Supplementary Information

IDENTIFICATION OF PATHOGENIC EPITOPES IN PAUCI-IMMUNE CRESCENTIC GLOMERULONEPHRITIS

Molecular Mimicry of Lysosomal Membrane Glycoprotein 2 (hLAMP-2) and the Bacterial Adhesin FimH

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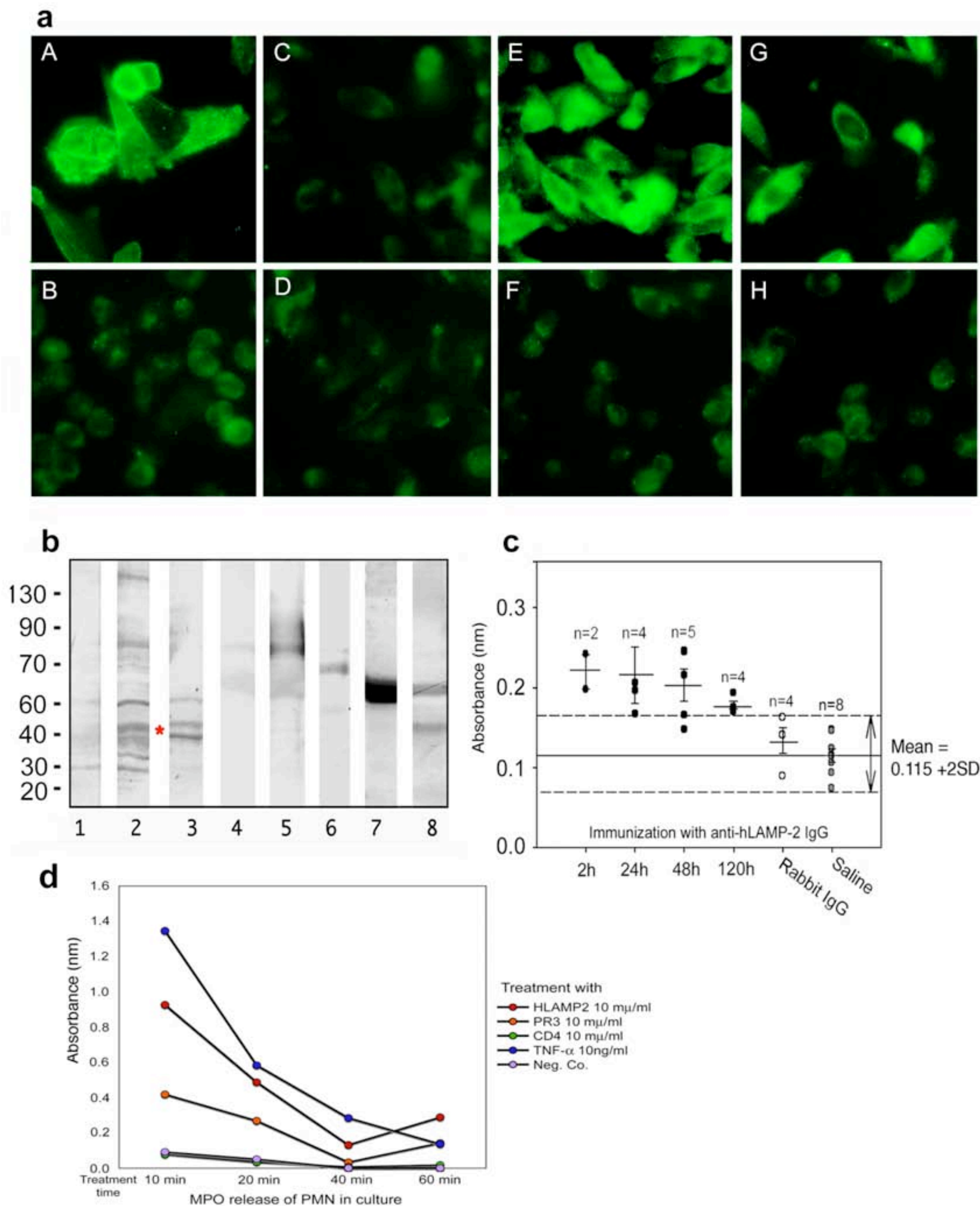
Supplementary Figure 1: Characterization of antibodies to hLAMP-2



(**a**) Parental ldlD (B, F, H) and ldlD cells stably expressing hLAMP-2 on the cell surface (ldlD/hLAMP-2H - A, C, D, E, G) were probed with a rabbit antiserum to hLAMP-2 (A), human serum from a healthy volunteer (C), or sera from two anti-hLAMP-2 positive patients with pauci-immune FNGN (E-F, G-H). Patients' IgG from hLAMP-2 positive sera bound specifically to ldlD/hLAMP-2H but not parental ldlD cells while sera from controls failed to bind to both parental and transfected cells. Anti-rabbit and anti-human IgG conjugated to FITC alone were used as control (B, D). Representative samples of 18 patients tested are shown.

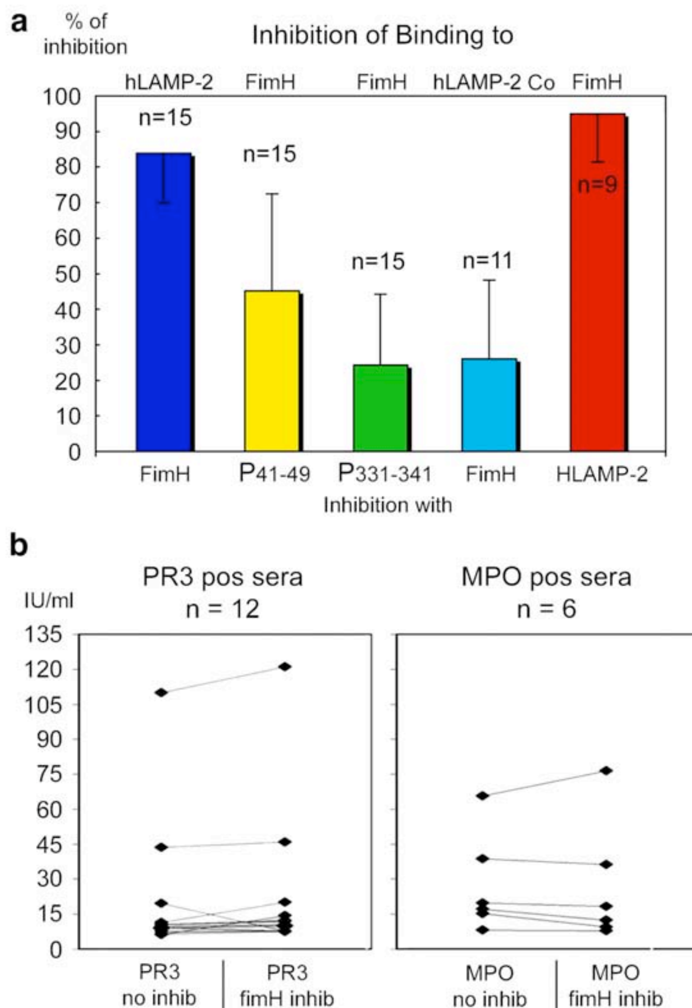
(**b**) Bacterial lysates and recombinant *E. coli* and mammalian expressed hLAMP-2 fusion proteins

were probed with a monoclonal antibody to hLAMP-2. Crude lysates of BL21/DE3 (lane 1) did not contain a band corresponding to the hLAMP-2 fusion protein that is visible in the BL21/DE3hl2sol cell lysate (lane 2) or with purified His-tagged hLAMP-2 (lane 3). Supernatants from CHO cells that express soluble hLAMP-2 was used in ELISA experiments either untreated (lane 5) and treated with PNGaseF to remove *N*-glycans (lane 6). Supernatant from untransfected CHO cells (lane 4) served as control. The purity of the hLAMP-2 GST-fusion protein used for all the inhibition experiments was confirmed by SDS-PAGE, (lane 7, Coomassie stain) and immunoblotting with hLAMP-2 specific IgG (lane 8).

(**c**) Sera of rats passively immunized with rabbit IgG to hLAMP-2 and culled at 2h, 24h, 48h and 120h were assayed by standard hLAMP-2 ELISA. Sera from animals injected with saline and normal rabbit IgG were used as controls. However antibodies to hLAMP-2 were detectable but at a lower concentration than after active immunization (see also **Supplementary Figure 3b**), and their values declined from 2h to 120h are evident.

(**d**) Purified human granulocytes were incubated for up to 60 minutes with 10 μg per ml of monoclonal antibodies to hLAMP-2 (H4B4), PR3 (1F11) or CD4: neutrophils treated with 10 ng per ml TNF-α were used as a positive controls. The supernatants were assayed for MPO concentrations using a standard ELISA. MPO is released into the supernatant in response to TNF-α and antibodies to hLAMP-2 and PR3 but is rapidly destroyed. The figure presents results from one experiment representative of three. The mean concentrations of MPO in the supernatants expressed as percentage of MPO released by TNF-α were: H4B4 - 83% (range: 80-85%); 1F11 - 57% (40-90%); CD4 -10% (0-21%). Results with both H4B4 and 1F11 were significantly different to control ($P < 0.05$ Wilcoxon).

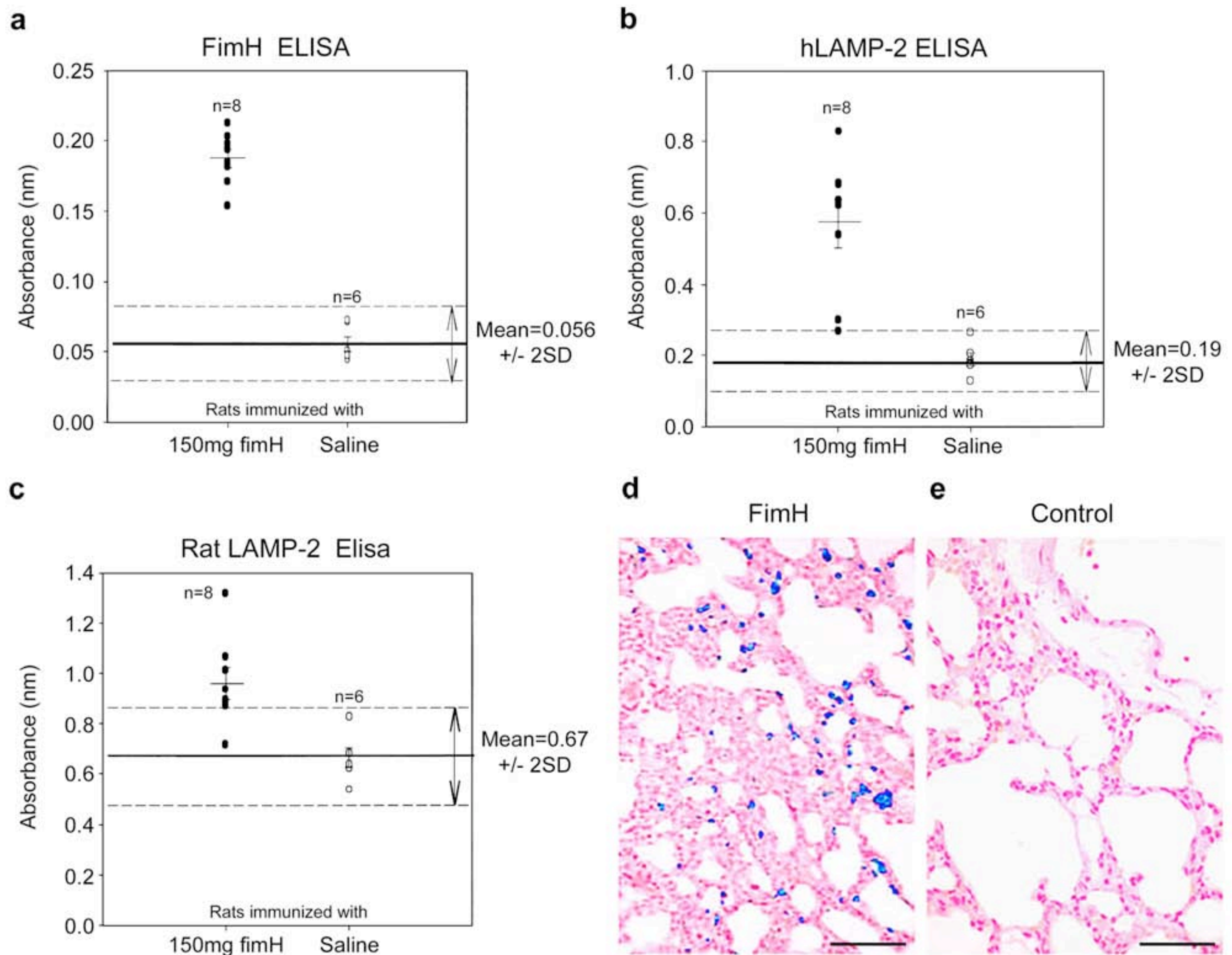
Supplentary Figure 2: Cross-reactivity of autoantibodies to hLAMP-2 with FimH



(**a**) 15 sera with hLAMP-2 specificity and 11 sera of healthy and hLAMP-2 negative disease controls were used to assess the relationship of antibodies to hLAMP-2 with fimH. Pre-incubation with fimH fusion protein specifically inhibited autoantibody binding to hLAMP-2 binding by 84% (±13.9). By contrast, fimH had no effect on hLAMP-2 binding in controls. Similarly, recombinant hLAMP-2 fusion protein inhibited patients' anti-hLAMP-2 positive sera from binding to fimH binding by 95% (±13.5). Binding to fimH was also inhibited by peptide $P_{41–49}$ (mean 45.1% ±27.1) but not by peptide $P_{331–341}$ is not significant ($P = 0.085$).

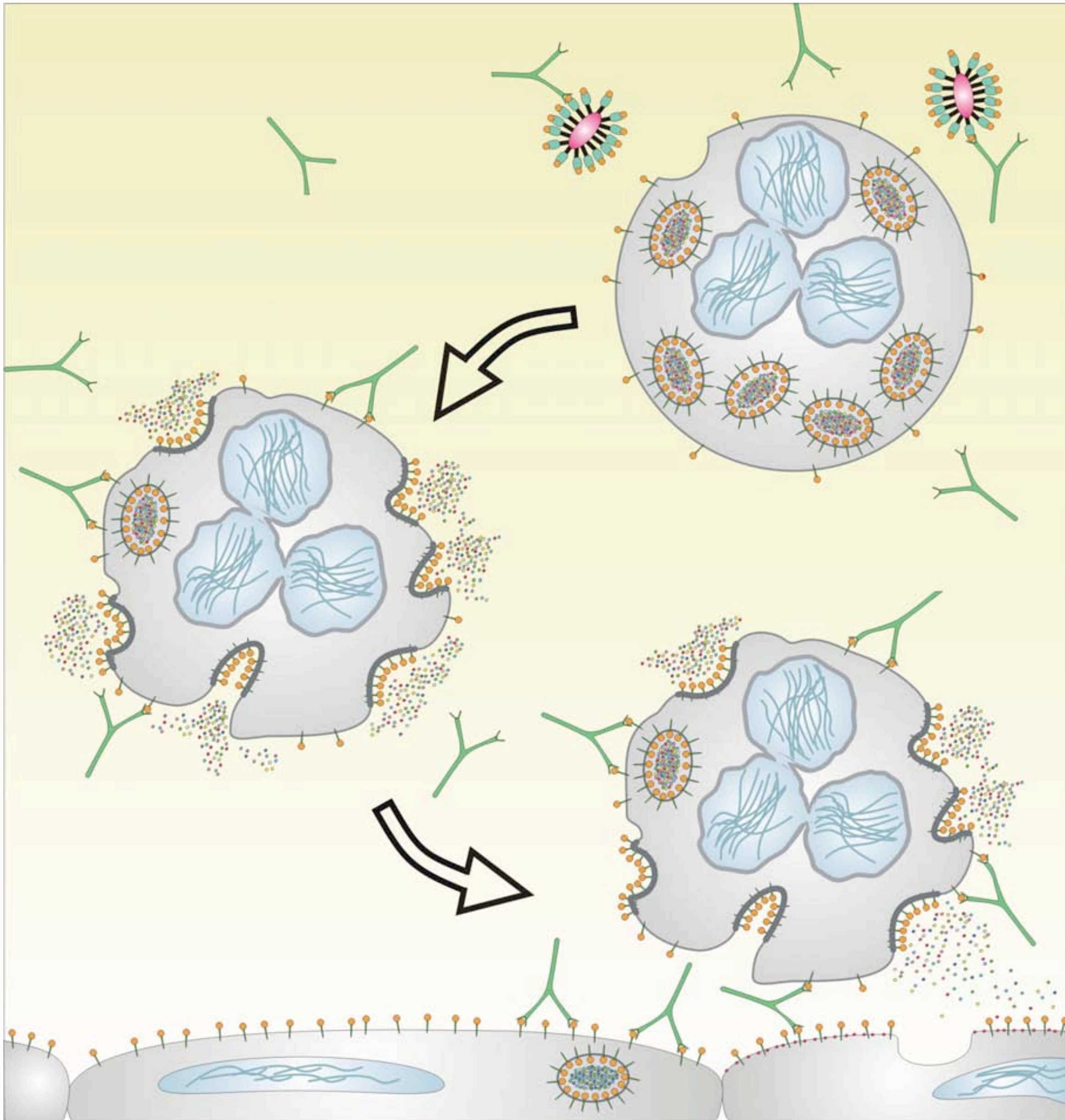
(**b**) The sera from 18 hLAMP-2 positive patients with pauci-immune FNGN also had autoantibodies to PR3 (n=12) or MPO (n=6). Pre-incubation with recombinant fimH did not inhibit binding of the autoantibodies to either of these ANCA antigens in specific ELISA.

Supplementary Figure 3: Antibody levels and lung pathology in FimH immunizsed rats



Eight rats were immunized with 150 micrograms of FimH using the protocol described in the methods and their sera taken at post mortem examination. The sera were assayed for antibodies to FimH (**a**), hLAMP-2 (**b**) and rat LAMP-2 (**c**) using specific ELISA. All the immunized rats developed antibodies to FimH. The antibodies cross-reacted with human and rat LAMP-2 in 7 of them. Lung histology revealed, in addition to neutrophilic capillaritis in one animal (see also Fig 4g), and haemosiderin deposition in a further rat (**d**), indicating protracted lung bleeding. (**e**) All control animals showed normal lung histology (Prussian blue reaction; Scale-bars d-e, 50 μm).

Supplementary Figure 4: Proposed mechanism of hLAMP-2 induced injury



In susceptible individuals, natural exposure to fimH during infection induces synthesis of antibodies to an epitope shared by shared by fimH ($P_{72\text{-}80}$) and hLAMP-2 ($P_{41\text{–}49}$) and that that these antibodies react with both antigens (1). We propose that the cross-reacting antibodies are pathogenetic and cause vasculitis in three ways: first by binding to hLAMP-2 on the granulocyte surface and activating neutrophils and triggering degranulation of lysosomal contents, similar to mechanisms described for canonical ANCA targets[23,24] (2), second by binding to hLAMP-2 on endothelial cells which they both activate and kill by inducing apoptosis; and third by cross-linking hLAMP-2 on the cell surface of granulocytes and microvascular endothelial cells and so promoting adherence (3) - the critical step in the pathogenesis of FNGN and vasculitis more generally.

Supplementary Table 1: Alignment of epitopes $P_{41–49}$ and $P_{331–341}$ with PR3 sequences

A

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                1                                                          50                                                         100
PR3;NP_002777   MAHRPPSPAL ASVLLALLLS GAARAAEIVG GHEAQPHSRP YMASLQMRGN PGSHFCGGTL IHPSFVLTAA HCLRDIPQRL VNVVLGAHNV RTQEPTQQHF
PR3;P24158      MAHRPPSPAL ASVLLALLLS GAARAAEIVG GHEAQPHSRP YMASLQMRGN PGSHFCGGTL IHPSFVLTAP HCLRDIPQRL VNVVLGAHNV RTQEPTQQHF

P41-49          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
P331-341        .......... .......... .......... .......... .......... .......... .......... .......... .......... ......QGKY

                101                                                        150                                                        200
PR3;NP_002777   SVAQVFLNNY DAENKLNDVL LIQLSSPANL SASVATVQLP QQDQPVPHGT QCLAMGWGRV GAHDPPAQVL QELNVTVVTF FCRPHNICTF VPRRKAGICF
PR3;P24158      SVAQVFLNNY DAENKLNDIL LIQLSSPANL SASVTSVQLP QQDQPVPHGT QCLAMGWGRV GAHDPPAQVL QELNVTVVTF FCRPHNICTF VPRRKAGICF

P41-49          .......... .......... .......... .......... .......HGT VTYNGS.... .......... .......... .......... ..........
P331-341        STAQDCS... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                201                                                        250
PR3;NP_002777   GDSGGPLICD GIIQGIDSFV IWGCATRLFP DFFTRVALYV DWIRSTLRRV EAKGRP
PR3;P24158      GDSGGPLICD GIIQGIDSFV IWGCATRLFP DFFTRVALYV DWIRSTLRRV EAKGRP

P41-49          .......... .......... .......... .......... .......... ......
P331-341        .......... .......... .......... .......... .......... ......
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B

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                1                                                          50                                                         100
cPR3-1;NP_002777 TRAASVQRSA SRVWSQARGP GQRCGRGGSG AALGLHTAQR RTDPVHVEGY PREEVREKAG GTSPDHEGVY SLDDAITDQG AT*VSEADAG LAARDESANV
cPR3-2;P24158    .......... .......... .......... .......... ....PGGETE AQHEATQLRG GRGTLAAALL PSWQPGARGG GTVTYEADAG LAARDESANV

P41-49           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
P331-341         .......... .......... .......... .......... .......... .......... .......... .........H GTVTYNGS.. ..........

                 101                                                        150                                                        200
cPR3-1;NP_002777 MWPAEEGHHG DIELLQDLGW GVVGTHAAPA HGQALGAVGH WLVLLWQLD* GDGRTEVGWA AQLDEENVVQ FVLRVVVVQK HLSHREVLLG GLLRPHVVGS
cPR3-2;P24158    MWPAEEGDHG DIELLQDLGW GVVGTHAAPA HGQALGAVGH WLVLLWQLDC GDGGTEVGWA AQLGQKAGGG ETLLPGHGGR PGRPPTTPTT PTTSKRRGCR

P41-49           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
P331-341         ........QG KYSTAQDCS. .......... .......... .......... .......... .......... .......... .......... ..........

                 201                                                        250                                                        300
cPR3-1;NP_002777 EHHVHQALGY VPQAVRGRQH EAGVDQGASA EVAARVPPHL QGGHVGPGVW LRLVPAHDLR SSGSTAQQQG QQHGRQGRAG GPVSHGGVQG APTIKLLL*P
cPR3-2;P24158    SLRLGPTTPF VPFVTIVFIH FLSFFLSFEM ESCSATQAGM QWR....... .......... .......... .......... .......... ..........

P41-49           .......... .......... .......... .......... .......... .......... .......... .........H GTVTYNGS.. ..........
P331-341         .......... .......... .......... .......... .......... .........Q GKYSTAQDCS .......... .......... ..........
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HLAMP-2 peptide epitopes P_{41-41} and $P_{331–341}$ were aligned with protein sequences of PR3 (A) and its complementary read cPR3 (B) published at Entrez [The National Center for Biotechnology Information (NCBI)]. The sequence in the upper line (B) corresponds to cPR3 published by Pendergraft[22] (NP_002777), the sequence in the lower line to a protein derived from the same complementary reading frame of an alternate PR3/myeloblastin sequence (P24158). There were no significant homologies to either PR3 or cPR3 nor any in the three possible reading frames of MPO or cMPO.

Supplementary Table 2: Bacterial strains used in for Immunoblot and inhibition experiments

Strain	Type	Host	Source
E. coli **23510** and 23522 (Migula) Castellani and Chalmers	O32:K.:H19	Calf, piglets and mice, facultative in humans	ATCC 23510 and 23522
E. coli **12900**	O157:H7, nontoxigenic	Human, cattle	ATCC 700728, NCTC 12900
Klebsiella pneumoniae subsp. *pneumoniae* (Schroeter) Trevisan	type 54	Human	ATCC 12657 and 12658
Klebsiellla pneumoniae	Wild type	Human	Isolated from sputum
Proteus vulgaris	Wild type	Human	Isolated from urine
Staphylococcus aureus	Wild type	Human	Isolated from tonsilary abscess

Proteins of bacterial strains detailed were used to assess patients' antibody profiles by Western blot analysis and selected *E. coli, Klebsiella, Proteus* and *Staphylococcus aureus* strains (bold) were used in inhibition assays.

Supplementary Table 3: Patients' characteristics

			hLAMP-2							hLAMP-2				
	Patient	Age/ gender	at presentation/ relapse	at remission	IF	ANCA ELISA	Clinical diagnosis/ Renal biopsy	Patient	Age/ gender	at presentation/ relapse	at remission	IF	ANCA ELISA	Clinical diagnosis/ Renal biopsy
GROUP B	1 **A** +	44 m	+ (on, rel)	neg	c	PR3	WG, FNGN	43 AQ	45 m	+ (on)	NA	c	PR3	WG, FNGN
	2 **B**	56 m	+ (rel)	neg	c	PR3	WG, FNGN	44 AR	60 f	+ (on)	neg	c	PR3	WG, FNGN
	3 **C**	66 m	+ (on)	NA	c/p	MPO	WG, FNGN	45 AS	50 f	+ (on)	neg	c	PR3	WG, FNGN
	4 **D**	75 f	+ (on, rel)	neg	c/p	MPO	MPA, FNGN	46 AT	70 f	+ (on, rel)	neg	c	PR3	WG, FNGN
	5 E	67 f	+ (on, rel)	neg	c	PR3	WG, FNGN	47 AU	78 m	neg	neg	p	MPO	MPA, FNGN
	6 F	46 m	+ (on, rel)	neg	c	PR3	WG, FNGN	48 AV	90 f	+ (on)	NA	p	MPO	MPA, FNGN
	7 **G**	60 m	+ (rel)	neg	c	PR3	WG, FNGN	49 AW	46 m	neg (ther)	NA	c	PR3	WG, FNGN
	8 **H** +	43 f	neg (plasma)	NA	c/p	PR3/ MPO	FNGN	50 AX	59 m	+ (on)	neg	p	MPO	MPA, FNGN
	9 **I**	66 f	neg (plasma)	NA	c	PR3	WG, FNGN	51 AY	58 m	+ (on)	neg	c	PR3	WG, FNGN
	10 J	44 m	neg	neg	c	PR3	WG, non renal	52 AZ	37 m	+ (on)	neg	c	PR3	WG, FNGN
	11 K	8 m	+ (rel)	neg	neg	neg	FNGN	53 BA	69 f	+ (on)	neg	p	MPO	MPA, FNGN
	12 K	48 m	+ (rel)	neg	c/p	MPO	MPA, FNGNre	54BB	38 f	+ (on, rel)	neg	c	PR3	WG, FNGN
	13 M	53 m	+ (rel)	neg	c	neg	WG, FNGN	55 BC	72 f	+ (on)	neg	c	PR3	WG, FNGN
	14 N	25 f	+ (rel, TX)	neg	c	PR3	WG, FNGN	56 BD	41 f	neg (ther)	NA	p	MPO	MPA, FNGN
	15 O +	65 f	+ (rel, TX)	neg	c/p	MPO	WG, FNGN	57 BE	57 f	+ (on, rel)	neg (1x pos)	c	PR3	WG, FNGN
	16 P	48 f	+ (on, rel)	neg	c	neg	FNGN	58 BF	70 f	+ (on)	neg	c	PR3	WG, FNGN
	17 Q	52 f	+ (rel)	neg	c/p	MPO	WG, FNGN	59 BG	56 f	+ (on)	neg	c/ANA	PR3	WG, FNGN
	18 R	57 f	+ (rel, TX)	neg	p	MPO	MPA, FNGNre	60 BH	77 f	+ (on)	NA	c	PR3/MPO	WG, FNGN
	19 **S**	80 f	+ (rel)	neg	c	neg	FNGNre	61 BI	30 m	+ (on)	neg	p	MPO	MPA, FNGN
	20 T	74 f	+ (rel)	neg	c/p	MPO/PR3	WG, FNGN	62 BJ	68 m	+ (rel)	neg	c	PR3	WG, FNGN
	21 U	53 m	± (on)+ (rel)	neg	c	PR3	FNGN	63 BK	64 f	+ (on, rel)	neg	c	PR3	WG, FNGN
	22 V	56 m	+ (rel, TX)	neg	p	neg	FNGN	64 BL	70 f	+ (on)	NA	p	MPO	MPA, FNGN
	23 W	59 m	+ (on)	neg	p	MPO	WG, FNGNre	65 BM	65 f	+ (on, rel)	neg (2x pos)	c/p	MPO	FNGNre
	24 **X**	72 m	+ (on, rel)	pos	p	MPO	CSS, FNGN	66 **BN** +	69 m	+ (on)	NA	c/p	MPO/PR3	WG, FNGN
	25 Y	22 f	+ (rel)	neg	c	neg	CSS, FNGNre	67 **BO**	66 m	+ (on)	NA	c/p	MPO	WG, FNGNre
	26 Z	58 m	+ (rel)	neg	p	MPO	FNGNre	68 BP	63 m	+ (rel)	NA	c	neg (AGBM)	WG, FNGN
	27 AA	50 m	+ (rel)	neg	p	MPO	WG, FNGN	69 BQ	73 f	+ (on)	NA	p	MPO	MPA, FNGN
	28 AB	43 f	+ (rel, TX)	neg (2x pos)	p	MPO	FNGN	70 BR	17 f	+ (rel)	NA	neg	neg	FNGN
	29 AC	29 m	+ (on, rel)	pos	c	PR3	MPA, FNGN	71 BS	63 m	+ (rel)	NA	c	PR3	WG, FNGNre
	30 AD	30 m	+ (rel)	neg	c	PR3	WG, FNGNre	72 BT	71 f	+ (on)	neg	c	PR3	FNGN
	31 AE	52 f	+ (rel)	pos	c/p	MPO/ PR3	FNGN, vasc.	73 BU	84 f	+ (on)	NA	p	MPO	FNGNre
	32 AF	35 f	± (rel)	neg	c/p	MPO/ PR3	WG, FNGN	74 **BV** +	35 m	+ (rel)	neg	c	PR3	WG, FNGN
	33 AG	54 m	+ (rel)	NA	p	MPO	MPA, FNGN	75 **BW** +	52 f	+ (rel TX)	NA	c/p	MPO	MPA, FNGNre

34 AH	57 m	+ (on)	NA	p	MPO	MPA, FNGN	76 BX	70 m	+ (rel)	NA	p	neg	WG, FNGNre
35 AI	65 f	+ (on)	NA	p	MPO	MPA, FNGN	77 BY	57 m	+ (on, rel)	neg (1x pos)	c	neg	FNGNre
36 AJ	44 m	+ (on)	neg	c	PR3	WG, FNGN	78 BZ	66 f	+ (on)	NA	c	PR3	WG, FNGNre
37 AK	58 f	+ (on)	NA	p	MPO	WG, FNGNre	79 CA	65 m	+ (rel)	NA	c	neg	MPA, FNGN
38 AL	42 m	+ (rel)	neg	c	PR3/ (MPO)	FNGNre	80 CB	22 f	+ (on, rel)	NA	c	PR3	WG, FNGN
39 AM	33 m	+ (on,)	neg	neg	neg	FNGN	81 CC	77 f	+ (on)	NA	c/p	MPO	MPA, FNGN
40 AN	65 f	+ (on, rel)	neg	c	PR3	WG, FNGNre	82 CD	44 f	+ (on)	NA	p	MPO	FNGN
41 AO	63 f	+ (on)	NA	p	MPO	MPA, FNGNre	83 CE	60 m	+ (rel)	neg	p	neg	MPA/PAN, vasc
42 AP	40 m	+ (rel)	neg	c/p	MPO	WG, FNGNre	84 CF	55 f	+ (on)	NA	c	neg	WG, FNGN

A cohort of 84 patients (44 female, 56.5±18.6 years; 40 male, 52±14.2 years) were included in this study. The prevalence of autoantibodies to hLAMP-2 was calculated from sera taken when the disease was active either at presentation or on relapse (rel, TX: serum from relapse in renal transplant). Serum was available from 54 of these patients when in remission that was defined as having no evidence of active vasculitis clinically for at least 2 months before the serum sample and three months thereafter. Sera from a subset of 32 patients (group B) were used in Western blot and immunofluorescence studies to determine hLAMP-2 specificity and glycosylation dependence of autoantibodies. Sera from eleven patients (designation in **bold**) were selected for SPOTs assay to determine epitope specificity of the autoantibodies to hLAMP-2. At least one serum from an active stage of disease and, where available, from remission, were assayed for the presence of ANCA and specificity for MPO and PR3. Clinical diagnoses, made according the the Chapel Hill criteria, were Wegener's granulomatosis (WG - 45 patients) including one patient with WG limited to the upper respiratory tract (WG - non renal); microscopic polyangiitis (MPA - 20) and Churg Strauss syndrome (CSS - 2). 17 patients had isolated focal necrotizing glomerulonephritis. Disease activity was determined clinically and presence of active renal disease (FNGN) or FNGN with signs of recurrent progression (FNGNre) were confirmed histologically. Tissue samples and serum from post mortem examination was available in 6 patients (+). Patient samples were taken for routine clinical management and their use conformed to legal regulations in Austria and the United Kingdom and was granted by the Research Ethics Committes of Grampian University Hospitals NHS Trust and the Medical University of Vienna. hLAMP-2 ELISA results: + = assay positive; neg = assay negative; NA: samples or data not available. ANCA IF: c = cANCA, p = pANCA. ANCA ELISA: sera assayed positive for myeloperoxidase (MPO) or proteinase 3 (PR3).