Anti–LAMP-2 Antibodies Are Not Prevalent in Patients With Antineutrophil Cytoplasmic Autoantibody Glomerulonephritis


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ABSTRACT
Lysosomal membrane protein 2 (LAMP-2) is a target of antineutrophil cytoplasmic autoantibodies (ANCA) in addition to the more commonly known targets proteinase 3 and myeloperoxidase. The prevalence of anti–LAMP-2 antibodies and their relationship to disease in ANCA glomerulonephritis are not well described. We measured anti–LAMP-2 reactivity in 680 sera samples (two academic centers) from patients with ANCA glomerulonephritis (n=329); those with ANCA-negative glomerulonephritis (n=104); those with fimbriated, gram-negative Escherichia coli urinary tract infection (n=104); disease controls (n=19); and healthy volunteers (n=124). With levels in healthy controls used to define a reference range, anti–LAMP-2 reactivity was present in 21% of ANCA sera from two of the centers; reactivity was present in 16% of the control group with urinary tract infection. Western blotting and immunofluorescence microscopy did not verify positivity. Titers of anti-myeloperoxidase and anti–proteinase 3 antibodies were 1500-fold and 10,000-fold higher than anti–LAMP-2 titers, respectively. There was no correlation between anti–LAMP-2 antibodies and disease activity. Furthermore, Wistar Kyoto rats injected with anti–LAMP-2 antibodies did not develop glomerulonephritis. In conclusion, antibodies that react with LAMP-2 may exist at very low titers in a minority of patients with ANCA disease. These data do not support a mechanistic relationship between anti–LAMP-2 antibodies and ANCA glomerulonephritis.


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triggered by bacterial infection led to production of autoantibodies to the human LAMP-2 (hLAMP-2) protein. The resulting anti-LAMP-2 autoantibodies were proposed to be pathogenic and able to cause GN in rats. Rats immunized with FimH peptide developed pauci-immune GN and antibodies to human LAMP-2. If fimbriated bacteria, with the relevant amino acid sequence of FimH, are proven to trigger ANCA disease in susceptible individuals, the therapeutic implications could be far-reaching.

The purported high prevalence of anti-LAMP-2 autoantibodies stimulated discussions on whether routine screening for anti-LAMP-2 autoantibodies should be initiated for all patients with ANCA disease. Before such steps are taken, the prevalence of these autoantibodies and their relationship with disease activity should be established in independent cohorts. To determine the diagnostic value of anti-LAMP-2 antibodies, the specificity and sensitivity of the antibody must be verified in multiple patient cohorts evaluated in multiple laboratories.

**RESULTS**

**Comparison of LAMP-2 Protein Substrates Used for Antibody Detection**

LAMP-2 is normally produced in all cell types. It contains oligosaccharide chains, some of which are polylactosaminoglycans, that are species-specific and complex. These are dispersed...
on two domains of the protein separated by a hinge-like structure containing O-linked oligosaccharides (Figure 1A).

A recombinant LAMP-2 protein consisting of the entire extracellular domain (aa 1-350) was used as substrate for studies at the UNC Kidney Center. The cDNA of human LAMP-2a was subcloned into a mammalian expression vector omitting the N-terminal signal sequence, the membrane-spanning domain, and the cytoplasmic tail (Figure 1B). Recombinant protein was expressed in human embryonic kidney (HEK) cells to make possible human-specific protein glycosylation. Affinity purified protein was of high quality, as determined by SDS-PAGE (Figure 1C), and was recognized by a commercial, polyclonal anti–LAMP-2 antibody by Western blot analysis (Figure 1D). In addition, a synthetic peptide was synthesized locally; this peptide contained the amino acids identified as the FimH-like epitope (Figure 1E). Purity of fast protein liquid chromatography–eluted peptide was indicated by a single peak (Figure 1F), and peptide composition was confirmed by mass spectrometry (Figure 1G).

A recombinant LAMP-2 protein commercially produced in a wheat germ cell-free system was used as substrate in studies conducted at Massachusetts General Hospital. Protein that translated this system is nonglycosylated, and thus the LAMP-2 substrate can be likened to the one “bacterially” produced and used by Kain et al. The amino acid sequence is only a portion of the extracellular domain (aa 30–127) but does contain the FimH-like epitope (Figure 1H).

Table 1 lists all sera samples analyzed in these studies. A total of 680 samples were screened for reactivity against LAMP-2.

Sera from Patients with PR3-ANCA or MPO-ANCA Have Little to No Reactivity to Recombinant LAMP-2 Protein Produced in HEK

Kain and coworkers from the Medical University of Vienna provided the UNC Kidney Center with sera (n=9) consisting of four samples known to be positive for LAMP-2 antibodies in their assay system and five known to be negative. In our assay system, four samples were positive and five were negative against HEK-expressed recombinant LAMP-2 (rLAMP-2) (Figure 2A), providing confidence that the systems are similar. We found that 21.1% of the 103 UNC patients with ANCA disease were deemed positive for reactivity against rLAMP-2 in this assay (greater than the mean + 2 SD of healthy controls; P=0.004; Figure 2A). Sera from otherwise healthy individuals with active urinary tract infections (n=104) produced antibodies against the gram-negative bacteria and contained antibodies reactive against rLAMP-2 at a frequency similar to that

<table>
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<tr>
<th>Table 1. Description of sera samples</th>
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<tr>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>Local North Carolina Community</td>
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<td>UNC Kidney Center</td>
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<td>Massachusetts General Hospital</td>
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<td></td>
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<tr>
<td>UNC Hospitals McLendon Clinical Laboratories</td>
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<tr>
<td>UNC Kidney Center</td>
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<tr>
<td>Medical University of Vienna</td>
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GDCN, Glomerular Disease Collaborative Network; BVAS = Birmingham Vasculitis Activity Score; MGH = Massachusetts General Hospital; FNGN, pauci-immune focal necrotizing glomerulonephritis.

aANCA samples are divided into MPO-, PR3-, or dual-positive; active; or remission. New-onset and never-frozen samples are included in the total number of samples.

bOne patient from the test cohort had insufficient data with which to determine disease activity.
found in the ANCA disease group ($P=0.10$). Samples from patients considered to have “new-onset” disease were obtained before therapy ($n=7$) or immediately after the first dose of glucocorticoids ($n=9$). In Figure 2A, open boxes in the ANCA disease cohort signify patients during active disease with a Birmingham Vasculitis Activity Score (BVAS) $>0$. Sera from patients with SLE were negative for rLAMP-2 reactivity (Figure 2A). rLAMP-2 reactivity was not significantly associated with disease onset or with fresh sera (the latter samples were tested the same day they were drawn and were never frozen).

As with most clinical testing, a second assay was used to validate ELISA results. All samples reactive with rLAMP-2 ($n=26$) were tested by Western blot analysis. MPO-ANCA–positive sera that were also positive for rLAMP-2 by ELISA were negative for LAMP-2 by Western blot (Figure 2B, lane 1) and were reactive with native MPO (lane 2). Blot 1 was re-probed with a commercially available anti-LAMP-2 antibody to verify that rLAMP-2 was present in lanes loaded with the protein preparation. PR3-ANCA sera and sera provided by Kain et al. were also negative for rLAMP-2 reactivity by Western blot analysis (data not shown).

Reactivity against rLAMP-2 by Indirect Immunofluorescence Microscopy

A third assay (indirect immunofluorescence) was used to validate positive rLAMP-2 reactivity. rLAMP-2 protein, overexpressed in HEK cells, stains with a polyclonal anti-LAMP-2 antibody to produce a cytoplasmic staining pattern consistent with preferential staining of lysosomes (Figure 3A, right). Low levels of endogenous LAMP-2 protein were detected in nontransfected cells (Figure 3A, left). None of the samples from healthy controls ($n=52$) or patients with ANCA disease ($n=103$) produced a staining pattern similar to the positive control staining, although some samples had low-intensity staining with other patterns. SLE samples ($n=10$) often stained cells in a variable, sometimes nuclear, pattern. Figure 3B shows representative samples from two healthy controls (one with nonspecific staining), two disease controls depicting nonspecific reactivity (SLE), and four high-titer ANCA disease sera. One MPO-ANCA sample shows irregular punctuate staining similar to that of control samples and differing from that of the polyclonal anti-LAMP-2 control. Many SLE samples had varying patterns of nuclear staining, apparently caused by antinuclear antibodies, but none had staining resembling the control positive for anti-LAMP-2.

Other investigators reported that heavy glycosylation of rLAMP-2 produced in HEK cells could alter antigenicity. To
address this issue, we acquired the same cell line used in studies by Kain et al., which is O-linked glycosylation deficient (CHO-LDL-D cells), and performed indirect immunofluorescence microscopy on rLAMP-2–overexpressing CHO-LDL-D cells. We could not detect reactivity to this substrate (Figure 3C). Furthermore, cells were grown in varying conditions to alter the glycosylation pattern of the overexpressed rLAMP-2 protein. O-linked glycosylation–deficient cells were grown in regular culture medium (αMEM) without additives to produce protein without hinge region O-linked glycosylation. Manipulation of rLAMP-2 produced in CHO-LDL-D did not produce seropositivity (data not shown). Three sera reacted to the nontransfected CHO-LDL-D cells (Figure 3D).

Reactivity against LAMP-2 Synthetic Peptide

We produced a synthetic peptide of the pathogenic epitope (HGTVTYNGS) identified by Kain et al. (Figure 4). Sera were tested for reactivity by peptide ELISAs. Sera from regional healthy controls were highly reactive, thereby raising the threshold for positivity to an optical density value of 1.03 (mean + 2 SD of healthy control). Only 4% of ANCA disease samples had results >1.03, which was not statistically significant (Figure 4). Urinary tract infection samples, SLE samples, and nine samples from Kain et al. were not significantly different from healthy control samples.

Clinical Associations with rLAMP-2 Seropositivity

Supplemental Table 1 summarizes the UNC Kidney Center evaluations. The total percentage of positive seroreactivity (i.e., greater than control mean + 2 SD) on any assay for our ANCA cohort was 22.1%. Only 3 of 103 patients produced antibodies reactive against both substrates, and all 3 had new-onset disease. Similar data collected for healthy individuals with urinary tract infection showed 16.2% total seroreactivity by any assay; only four samples were positive on more than one ELISA (3.8%).

LAMP-2 reactivity was not associated with BVAS score, ANCA titer, PR3 or MPO ANCA seropositivity, disease type, or disease course (Supplemental Table 2). Of
female patients with GN were more likely to have rLAMP-2 reactivity than women from the local community with urinary tract infection.

**Injection of High-Titer Rabbit Anti–hLAMP-2 Antibodies Did Not Cause GN in Wistar Kyoto Rats**

To support the hypothesis that LAMP-2 autoantibodies are causal in human disease, Kain et al. demonstrated that injection of antibodies raised against the LAMP-2 peptide in a rabbit caused crescentic GN in Wistar Kyoto rats.\(^2\) We attempted to reproduce these results. Total IgG from a LAMP-2-peptide (HGTVTYNGS)–immunized rabbit was highly reactive with rLAMP-2 protein and LAMP-2 peptide and was cross-reactive with FimH peptide (Figure 5A). IgG from the immunized rabbit was reactive with rat leukocytes by immunofluorescence, but the preimmune serum from this rabbit was not (data not shown). Animals were injected with normal rabbit IgG (\(n=5\)) or with rabbit IgG reactive against human LAMP-2 peptide (\(n=5\)). Twenty-four hours after injection, circulating rabbit-specific IgG was detected in the five rats immunized with anti–LAMP-2 IgG (Figure 5B). Urine specimens were examined on days 1, 3, and 5 (Figure 5C), and none of the rats developed hematuria, proteinuria, or leukocyturia. Histologic examination of tissues (by J.C.J.) revealed no histologic abnormalities, including GN.

**Massachusetts General Hospital Cohort Study**

**Sera Reactivity with Commercially Produced rLAMP-2**

rLAMP-2 protein used as a substrate in this set of experiments was a recombinant protein (aa 30–127) produced in wheat germ cell-free system, in which glycosylation does not occur. Initial direct ELISAs did not identify any positive optical density above background with use of many concentrations of LAMP-2 antigen (20–800 ng per plate). Thus, an indirect assay was developed that was similar to indirect assays used for titration of PR3 sera.\(^8,13,14\) Of 72 blood bank sera, 104 ANCA-negative GN sera, 108 MPO-positive sera, and 118 PR3-positive sera evaluated for antibodies to rLAMP-2, 13.9%, 28.8%, 22%, and 20%, respectively, had detectable titers above background, although the titers were usually low, with minimal optical density values above background. The mean titers were 3.7 for blood bank sera, 6.3 for ANCA-negative sera, 4.6 for MPO-positive sera, and 3.6 for PR3-positive sera. The median titer for all groups was zero. Figure 6 shows the distribution and frequency of titers (log\(_{10}\)). There were no statistically significant difference in titers among these four groups of samples by Kruskal-Wallis test or ANOVA with post hoc tests (Supplemental Table 3).

**Indirect Immunofluorescence and Western Blot Analysis of rLAMP-2–Positive Sera**

Ten of the highest-titer rLAMP-2–positive sera without MPO-ANCA or PR3-ANCA antibodies were tested by indirect immunofluorescence to determine whether a specific pattern
Figure 6. Data generated at Massachusetts General, Boston, showed no significant seroreactivity against recombinant/non-glycosylated LAMP-2 protein. Sera samples (blood bank sera [BBS] n=72], ANCA-negative sera [n=104], MPO-ANCA–positive sera [n=108], and PR3-ANCA–positive sera [n=118]) were evaluated for antibodies against LAMP-2. The distribution and frequency of titers (log10) were defined by regression of optical densities versus antigen concentration, yielding an optical density of 0.4. The curves were linear to an optical density of 0.8. There was no statistical difference in titers among the four groups by both Kruskal-Wallis and ANOVA tests.

Comparison of LAMP-2 Titers with PR3-ANCA and MPO-ANCA in Dual-Positive Sera Staining Pattern of Anti–LAMP-2 Antibodies on Human Neutrophils

If anti–LAMP-2 antibodies coexist in patients’ sera with PR3-ANCA and MPO-ANCA as reported,2 how would they affect the results of a routine clinical immunofluorescence assay? Normal human neutrophils were stained with PR3-ANCA sera, resulting in the distinctive cytoplasmic staining pattern (Figure 7). Normal human neutrophils stained with anti–LAMP-2 antibody showed a similar cytoplasmic stain (Figure 7).
patients had antibodies binding the region of LAMP-2 that contained an amino acid sequence homologous to FimH. We synthesized a peptide containing the FimH-like sequence of LAMP-2 and screened sera for reactivity. Results indicated that both healthy controls and patients had similar reactivity in that assay.

Two recombinant LAMP-2 proteins were used as substrates for analyses presented here: one produced in a HEK line, which posttranslationally adds glycosylation moieties to the protein and one produced in a wheat germ cell-free system, rLAMP-2 (aa 30–127) protein. In the latter, glycosylation does not occur, —fluorescence staining. All samples were negative under

Table 2. Statistical analysis with 95% confidence intervals

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive/Total (n/n)</th>
<th>Proportion (%)</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Carolina</td>
<td>22/103 (21.36)</td>
<td>13.3%–29.0%</td>
<td></td>
</tr>
<tr>
<td>Massachusetts</td>
<td>48/226 (21.24%)</td>
<td>15.9%–26.6%</td>
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</table>

Table 3. Summary of LAMP-2 seroreactivity

<table>
<thead>
<tr>
<th>Population</th>
<th>ELISA Substrates</th>
<th>UNC</th>
<th>MGH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC/BBS</td>
<td>R-LAMP-2 (glycosylated)</td>
<td>2/52 (4.0)</td>
<td>10/72 (14)</td>
<td>12/124 (9.7)%</td>
</tr>
<tr>
<td></td>
<td>R-LAMP-2 (nonglycosylated)</td>
<td>0/52 (0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANCA GN</td>
<td>R-LAMP-2 (glycosylated)</td>
<td>22/103 (21.3)</td>
<td>48/226 (21)</td>
<td>75/329 (22.8)%</td>
</tr>
<tr>
<td></td>
<td>R-LAMP-2 (non-glycosylated)</td>
<td>4/101 (3.8)%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANCA-negative GN</td>
<td>R-LAMP-2 (glycosylated)</td>
<td>0/2 (0)</td>
<td>30/104 (29)</td>
<td>30/106 (28.0)%</td>
</tr>
<tr>
<td></td>
<td>R-LAMP-2 (non-glycosylated)</td>
<td>30/104 (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-LAMP-2-peptide</td>
<td>4/104 (3.8)</td>
<td>17/105 (16.2)%</td>
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</tbody>
</table>

To explore the pathogenic potential of anti–LAMP-2 antibodies, Wistar Kyoto rats were injected with anti–LAMP-2 antibodies produced by immunizing a rabbit with the pathogenic peptide. Histologic evaluation indicated no evidence of renal disease in injected animals, in contrast to what was previously reported. We acknowledge that such factors as the supplier of the particular strain of Wistar Kyoto rats and housing and food in the UNC animal facility may have influenced the susceptibility.

Similar to the results of Kain and colleagues, we found that individuals with a fimbriated bacterial infection may produce antibodies reactive with LAMP-2. However, we did not find the frequency of anti–LAMP-2–positive sera in the ANCA GN group that had been described previously. Several reasons may explain the differences. The most obvious disparity is geographic differences in the patient cohorts. Although we analyzed two independent patient groups, both of these are from the eastern United States. A second explanation is that our reagents and methods are not the same as those of Kain et al., even though every effort was made to duplicate their results, including using sera they provided as controls. Moreover, we exchanged unidentified sera samples to test the concordance between our assay and the one used by Kain and colleagues, and the overall results were similar. There was some discordance on sample-to-sample comparisons, but this did not exceed what would be expected in comparing assays that are not optimized.

In conclusion, the mechanistic association between fimbriated, bacterial infections and ANCA disease has exciting appeal, but we have not been able to confirm the evidence for this. We found very low titers of anti–LAMP-2 antibodies in human sera, although there was no difference when comparing healthy individuals with gram-negative bacterial infections, ANCA-negative GN sera and MPO-ANCA, or PR3-ANCA–positive sera. There was no correlation with disease activity or across demographic characteristics. We conclude that anti–LAMP-2 antibodies are identifiable, low-titer, natural, or induced antibodies occasionally found in the population.

CONCISE METHODS

Study Cohorts

UNC at Chapel Hill
To test for the presence of LAMP-2 autoantibodies in patients with pauci-immune GN, we chose a cohort of 103 patients with biopsy-proven
disease composed of 53 women and 50 men: 48 with MPO, 53 with PR3, and 2 with both MPO and PR3; the mean age was 53 ± 18.6 years. The BVAS, the measurement of disease activity in patients with vasculitis, was used after chart review to cumulatively define disease status of the patient cohort. As determined by BVAS, 45 of these patients had active disease (BVAS > 0) and 57 were in remission (BVAS = 0). For control populations, we tested 52 healthy individuals and 104 patients with urinary tract infection.

Massachusetts General Hospital
ANCA patient and control patient cohorts from Massachusetts General Hospital were tested in parallel for LAMP-2 autoantibodies. The cohort consisted of 226 ANCA samples, 104 ANCA-negative GN samples, and 72 blood bank controls. Sera analyzed at Massachusetts General Hospital, from patients in-house (45.6% of total samples), were considered to represent "active" disease because they were collected at initial presentation; two relapsed cases were the exceptions. The remaining 54.4% of samples were from outside referral sources and were positive for ANCA disease, but additional clinical data were not available. Positive ANCA sera were divided among patients with rapidly progressing renal failure (62.5%), patients with pulmonary involvement and hemoptysis (25%), and patients with both rapidly progressing renal failure and pulmonary disease/hemoptysis (12.5%). Of the patients with rapidly progressing renal failure only, 40% were treated with prednisone and cyclophosphamide, and 60% were treated with prednisone and cyclophosphamide, followed by rituximab. Of the patients with pulmonary ANCA disease, half were treated with prednisone and cyclophosphamide and half with prednisone and cyclophosphamide, followed by rituximab. All patients with both pulmonary and renal disease were treated with prednisone and cyclophosphamide, followed by rituximab. Patients not receiving rituximab were usually treated before 2008. One patient was diagnosed with neuropathic vasculitis and received prednisone, cyclophosphamide, and rituximab (not included in percentages).

Protein, Peptides, and Antibodies
UNC at Chapel Hill
For the recombinant protein, the extracellular domain of LAMP-2 was amplified by PCR corresponding to AA (1-359) and cloned into a pcDNA2.1 His-Tag plasmid construct (Invitrogen, Carlsbad, CA) modified with a BM40 secretion signal. Protein was produced using the HEK-293F expression system (Invitrogen). Protein from HEK-293F cell supernatant was purified using a His-Trap Column (GE Healthcare, Piscataway, NJ) by fast protein liquid chromatography. The LAMP-2 epitope (41-49) peptide and the FimH peptide were synthesized (331-341). A rabbit was immunized with the LAMP-2 peptide to produce high-titer anti-LAMP-2 total IgG for rat studies. For assay positive controls, we used a commercial polyclonal antibody raised against native full-length LAMP-2 (Abnova, Taipei City, Taiwan).

Massachusetts General Hospital
A recombinant LAMP-2(aa 30–127) protein commercially produced in a wheat germ cell-free system (Abnova) was used for ELISA and Western blot analysis. Protein translated in this system is nonglycosylated. All peptides were reconstituted to a stock solution of 1 mg/ml in 5% DMSO (Sigma) and sterile PBS. All reagents were endotoxin free. Peptides were divided into aliquots to prevent repeated freezing and thawing and were stored at −20°C until required. The peptides were handled in a sterile tissue culture hood to preserve sterility.

ELISAs and Western Blot Analyses
UNC at Chapel Hill
For the recombinant protein ELISA, LAMP-2 was coated on a Costar 96-well high-binding enzyme immunoassay/RIA plate (Corning, Lowell, MA) at 4°C overnight (10 µg/ml), blocked for 2 hours in 3% BSA (ThermoFisher Scientific, Waltham, MA), and probed with patient serum at 1:20 in 1% BSA. Reactive IgG was detected by alkaline phosphatase–conjugated goat antihuman IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA). Optical density at 405 nm was measured using a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). For the LAMP-2 and FimH peptide ELISAs, the peptides were first cross-linked on themselves to enhance plate binding using 10% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS (Invitrogen) at a concentration of 5 mg/ml for 2 days at room temperature. Nunc Polysorp plates (ThermoFisher Scientific) were irradiated for 20 minutes in a UV Stratalinker (Stratagene, La Jolla, CA) and coated with cross-linked peptide overnight at 4°C (50 µg/ml). The preceding steps of the protocol were carried out in the same manner as in the rLAMP-2 ELISA above. Western blots were used to confirm positive results on the recombinant ELISA by probing with serum in 1% blotto at 1:100 overnight (4°C) on 10 µg of rLAMP-2 antigen and MPO (Elastin Products Company, Owensville, MO) or recombinant PR3, depending on the patient’s diagnosis.

Massachusetts General Hospital
Screening of many normal and ANCA sera did not identify any positive optical density above background in a direct ELISA using many concentrations of LAMP-2 antigen (20–800 ng per plate), and an indirect assay was developed that was similar to one of the indirect assays used for titration of PR3 sera. The optimal amount of LAMP-2 antigen was determined to be about 200 ng per well, 0.035 ml of LAMP-2 antigen at 6.125 µg/ml. The remainder of the assay was conducted as previously published using phosphatase-conjugated goat anti-human IgG (heavy and light chains) (KPL Laboratories, Gaithersburg, MD), with subsequent development with p-nitro-phenyl phosphate (KPL Laboratories) at 15 minutes. Titers were defined by regression of optical densities versus multiple dilutions as the dilution giving an optical density of 0.4. The curves were linear to an optical density of 0.8. Indirect immunofluorescence to screen for pANCA and cANCA was performed as previously described. Western blots were performed as previously described.

Immunofluorescence Microscopy Assays
A cytospin was used to mount HEK-293F cells (Invitrogen) transfected with LAMP-2 onto microscope slides (ThermoFisher Scientific). Cells were fixed using acetone (ThermoFisher Scientific) and were probed with patient serum at 1:100 for 1 hour. Bound IgG was detected by immunofluorescence microscopy using FITC-conjugated goat antihuman IgG (Jackson ImmunoResearch). LAMP-2–transfected O-linked glycosylation-deficient CHO-LDL-D cells were also used as a
substrate. CHO-LDL-D cells (ATCC, Manassas, VA) were obtained with the permission of Dr. Monty Krieger. Cells were grown in varying conditions to alter the glycosylation pattern of the recombinant LAMP-2. To knock out O-linked glycosylation, cells were grown in Ham F12 medium (Invitrogen) supplemented with 5% FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). For fully glycosylated LAMP-2, 20 \( \mu \)mol/L galactose and 200 \( \mu \)mol/L N-acetylglactosamine (Sigma-Aldrich) was added to the O-linked knock-out medium. To knock out both forms of glycosylation, cells were grown in 50% OPTI-MEM, 47% αMEM, and 3% dialyzed FBS (Invitrogen) (M. Krieger, personal communication). CHO cells were grown on 8-well Chemically Coated 2nd generation (Lab-Tek II CC2)–treated microscope slides (ThermoFisher Scientific) to 70% confluence and were fixed with 4% paraformaldehyde, permeabilized with pure methanol at -20°C (ThermoFisher Scientific) for 10 minutes, and blocked with 5% goat serum in 0.05% triton X-100 (ThermoFisher Scientific).

Patient serum was diluted at 1:100, and FITC-conjugated goat anti-human IgG, preimmune and postimmunization with LAMP-2 peptide (Sigma) at a concentration of 1:100. Rabbit total IgG was transferred with Chemstrip 10 MD urine test strips (Roche). Antibodies and sera samples were collected on days 0, 1, 3, and 5, and urinalysis was performed with Chemstrip 10 MD urine test strips (Roche). Antibodies to LAMP-2 were produced in a rabbit (Robinson services) by immunizing with LAMP-2 peptide (HGTVTYNGS) in Freund complete adjuvant; subsequent boosts were in incomplete Freund adjuvant.

A rat ANCA test was performed on a rat total leukocyte preparation from healthy rats. Total leukocytes were placed onto slides using a cytospin and stained with a positive control: LAMP-2 antibody produced in a rabbit (Sigma) at a concentration of 1:100. Rabbit total IgG, preimmune and postimmunization with LAMP-2 peptide (HGTVTYNGS), was purified from sera with sepharose protein A/G beads (Santa Cruz Biotechnologies), and used at 3 mg/ml, followed by a goat antirabbit FITC-conjugated secondary (Jackson Immunology) at 1:200. All slides were reviewed by J. Charles Jennette.

**In Vivo Testing of LAMP-2 Antibody Pathogenicity**

Total IgG was purified from rabbit serum using a Hi-Trap Protein G column (GE Healthcare) using fast protein liquid chromatography. Wistar Kyoto rats were obtained from Harlan Sprague–Dawley, and were age- and weight-matched at about 80 g. Ten grams of anti-LAMP-2 high-titer total IgG or normal rabbit IgG was transferred by tail vein injection into rats (five rats per group). Rats were killed after 5 days. Kidneys were harvested for histologic analysis, fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin and periodic acid–Schiff stains. To ensure successful transfer of rabbit IgG, serum was obtained 24 hours after injection and coated on a Costar 96-well high-binding enzyme immunoassay/RIA plate overnight at 4°C and blocked with 3% BSA for 1 hour. Bound IgG was detected using alkaline phosphatase–conjugated goat antirabbit IgG (Jackson ImmunoResearch). Urine samples were collected on days 0, 1, 3, and 5, and urinalysis was performed with Chemstrip 10 MD urine test strips (Roche). Antibodies to LAMP-2 were produced in a New Zealand white rabbit (Robinson services) by immunizing with LAMP-2 peptide (HGTVTYNGS) in Freund complete adjuvant; subsequent boosts were in incomplete Freund adjuvant.

A rat ANCA test was performed on a rat total leukocyte preparation from healthy rats. Total leukocytes were placed onto slides using a cytospin and stained with a positive control: LAMP-2 antibody produced in a rabbit (Sigma) at a concentration of 1:100. Rabbit total IgG, preimmune and postimmunization with LAMP-2 peptide (HGTVTYNGS), was purified from sera with sepharose protein A/G beads (Santa Cruz Biotechnologies), and used at 3 mg/ml, followed by a goat antirabbit FITC-conjugated secondary (Jackson Immunology) at 1:200. All slides were reviewed by J. Charles Jennette.

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**DISCLOSURES**

None.

**REFERENCES**


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