49.2%) versus 12.5% (10.2–14.1%) respectively ($P$ 0.05). The results for untreated neutrophils was 8.5% (7.1–9.3%). A monoclonal antibody to proteinase-3 (1F11) also induced neutrophil shape change but to a significantly lesser degree (mean 16.5%, range 13.4–21.1% for 10 μg ml$^{-1}$ and mean 30.6%, range 21.3–36.5% for 20 μg ml$^{-1}$; each with $P < 0.05$ compared to H4B4). These results were confirmed by staining for actin condensation[26] (Fig. 2a–d). H4B4 also induced neutrophil degranulation (Supplementary Fig. 1d). Incubation with 10 μg ml$^{-1}$ H4B4, 1F11 or CD4 released 83% (range 80–85%), 57% (40–90%) or 10% (0–21%) myeloperoxidase, respectively (expressed as a percentage of myeloperoxidase released by treatment with 10 ng ml$^{-1}$ tumor necrosis factor-α (TNF-α)). H4B4 and 1F11 treatments were both significantly different from control treatment ($P < 0.05$).

We then incubated the same monoclonal antibodies with purified human dermal microvascular blood endothelium[27], the principal target of injury in pauci-immune FNGN (Fig. 2e–h). H4B4 significantly increased the number of cells expressing CD62E (E-selectin) ($P <0.01$) (Fig. 2e,f). It also induced apoptosis, as determined by the proportion of endothelial cells expressing cleaved caspase-3 (Fig. 2g,h), and significantly reduced their number after 20 h of culture (464 ± 195 cells and 366 ± 76 cells per ten high-power fields for 10 μg ml$^{-1}$ and 20 μg ml$^{-1}$, respectively) compared to treatment with the antibodies to CD4 (623 ± 89 cells and 680 ± 78 cells for 10 μg ml$^{-1}$ and 20 μg ml$^{-1}$, respectively) and proteinase-3 (615 ± 220 cells and 600 ± 10 cells for 10 μg ml$^{-1}$ and 20 μg ml$^{-1}$, respectively) ($P < 0.05$). Thus, antibodies to human LAMP-2 kill microvascular blood endothelium directly without the need for neutrophils or an external source of antigen.

### Autoantibodies to LAMP-2 recognize two major epitopes

Using our previously validated SPOTscan analysis[28], we characterized the epitopes recognized by autoantibodies from individuals with pauci-immune FNGN and identified two common human LAMP-2 epitopes, P$_{41–49}$ (HGTVTYNGS) and P$_{331–341}$ (QGKYSTAQDCS). These were recognized by autoantibodies from nine (82%) and seven (64%) of eleven randomly selected subjects, respectively, and five sera recognized both epitopes (Fig. 3a). Sequential alanine substitution confirmed the specificity of both interactions and identified the single tyrosine residue in each epitope as crucial for strong antibody binding (data not shown). Subsequent post-treatment sera from five of the subjects with pauci-immune FNGN in which antibodies to LAMP-2 were no longer present (LAMP-2–negative sera) did not recognize either epitope, nor did sera from five healthy controls (data not shown).

The specificity of the subjects' antibodies for both human LAMP-2 epitopes was confirmed by competition ELISA. In these assays, a synthetic peptide corresponding to P$_{41–49}$ specifically inhibited auto-antibody binding to human LAMP-2 by a mean of 53% (range 17–100%) and a peptide corresponding to P$_{331–341}$ inhibited by a mean of 73% (range 29–100%). Neither peptide affected background binding in ELISAs of human LAMP-2–negative sera from treated subjects (P$_{41–49}$ median inhibition 2%, range 0–11%; P$_{331–341}$ median inhibition 4%, range 0–20%) or healthy controls (P$_{41–49}$ median inhibition 9%, range 0–13%; P$_{331–341}$ median

inhibition 9%, range 0–18%) (Fig. 3b). The degree of inhibition confirms that $P_{41-49}$ and $P_{331-341}$ are the two major epitopes recognized by autoantibodies to human LAMP-2 in FNGN.

## LAMP-2 epitope $P_{41-49}$ is homologous to bacterial FimH

Neither human LAMP-2 epitope had homologies to myeloperoxidase or proteinase-3 or to their complementary sequences (Supplementary Table 1 online). However, the $P_{41-49}$ epitope has 100% homology with amino acids 72–80 of mature FimH (with eight of the nine amino acids identical), an adhesin located at the tip of type 1 fimbriae and crucial for attachment to host epithelia of Gram-negative pathogens such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Table 1). The homologous sequence lies on the surface of FimH in the mannose-binding pocket, an essential region for mannose binding and accessible to the host immune system (Fig. 3c). The $P_{331-341}$ epitope was not homologous to FimH but had looser homologies to other bacterial and viral proteins (Table 1).

We next investigated whether autoantibodies from individuals with pauci-immune FNGN cross-reacted with FimH. Lysates from type 1 fimbriated bacteria inhibited autoantibody binding to human LAMP-2, whereas lysates from non–FimH-expressing bacteria (detailed in Supplementary Table 2 online) had no effect (Fig. 3d). In western blots, IgG from human LAMP-2–positive sera that recognized the $P_{41-49}$ epitope consistently bound to the 28 kDa FimH and 32 kDa pre-FimH proteins in lysates of *E. coli* 12900, *K. pneumoniae* and *P. mirabilis* but did not react with proteins from FimH-negative bacteria (Fig. 3e). Finally, a purified recombinant FimH fusion protein specifically inhibited autoantibodies from subjects with FNGN, but not control sera, from binding human LAMP-2 in 14 of 15 (93.3%) subjects tested (median inhibition 83.9%, range 75–100%). Similarly, human LAMP-2 specifically inhibited binding of sera from FNGN-affected individuals to FimH (median inhibition 95%; range 59–100%). This competition was dependent on the homologous sequence, as peptide $P_{41-49}$ but not peptide $P_{331-341}$ was inhibitory (Supplementary Fig. 2a online). Neither human LAMP-2 nor FimH inhibited antibodies to myeloperoxidase or proteinase-3 from binding to their respective antigens in standard ELISAs (Supplementary Fig. 2b). This proves that autoantibodies to human LAMP-2 from individuals with FNGN cross-react with FimH.

## FimH immunization induces antibodies to LAMP2 and FNGN

Rat and human LAMP-2 are identical at six of the nine $P_{41-49}$ epitope residues, whereas this sequence in mice is very different[29]. Consequently, we immunized ten WKY rats with recombinant FimH fusion protein emulsified in Titermax, an adjuvant free of bacterial proteins. All of the rats developed antibodies to FimH that cross-reacted with human LAMP-2 (Fig. 4 and Supplementary Fig. 3a, b online). Two rats immunized with synthetic $P_{41-49}$ human LAMP-2 peptide also developed cross-reactive antibodies, but assays were negative after immunization with Titermax alone (Fig. 4a,c). None of the rats developed antibodies to myeloperoxidase or proteinase-3 (Fig. 4a).

Sera from FimH-immunized rats were positive in standard ANCA assays using human (Fig. 4b) and rat (data not shown) neutrophils, and they cross-reacted with rat LAMP-2 (Supplementary Fig. 3c). IgG from rats immunized with FimH and affinity-purified on peptide $P_{41-49}$ bound specifically to lysates of human umbilical vein endothelial cells (HUVECs) and human dermal blood micro-vascular endothelial cells (Fig. 4c). Immunoelectron microscopy (Fig. 5) showed that these antibodies targeted an antigen located on the endothelial plasma membrane, as well as within multivesicular bodies (Fig. 5d)—a binding pattern identical to that of authentic antibodies to human LAMP-2 (Fig. 5e). Similarly, rabbit IgG specific for FimH bound to glomerular endothelium and lysosomes in normal human kidney (Fig. 5a–c). Sera from rats immunized with Titermax alone were negative (data not shown).

Nine of the FimH-immunized rats also developed antibodies to rat LAMP-2 and pauci-immune FNGN similar to the human disease (Fig. 4e,f), with 15–31% (mean 24%) of rat glomeruli affected by crescents without detectable immunoglobulin deposits, despite circulating antibodies to rat LAMP-2 (Fig. 4h). Two rats also developed hemorrhagic pulmonary vasculitis (Fig. 4g and Supplementary Fig. 3d). Rats immunized with peptide $P_{41–49}$ alone developed milder disease, whereas controls injected with only Titermax remained healthy (Fig. 4d). Thus, immunizing rats with FimH breaks immunological tolerance to LAMP-2 and provokes synthesis of autoanti-bodies to it through molecular mimicry. These antibody responses are accompanied by pauci-immune FNGN and pulmonary vasculitis.

### Infection with fimbriated pathogens is common before FNGN

Finally, data available from samples sent by primary care physicians showed that nine of the most recent thirteen (69%) subjects in our cohort had microbiologically confirmed diagnoses of infection with FimH-expressing bacteria during the 12 weeks before presentation with pauci-immune FNGN (eight *E. coli* and one *K. pneumoniae* infection), indicating that they had been exposed to the relevant pathogens. The autoantibodies in these individuals bound the human LAMP-2 region containing the cross-reactive $P_{41–49}$ epitope. By contrast, subjects recovering from systemic infections with the relevant bacteria without evidence of renal disease had high titers of antibodies to FimH that did not recognize the $P_{41–49}$ epitope or cross-react with human LAMP-2 (data not shown).

## DISCUSSION

The results presented here lead us to propose a previously unrecognized molecular explanation for the origin and development of injury in pauci-immune FNGN. We show that (i) autoantibodies to human LAMP-2 are present in over 90% of individuals with active pauci-immune FNGN; (ii) antibodies to human LAMP-2 injure human microvascular endothelium *in vitro* and induce FNGN when administered to rats; (iii) autoantibodies from individuals with pauci-immune FNGN commonly bind an epitope shared by human LAMP-2 and the bacterial adhesin FimH and cross-react with it; (iv) immunization with FimH induces pauci-immune FNGN associated with antibodies that bind human and rat LAMP-2; and (v) individuals with pauci-immune FNGN commonly have infections with FimH-expressing bacteria shortly before presentation of FNGN. Thus, FimH-triggered autoimmunity to human LAMP-2 could be responsible for the initiation and development of injury in pauci-immune FNGN, either operating alone or synergistically with antibodies to myeloperoxidase and proteinase-3.

Our hypothesis depends on the high prevalence of antibodies to human LAMP-2 in individuals with active pauci-immune FNGN. Consequently, we validated the results of our standard ELISA, not only by western blotting but also in other assays using mammal-expressed human LAMP-2. Thus, we can be certain that over 90% of our subject cohort with active pauci-immune FNGN had circulating autoantibodies to human LAMP-2. This exceeds the prevalence of antibodies to myeloperoxidase and proteinase-3, which, as in other series, were each present in around half of the subjects. Thus, autoantibodies to human LAMP-2 are the first to be found in all types of pauci-immune FNGN. Furthermore, they bind fully glycosylated human LAMP-2 on the cell surface, suggesting that they could cause injury.

The frequent co-existence of autoantibodies to LAMP-2 with those to myeloperoxidase and proteinase-3 precluded the use of patients' sera for testing whether the autoantibodies to LAMP-2 cause injury. However, we showed that pathologically plausible concentrations of monoclonal and polyclonal antibodies to human LAMP-2 cause injury both *in vitro* and *in vivo* without the need for additional stimuli—such as LPS or cytokines—that are commonly required in such experiments[7,30,31]. Uniquely among autoantibodies found in pauci-immune FNGN, the monoclonal human LAMP-2–specific antibody H4B4 injured human blood

microvascular endothelium, the primary target of damage in pauci-immune FNGN. Although the molecular mechanisms for this have yet to be defined, activation of endothelial caspase-3 is consistent with the role of LAMP-2 in apoptotic and autophagocytic cell death[32,33]. Antibodies to human LAMP-2 cause pauci-immune FNGN with up to 25% crescents when injected into WKY rats. Taken together, our data provide strong, though indirect, evidence that autoantibodies to human LAMP-2 contribute to glomerular injury in individuals with pauci-immune FNGN.

The data raise the intriguing question as to why autoantibodies to human LAMP-2 so commonly occur together with antibodies to either myeloperoxidase or proteinase-3. An essential synergistic effect on injury provides an obvious explanation, but an alternative (and not mutually exclusive) possibility is that the autoantibodies to human LAMP-2 dysregulate the role of LAMP-2a in presentation of cytoplasmic antigens, including lysosomal proteins[15,34,35]. This, in turn, could facilitate the generation of autoantibodies to myeloperoxidase and proteinase-3.

Characterization of the epitopes recognized by autoantibodies from subjects with pauci-immune FNGN provided our most unexpected result. It uncovered a hitherto unsuspected homology between one of these epitopes ($P_{41-49}$) and the bacterial fimbrial adhesin FimH. The homology has profound functional consequences, first because auto-antibodies that recognize $P_{41-49}$ cross-react with FimH, as proved by our reciprocal inhibition experiments, and second because WKY rats immunized with FimH develop antibodies to the shared epitope. The immunized rats also developed autoantibodies to rat LAMP-2 and pauci-immune FNGN with pulmonary small vessel vasculitis similar to the human disease. This clearly demonstrates that FimH can induce FNGN by operating as a molecular mimic for LAMP-2, at least when administered together with adjuvant. These results identify $P_{41-49}$ as a pathogenic epitope and support our proposal that autoimmunity to LAMP-2 causes FNGN, even in the absence of antibodies to myeloperoxidase or proteinase-3.

Molecular mimicry has frequently been proposed as a mechanism for autoimmune disease, but convincing evidence has been difficult to obtain, as has been previously reviewed[21], except possibly in neurological diseases in the presence of cross-reacting antibodies[36,37]. Our demonstration that human LAMP-2 cross-reacts with FimH and that immunization with FimH induces antibodies to LAMP-2 and FNGN shows the molecular mimicry between the two proteins. This conclusion is supported by two additional pieces of data: it has previously been shown that FNGN is induced in rats immunized with attenuated *E. coli* but not with *Staphylococcus aureus*[38], and we have found that nine of thirteen subjects had microbiologically proven infections with FimH-bearing organisms shortly before the clinical appearance of FNGN. Although this provides some of the most complete evidence yet for molecular mimicry as mechanism of human autoimmune disease, it doesn't identify the exceptional circumstances needed for FimH to induce the cross-reactive auto-antibodies. FimH is required for bacterial colonization of bladder epithelium[39,40] and so has been proposed as an attractive target for immunization programs for urinary tract infections in humans[41,42]. Our data clearly question the safety of this approach.

In conclusion, we propose that infection with fimbriated bacteria induces autoantibodies to human LAMP-2 through molecular mimicry and that these antibodies bind microvascular endothelium and cause injury (Supplementary Fig. 4). This presents a new model for the pathogenesis of pauci-immune FNGN and related ANCA-associated diseases and could provide the foundation of a new therapeutic strategy for this devastating disease.

# METHODS

## Study subjects and controls

We studied sera from 84 individuals with biopsy-proven active pauci-immune FNGN, diagnosed according to Chapel Hill definitions[43]. We assessed disease activity with a validated renal biopsy scoring system[44] supplemented by clinical indices (Supplementary Table 3 online). Remission sera were available from 54 individuals who had no clinical evidence of disease activity for at least 2 months before and after the samples were taken. Control sera were from individuals with glomerulonephritis other than FNGN ($n = 30$); ANCA-positive individuals with other types of FNGN ($n = 8$; two each of Goodpasture's, systemic lupus erythematosus, immune-complex glomerulonephritis and thrombotic thrombocytopenic purpura and hemolytic uremic syndrome); individuals with localized vasculitis without ANCA or renal involvement ($n = 20$); and healthy volunteers ($n = 50$). Subject samples were taken for routine clinical management, and use of human samples conformed to legal regulations in Austria and the United Kingdom and were approved by the Ethics Committees of the Medical University of Vienna and the Grampian Research Ethics Committee. Informed consent was sought for the use of the samples, where required.

## Reagents

We used the following monoclonal (except where stated) antibodies: human LAMP-2 (clone H4B4, Developmental Studies Hybridoma Bank), proteinase-3 (clone 1F11, HyTest), CD4 (clone RPA-T4, BD-Pharmingen), actin (Chemicon) and E-selectin (CD62E, HyCult Biotechnology) and rabbit antibody to cleaved caspase-3 (Cell Signaling Technology).

## Generation of recombinant human LAMP-2

We amplified the extracellular domain of human LAMP-2 from bases −83 to 1026 of the open reading frame by PCR and cloned it into pRSETA or pGEX6P1 (His-tagged (Invitrogen) or GST fusion (Amersham, respectively) for bacterial protein expression and into pCDNAI (Invitrogen) for mammalian expression. We confirmed the sequences and cloning sites of all constructs as well as the size and immunoreactivity of recombinant human LAMP-2 by methods described previously[10,45,46] (Supplementary Methods online).

## Enzyme-linked immunosorbent assay for human LAMP-2 and canonical autoantibodies to neutrophil cytoplasmic antigens

We assayed serum samples at a dilution of 1:100 on ELISA plates coated with purified human LAMP-2 extracellular domain. We calculated the 99.5% confidence limit for the upper limit of normal as three standard deviations above the mean absorbance for healthy individuals. We confirmed ELISA results by western blotting and indirect immunofluorescence on ldlD[47] cells expressing human LAMP-2 on their surface (Supplementary Methods). We assayed ANCA staining patterns by immunofluorescence in accordance with published guidelines[10,48]. We tested sera from an active and inactive stage of disease with commercially available ELISAs for myeloperoxidase and proteinase-3 (Varelisa, Phadia).

## Human LAMP-2 epitope analysis

We characterized human LAMP-2 epitopes recognized by subjects' IgG by SPOTscan analysis (Sigma-Genosys)[28] (Supplementary Methods), validated positive results by sequential alanine substitution (SPOTsalogue, Sigma-Genosys) and confirmed them by specific inhibition with synthetic peptides (gift from A. Jungbauer).

### Recognition of bacterial proteins by autoantibodies

We probed lysates of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Pseudomonas aeruginosa* and *S. aureus* (Supplementary Table 2) by western blotting with sera from subjects with pauci-immune FNGN and control sera. We analyzed FimH binding and cross-reactivity of human LAMP-2 autoantibodies by reciprocal cross-inhibition ELISA, using equimolar concentrations of soluble human LAMP-2, FimH fusion protein[49] or synthetic human LAMP-2 peptides $P_{41-49}$ or $P_{331-341}$ (Supplementary Methods).

### Interaction of antibodies to human LAMP-2 with human granulocytes and microvascular endothelium

We incubated human neutrophils[10,26] for 30 min with 10 μg ml$^{-1}$ or 20 μg ml$^{-1}$ antibodies to human LAMP-2, proteinase-3 or CD4 or with TNF-α (2 ng ml$^{-1}$), assessed shape change[25] and F-actin condensation[26] and measured myeloperoxidase concentrations in supernatants (Immundiagnostik). We incubated purified human dermal microvascular blood endothelial cells, grown to 60% confluence[27], with the monoclonal antibodies described above for 16 h and assessed expression of E-selectin and activated caspase-3 by immunofluorescence (Supplementary Methods). All antibodies contained less than 2 pg ml$^{-1}$ LPS (Limulus Amebocyte Lysate, Cambrex BioScience).

### Immunization with antibodies to LAMP-2

We injected WKY rats intravenously with 10 mg of IgG to human and rat LAMP-2 extracellular domain purified using endotoxin-free reagents. Control rats received normal rabbit IgG or saline. We culled the rats at specified time points up to 120 h (Supplementary Methods) and assessed glomerular injury by hematuria (Combur-7 test, Roche Diagnostics), albuminuria (QuantiChrom BCG Albumin Assay Kit, BioAssay Systems), histomorphology and immunohistochemistry.

### Immunization with FimH fusion protein or the cross-reactive epitope

We immunized WKY rats with 150 μg purified FimH fusion protein emulsified in Titermax Gold (Sigma-Aldrich) or with peptide $P_{41-49}$ conjugated to rat serum albumin. Controls received PBS in Titermax alone. We culled the rats on day 39 and assessed glomerular injury as described above (Supplementary Methods).

### Analysis of rat sera

We assayed ANCAs by immunofluorescence on human and rat granulocytes and antibodies to human LAMP-2, FimH, human myeloperoxidase and proteinase-3 by ELISA. We used dot-blot analysis to test whole sera for antibodies to human LAMP-2, rat LAMP-2, FimH, human myeloperoxidase and proteinase-3, serum albumin and glomerular basement membranes, and we probed rat IgG, affinity-purified on peptide $P_{41-49}$ and $P_{331-341}$ (ref. [50]) for reactivity with lysates of blood microvascular endothelial cells or lymphatic endothelial cells (Supplementary Methods). We probed rat and rabbit IgG specific for FimH and LAMP-2 on 4-μm-thick unfixed frozen sections or ultrathin frozen sections of PLP-fixed normal human kidneys[10].

### Statistical analyses

We used two-way analysis of variance followed by a multiple range test (Tukey analysis) to estimate the significance of results from ELISA. We used the Wilcoxon rank sum test after an initial Kruskal Wallis test for the *in vivo* and *in vitro* experiments.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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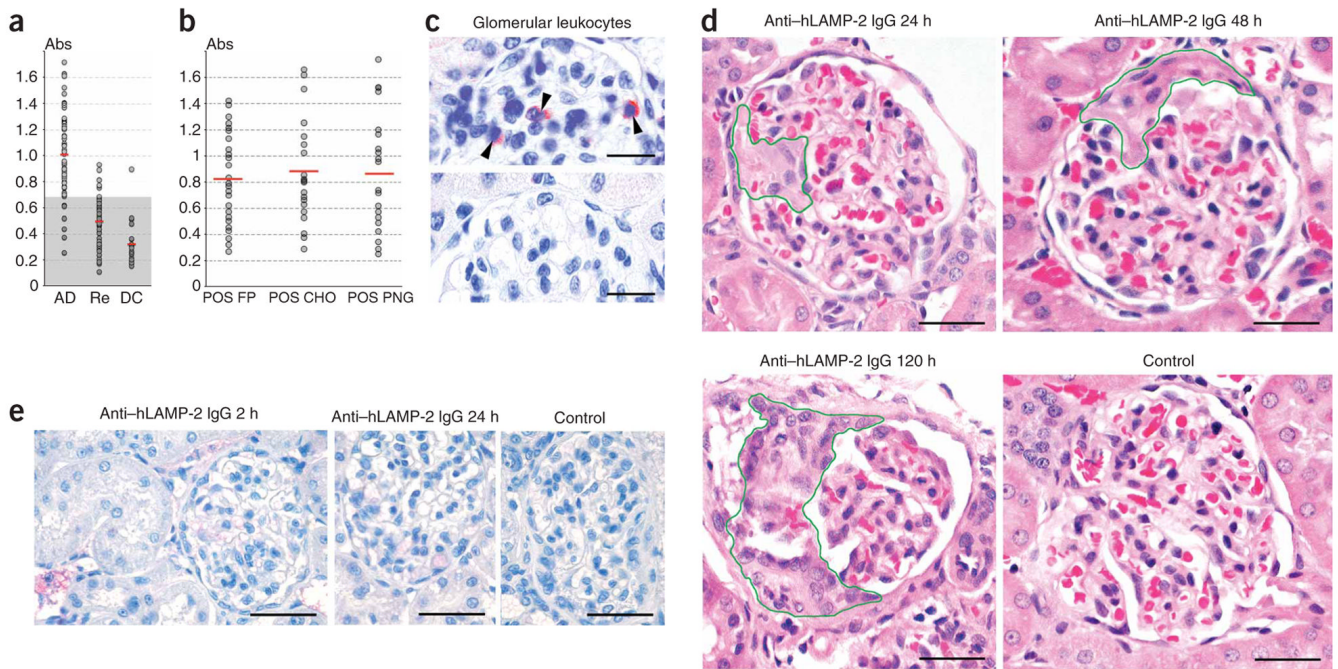
**Figure 1.**
Antibodies to human LAMP-2 (hLAMP-2) in pauci-immune FNGN. (**a**) Absorbance values of a specific ELISA to assay autoantibodies to LAMP-2 measured in sera from individuals with pauci-immune FNGN and active disease (AD), from the same individuals after treatment when in remission (Re) or from ANCA-negative disease controls (DC) with other types of glomerulonephritis. The shaded area indicates the 99.5% confidence interval for the normal range. (**b**) Absorbance values of sera from 32 individuals with autoantibodies to LAMP-2 assayed in three different ELISAs with the following substrates: purified unglycosylated, *E. coli*–expressed human LAMP-2 fusion protein (POS FP); glycosylated mammalian expressed human LAMP-2 (POS CHO); and PNGaseF-treated mammalian-expressed human LAMP-2 (POS PNG). The mean value for each ELISA is indicated as is the upper limit of normal. (**c**) Glomerular CD45 positive leukocytes (arrowheads) in WKY rats injected 24 h earlier with 10 mg of rabbit antibodies to LAMP-2 (anti-hLAMP2 IgG, top) or 10 mg normal rabbit IgG (bottom). Scale bar, 25 μm. (**d**) Morphology of representative glomeruli from WKY rats injected intravenously with IgG to rat LAMP-2. Segmental necrosis is present after 24 h, cellular crescents after 48 h and segmental scarring with fibrocellular crescents and adhesions after 120 h. Scale bars, 25 μm. (**e**) Direct immunoperoxidase staining of representative glomeruli from WKY rats injected with rabbit IgG to LAMP-2. Small quantities of deposited IgG is seen in blood vessels and occasional circulating leukocytes after 2 h but not at later time points. Scale bar, 50 μm.
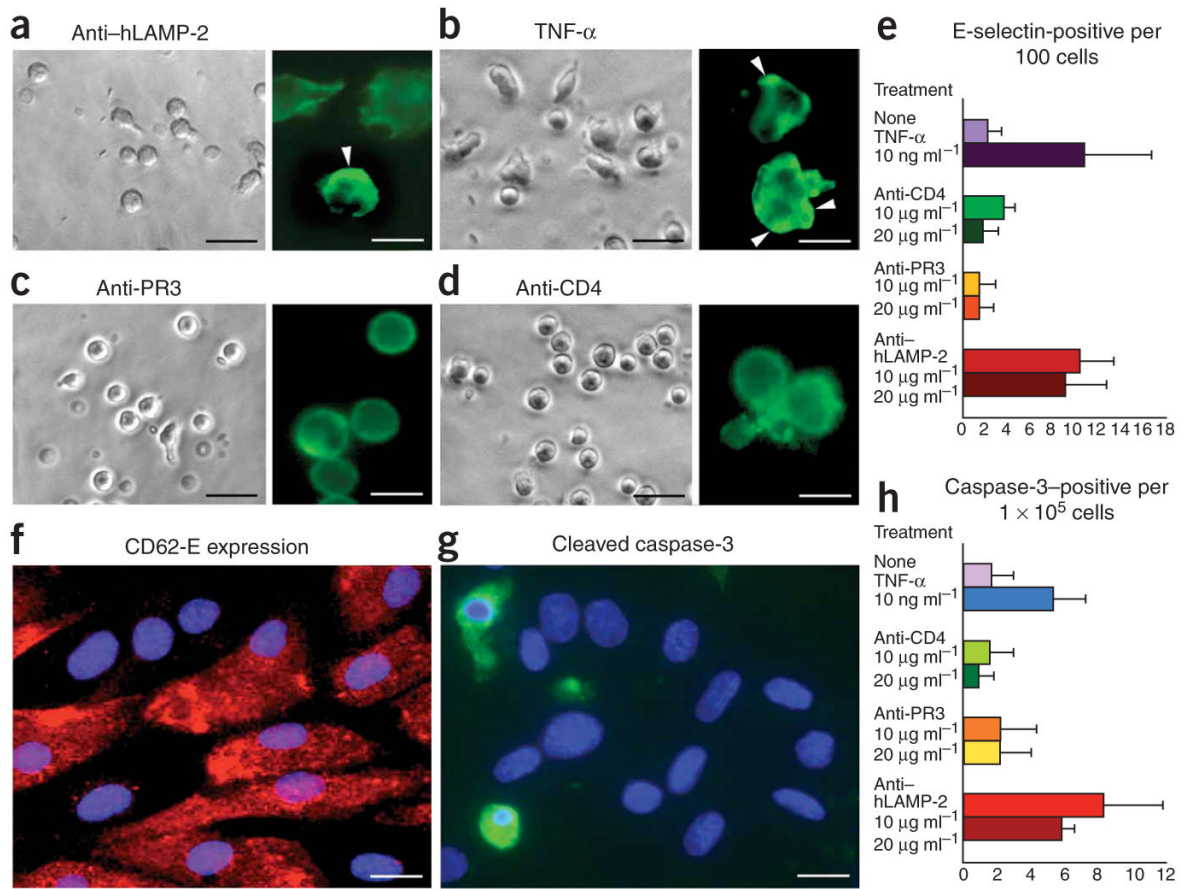
**Figure 2.**
Antibodies to hLAMP-2 activate neutrophils and kill human microvascular endothelium. (**a**–**d**) Purified human neutrophils were incubated with 10 μg ml$^{-1}$ H4B4, a monoclonal antibody to human LAMP-2 (**a**); 2 ng ml$^{-1}$ TNF-α (**b**), 10 μg ml$^{-1}$ 1F11, a monoclonal antibody to proteinase-3 (**c**) or 10 μg ml$^{-1}$ of monoclonal antibody to CD4 (**d**). H4B4 and TNF induced significantly more shape change than the other two treatments, and the insets confirm that the shape change is associated with actin condensation (insets in **a**–**d**; scale bars, 50 μm). When the same antibodies were incubated with purified human dermal microvascular blood endothelial cells (BECs), H4B4 significantly increased CD62E (E-selectin) expression ($P < 0.01$) whereas 1F11 and CD4 had no effect (**e, f**). H4B4 also significantly increased the number of cells expressing activated caspase-3 ($P < 0.05$), a marker of apoptosis (**g, h**). Scale bars, 50 μm. Values in **e** and **h** represent means ± s.d.
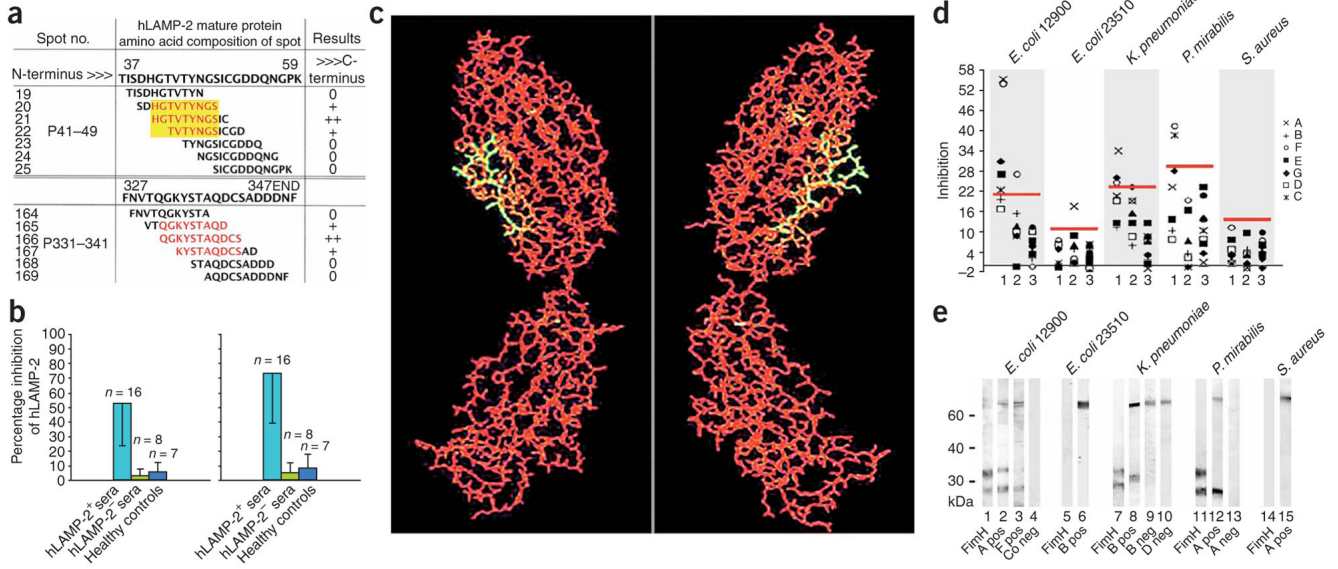
**Figure 3.**
Autoantibodies to hLAMP-2 cross-react with the bacterial adhesin FimH. (**a**) Two epitopes, $P_{41-49}$ (HGTVTYNGS) and $P_{331-341}$ (QGKYSTAQDCS) recognized by autoantibodies to hLAMP-2 in active FNGN are shown in red. The yellow box illustrates the homologous sequence shared by human LAMP-2 and FimH. Depicted are examples of overlapping peptides synthesized onto a SPOTs membrane aligned to the human LAMP-2 sequence. (**b**) Inhibition ELISA used to determine whether synthetic peptides corresponding to $P_{41-49}$ (left) and $P_{331-341}$ (right) prevented autoantibodies in active pauci-immune FNGN from binding to human LAMP-2. Both peptides significantly inhibited binding ($P < 0.001$). Values represent means ± s.d. (**c**) Three-dimensional projection of the structure of the FimH mannose binding pocket. Amino acids 72–80 of FimH, homologous to $P_{41-49}$ (yellow), are exposed on the surface. (**d**) ELISA was used to determine whether bacterial lysates from FimH-expressing (*E. coli* 12900, *K. pneumoniae.*, *P. mirabilis*) or non-expressing (*E. coli* 23510, *S. aureus*) organisms inhibit binding of antibodies to LAMP-2 from subjects (A–G) with active FNGN (lane 1), FNGN in remission (lane 2) or healthy controls (lane 3). Red bars, 99.5% confidence interval for inhibition of binding. (**e**) Western blot showing binding of antibodies from subjects with FNGN to lysates of bacteria that do (lanes 1–4, 7–10 and 11–13) or do not (lanes 5–6 and 14–15) express FimH. Two sera with specificity for hLAMP-2 (A pos, B pos and F pos) recognize the same bands as an antibody to FimH (fimH), whereas negative sera (A neg, B neg and D neg) and controls (Co neg) do not.
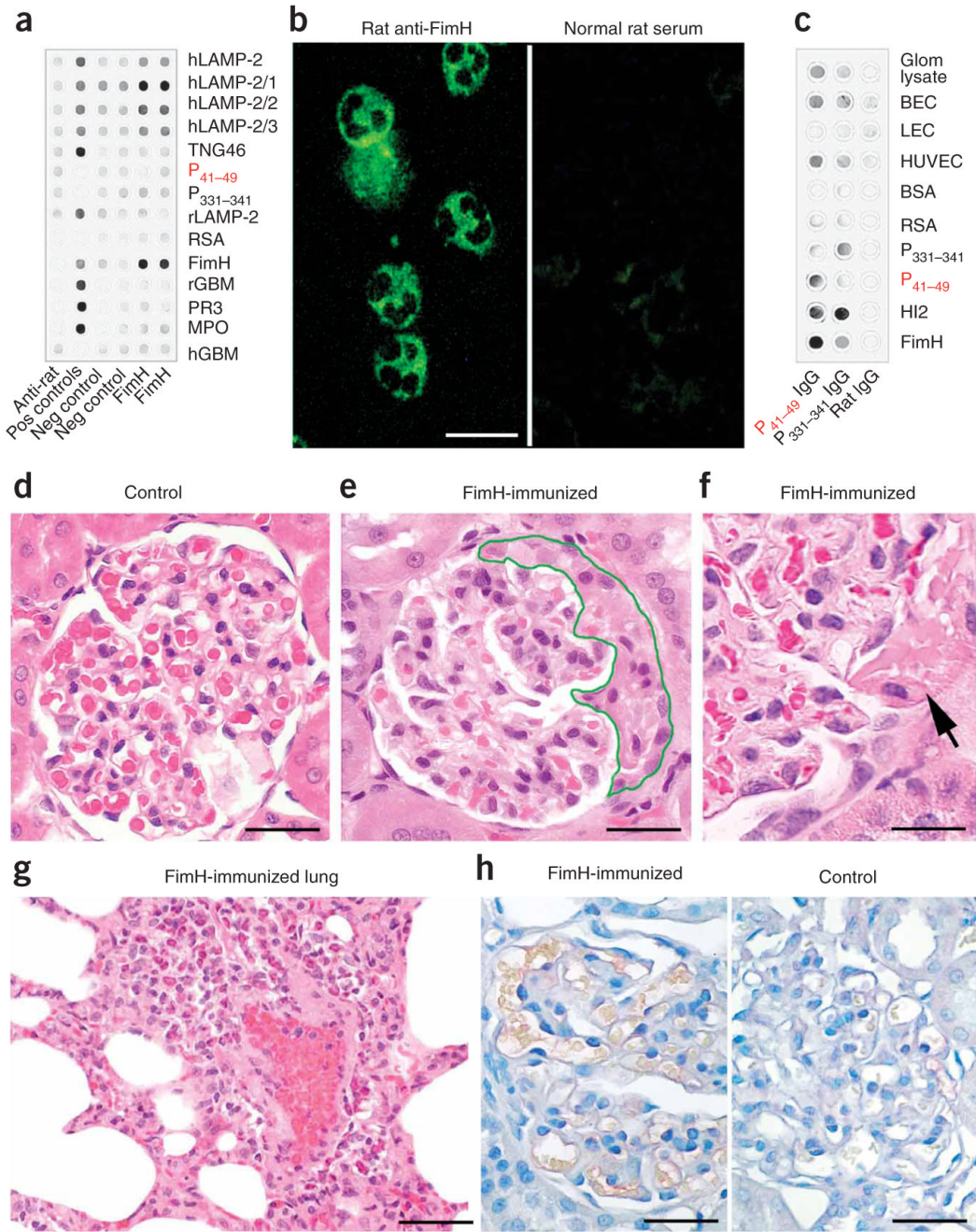
**Figure 4.**
Immunization with FimH induces antibodies to LAMP-2 and FNGN in rats. (**a**) Sera from
FimH-immunized rats and controls were probed for FimH, full-length and N-terminal
hLAMP-2 and 100–amino acid (hLAMP-2/1) fusion proteins. The specificity of rat IgG for
human or rat glomerular basement membrane (rGBM and hGBM), proteinase-3 (PR3) or
myeloperoxidase (MPO), *trans*-Golgi network protein 46 (TGN46) or rat serum albumin
(RSA) was tested with purified proteins. Specific antibodies to these proteins, where available,
were used as positive controls, and anti–rat IgG alone as negative control. (**b**) Sera from FimH-
immunized WKY rats bound neutrophil granules, resulting in a cytoplasmic staining pattern
similar to that observed with sera from subjects with FNGN (left). Sera from control rats

remained negative. Scale bar, 25 μm. (**c**, **d**) IgG specific for $P_{41-49}$ or $P_{331-341}$, purified from rat serum after immunization with FimH was probed on purified FimH and hLAMP-2 fusion proteins, peptides $P_{41-49}$ and $P_{331-341}$, and proteins from isolated glomeruli, microvascular blood endothelial cells (BECs), HUVECs and lymphatic endothelial cells (LECs) (**c**). Purified normal rat IgG was used as control. Glomerular histology from un-immunized control animals appears normal (**d**). WKY rats immunized with FimH show glomerular crescent formation (**e**), segmental necrosis of capillaries (**f**) and marked neutrophilic capillaritis of the lung (**g**). Direct immunoperoxidase staining shows no glomerular IgG deposition in glomeruli from FimH immunized nephritic rats (**h**; left) nor from adjuvant treated controls (**h**; right). Scale bars in **d**–**h**, 50 μm.
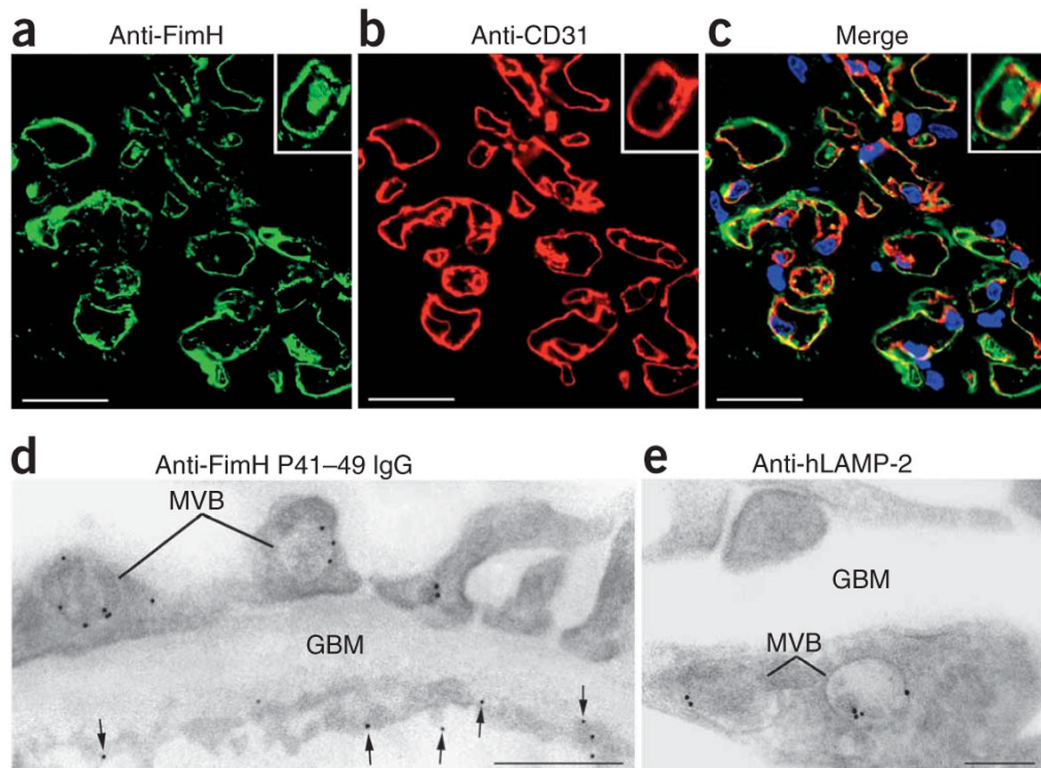
**Figure 5.**
Antibodies induced by FimH immunization bind human glomerular endothelium. (**a**, **b**) Double labeling of frozen sections of normal human glomeruli with purified rabbit IgG specific for the FimH mannose binding pocket (green, **a**) and with an antibody to CD31 (red, **b**). (**c**) Overlay of **a** and **b** (yellow; scale bars, 25 μm). (**d**, **e**) IgG with specificity for peptide $P_{41-49}$, purified from rat sera after immunization with FimH, was used for indirect immunolabeling of ultrathin frozen sections of human glomeruli. The staining pattern on endothelial cell membrane (arrows) as well as within multivesicular bodies (MVB) of endothelial and epithelial (E) is compared to that of a monoclonal antibody to hLAMP-2 (**e**). GBM, glomerular basement membrane. Scale bar in **d**, 500 nm; scale bar in **e**, 200 nm.

**Table 1**

Homologies in hLAMP-2 peptides $P_{41-49}$ and $P_{331-341}$ to bacterial and viral proteins

| Protein | AA | | | | | | | | | | AA | Species | Accession code or GenBank GI number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LAMP-2 $P_{41-49}$ | 69 | H | G | T | V | T | Y | N | | S | 77 | *Homo sapiens* | gi: 307110 |
| FimH | 94 | | G | T | V | K | Y | N | | S | 101 | *E. coli* O18:K1:H7, O2:MT78, LF82 | gi: 1094 6257 |
| Minor fimbrial subunit | 94 | | G | T | V | K | Y | S | | S | 101 | *E. coli* K12, O157:H7 | NP 418740, AAC35864 |
| Fimbrial protein | 26 | | G | T | V | T | F | N | | K | 33 | *Yersinia pestis* | 16123128, 15980903 |
| Fimbrial adhesin | 96 | | G | T | V | K | Y | N | | T | 103 | *Klebsiella pneumoniae* | AAA25063 |
| FimH | 185 | | G | T | I | S | Y | S | | K | 192 | *Salmonella typhimurium* | NP 455136 |
| Uroepithelial adherence protein | 25 | | Y | T | I | T | F | T | | K | 32 | *Proteus mirabilis* | AAA68979 |
| Glucosyltransferase | 401 | | G | T | V | T | F | N | | Q | 407 | *Streptococcus mutans* | BAB83942 |
| ORF 1 | 170 | | G | T | V | S | Y | N | | S | 177 | *Staphylococcus warneri* | BAB83942 |
| Putative ABC transporter | 63 | | E | T | I | T | Y | N | | S | 69 | *Streptomyces coelicolor* | gi: 9967662 |
| ORF | 171 | | G | T | V | S | Y | N | | S | 177 | *Staphylococcus warnerii* | gi: 7804481 |
| Putative outer membrane protein | 593 | | G | T | V | T | Y | N | | S | 599 | *E. coli* CFT073 | gi: 26248725 |
| Outer membrane protein | 261 | | | | V | T | Y | N | | S | 266 | *Bacteroides thetaiotaomicron* | gi: 1100065 |
| Myxobacterial hemagglutinin | 183 | | G | T | M | T | Y | N | | S | 189 | *Myxococcus xanthus* | gi: 126790 |
| Envelope glycoprotein | 139 | | G | T | V | S | Y | N | | S | 144 | HIV type 1 | gi: 17046905 |
| Virion protein | 6 | H | S | S | V | S | Y | N | | | 13 | Human herpesvirus 3 | gi: 9625913 |
| Glycoprotein I | 170 | Q | G | T | L | A | Y | N | | S | | Human herpesvirus 1 | gi: 9629448 |
| LAMP-2 $P_{331-341}$ | 357 | | G | K | Y | S | T | A | C | D | 366 | *Homo sapiens* | gi: 307110 |
| ORF | 71 | | | | Y | S | T | V | C | D | 77 | *E. coli* O157:H7 | gi: 15804774 |
| DMSO reductase | 193 | | G | S | Y | S | T | A | | D | 199 | *E. coli* | gi: 1742611 |
| E1 protein | 163 | | | | Y | N | T | A | C | D | 169 | Hepatitis C virus | gi: 1469841 |
| Polyprotein | 143 | | | | | T | T | A | C | D | 147 | Hepatitis C virus | gi: 1173700 |
| Envelope protein 1 | 88 | | | T | Y | T | T | A | C | D | 92 | Hepatitis C virus | gi: 1737397 |
| vif protein | 91 | Q | G | R | Y | S | T | Y | | G | 96 | HIV type 1 | gi: 17981621 |
| E7 protein | 2 | H | G | K | Y | S | T | Y | | G | 7 | Human papillomavirus type 74 | gi: 1491799 |

Amino acid numbers correspond to those of preproteins published at Entrez, The National Center for Biotechnology Information (NCBI). AA, number of amino acids. ORF, open reading frame.