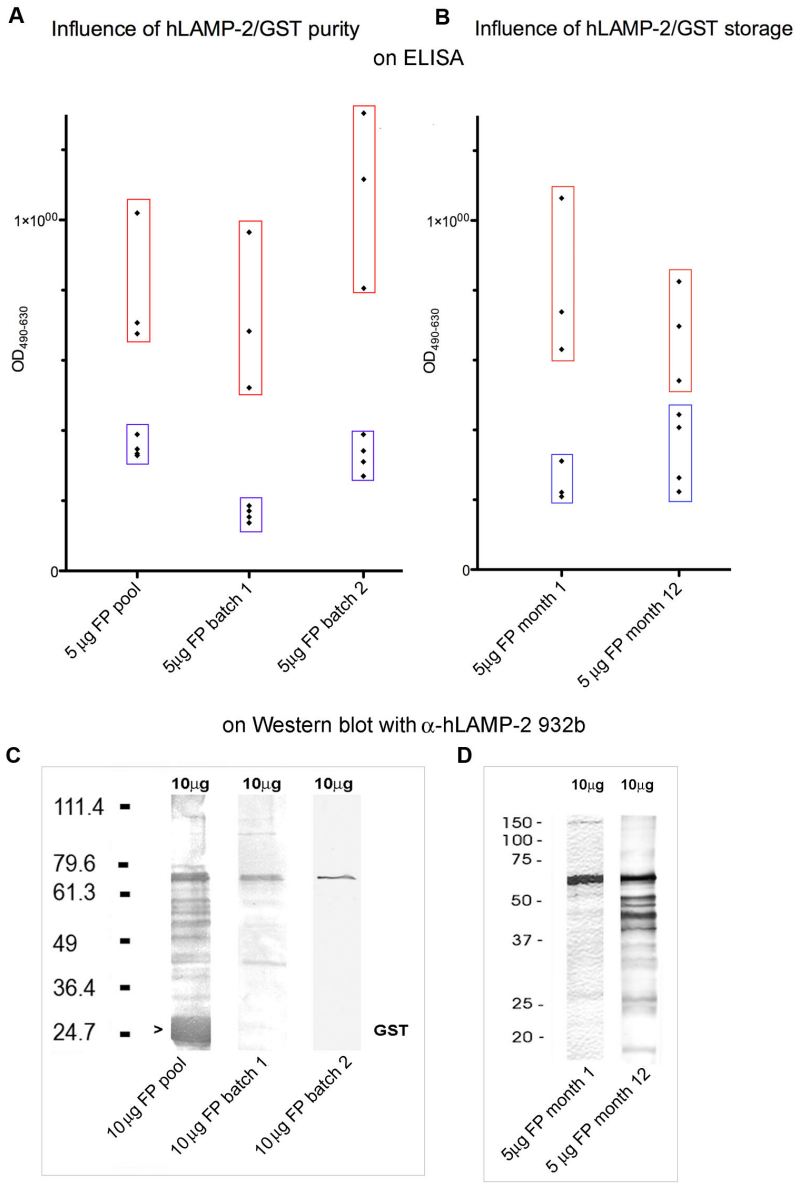
Multiple assays for antibodies to hLAMP-2 confirm their high prevalence in active pauci-immune focal necrotizing glomerulonephritis

Supplementary material

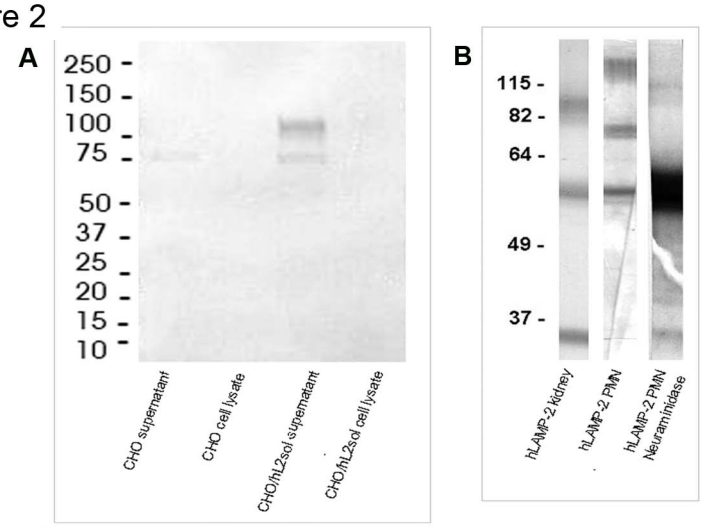
Supplementary Figure 1

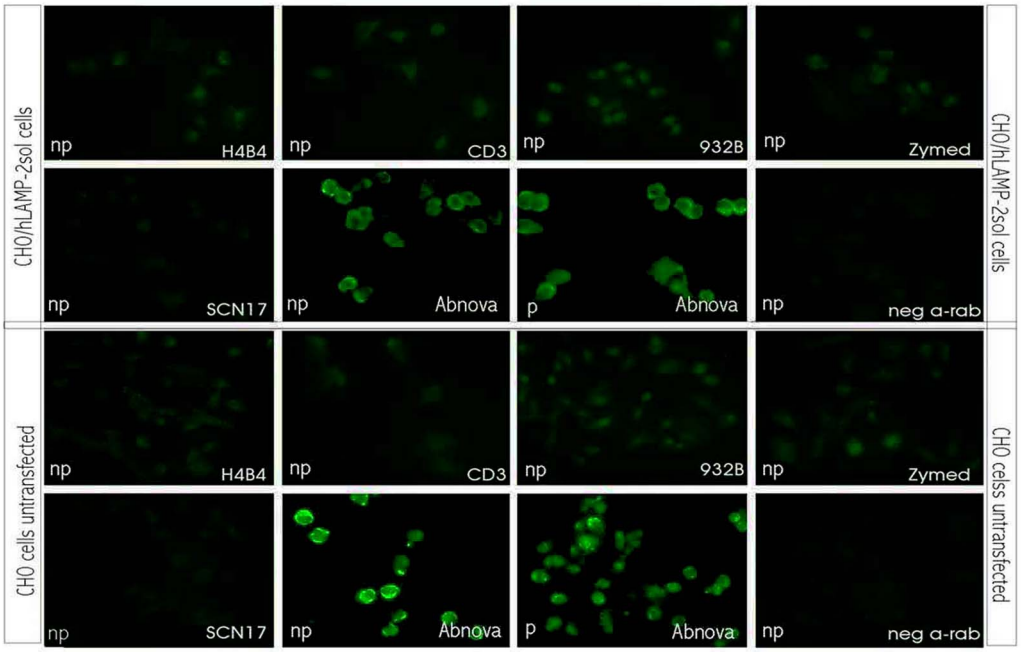


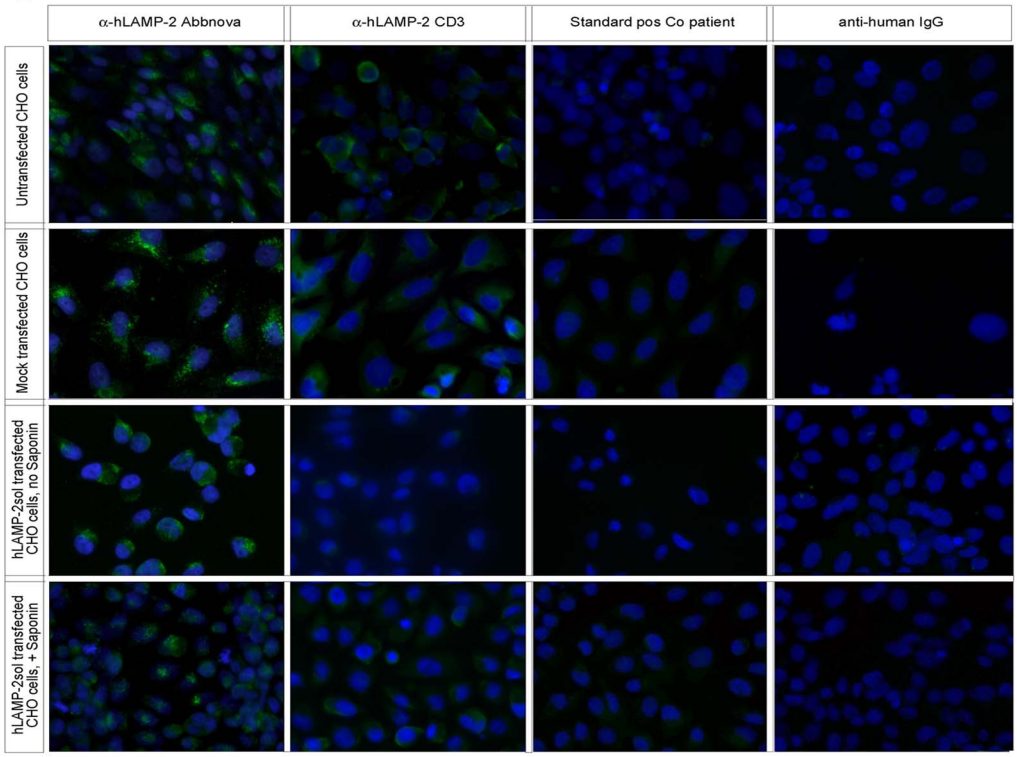
Supplementary Figure 1: Influence of purity and storage of hLAMP-2/GST fusion protein on ELISA and Western blot

Recombinant hLAMP-2/GST was prepared in batches and assessed as substrate for the ELISA using three positive and four negative sera as assay controls (A and B) and related to the purity of the preparations assessed by western blotting after separation on SDS-PAGE (C and D). Large scale cultures and storage of pellets before purification increases degradation and contamination with other proteins and reduces separation between positive and negative sera in the ELISA (A and C). Consequently hLAMP-2/GST was routinely prepared in batches of less than 10mg and tested for purity before use. Some batches (Batch 1) contained minor degrees of degradation which also affected binding in the ELISA (Batch 1 in A and C) whereas others were free from degradation (Batch 2 in A and D). Only hLAMP-2/GST batches without detectable degradation were used to coat ELISA plates. hLAMP-2/GST preparations were used within 3 months because it degrades when stored for long periods even at -80°C (B and D).

Supplementary Figure 2







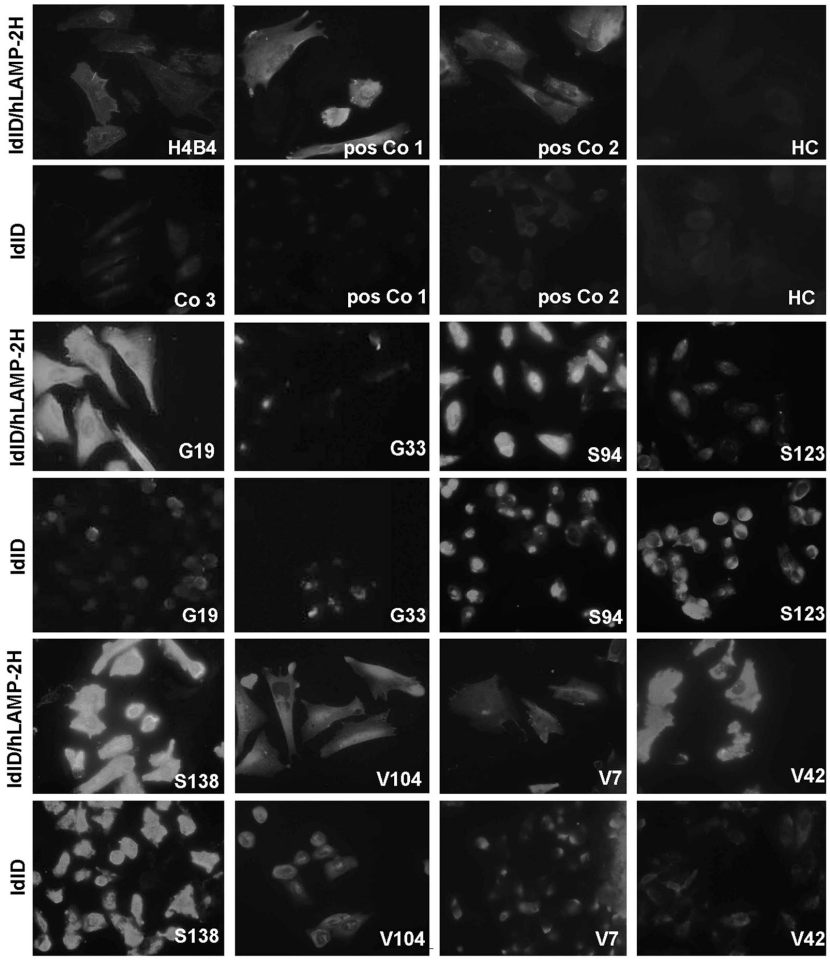
Supplementary Figure 2: Generation of glycosylated soluble hLAMP-2sol in CHO DG44 cells

We transfected CHO DG44 cells with the hLAMP-2sol extracellular domain to assess the complexity of glycosylation in western blots using a human specific antibody (932B) that does not cross-react with hamster LAMP-2 to detect the protein. The resulting soluble protein is entirely secreted into the supernatant of stably transfected cells and cannot be detected in the cell lysates. It has a molecular mass of approximately 110 kDa (A, lane 3) which is similar to native human glomerular hLAMP-2 (B, lane 1) reflecting glycosylation of equivalent complexity but lower than neutrophil hLAMP-2 (B, lane 2) even after removal of sialic acid residues (B, lane 3). The hLAMP-2 extracellular domain cannot be detected in the cell pellet (A, lane 4) confirming it is exclusive release from the ldld cells by the constitutive secretary pathway, as would be expected for a soluble protein. Similarly hLAMP-2 extracellular domain cannot be detected in either cell pellet or supernatant of untransfected cells by anti-hLAMP-2 antibodies that do not cross-react with hamster LAMP-2 (A, lanes 1 and 2).

CHO parental cells and cells transfected with hLAMP-2 extracellular domain (CHO/hLAMP-2sol) were probed by IIF using a set of antibodies to hLAMP-2. The commercially available anti-hLAMP-2 antibodies tested (monoclonal (H4B4) and polyclonal antibodies (SCN17 and Abnova) cross-react with hamster LAMP-2 in a lysosomal staining pattern after permeabilsation (p) in both, stably transfected (CHO/hLAMP-2sol) and untransfected cells (CHO) (C). A monoclonal antibodies (CD3) and rabbit anti-hLAMP-2 (932B) that are specific for human LAMP-2 do not bind to untransfected CHO cells (C; CHO untransfected) nor did they react with CHO cells carrying the transgene for the soluble extracellular domain of hLAMP-2 that is entirely secreted into the cell culture supernatant (C; CHO/hLAMP-2sol) as demonstrated in panel A.

These results were confirmed in transiently transfected cells (D). In this experiment, CHO DG44 cells were transfected with either hLAMP-2 extracellular domain in pCDNA3 (CHO/hLAMP-2sol) or pCDNCA3 alone (mock transfected cells). Cells were stained either untreated or after after fixation with 4%PFA and permeabilization with saponin. Staining with the human specific monoclonal CD3 anti-LAMP-2 antibody was uniformly negative as was a standard human positive control serum. By contrast, the rabbit anti-hLAMP-2 IgG (Abnova) that cross-reacts with hamster LAMP-2 and binds to untransfected CHO DG44 cells showed increased binding after transient transfection with CHO/hLAMP-2sol and after mock transfection. A polyclonal antibody to rat LAMP-2 (C) and secondary antibody alone do not detect either human or hamster LAMP-2 and was used as a further negative control (C and D).

Supplementary Figure 3



Supplementary Figure 3: Staining pattern of human sera on ldlD and ldlDhLAMP-2H cells

ldlD parental cells and cells stably transfected to express hLAMP-2 on the cell surface were grown on 8 well chamber slides and used to assay the presence of antibodies to hLAMP-2 in patients' sera in a dilution of 1:40. Slides were viewed and assessed independently by two viewers without knowledge of clinical data or results of ELISA or Western blot results. Pictures were taken at fixed exposure time that was determined as the average exposure time measured using one serum each moderately (pos Co 1) and a weakly (pos Co 2) reactive with hLAMP-2 that had been shown to be positive (G19), weakly positive (V104) or negative (V42) by anti-LAMP-2 antibody ELISA. Also negative (V7) or low positive (G33) ELISA results were thus confirmed as negative.

Comparison by IIF of ldlD/hLAMP-2H cells and untransfected controls enabled us to discount results form interference from antibodies that bound nuclei or other intracellular organelles or that bound non-specifically to ldld cells. Sera that bound equally well to both ldldD parental and ldlD/hLAMP-2H cells equally were scored negative (S138), as were sera with strong nuclear or cytoplasmic background staining in untransfected cells (S94 and S123). Percentage of positive cells and staining intensity was assessed in each experiment with moAb H4B4. All sera from healthy controls (HC) or with repeatedly negative ELISA assays (Co 3) were negative.