CHARACTERIZATION OF ANTIGENIC DETERMINANTS AND THEIR CORRELATION WITH DISEASE ACTIVITY IN ANCA GLOMERULONEPHRITIS

Aleeza Jane Roth

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular and Cellular Pathology in the Department of Pathology and Laboratory Medicine, School of Medicine.

Chapel Hill
2012

Approved by:

Dr. Ronald J. Falk, M.D.

Dr. J. Charles Jennette, M.D.

Dr. Patrick Nachman, M.D.

Dr. Witold Winnik, Ph.D.

Dr. Craig Fletcher, D.V.M., Ph.D.

ABSTRACT

ALEEZA J ROTH: Characterization of antigenic determinants and their correlation with disease activity in ANCA glomerulonephritis
(Under the direction of Dr. Ronald J. Falk, M.D.)

This dissertation focuses on the interaction of antineutrophil cytoplasmic autoantibodies (ANCA) and two of their target autoantigens, myeloperoxidase and lysosomal membrane protein 2. The results of these interactions reveal novel epitopes associated with disease activity in granulomatosis with polyangiitis (GPA).

The relationship between lysosomal membrane protein 2 (LAMP-2), a putative 3rd autoantigen, and ANCA disease is described in Chapter 1. In 2008, LAMP-2 was purposed to be a novel autoantigen which predicted relapse in ANCA disease. Exhaustive efforts uncovered that antibodies directed to LAMP-2 were not specific to ANCA disease nor were they relevant to disease activity in our US patient cohort.

Chapter 2 investigates the binding sites of myeloperoxidase (MPO) specific autoantibodies in sera samples from patients with ANCA glomerulonephritis and healthy controls. We prove that anti-MPO autoantibodies are restricted to a limited number of epitopes on MPO and circulating autoantibodies target the same epitopes during relapse and remission. In addition, to identify MPO epitopes of interest, a conformation dependent proteomics assay was used in conjunction with traditional immunologic methods. An ANCA disease associated epitope was uncovered (Chapter 3) with an association with disease activity. The discovery of this epitope has lead to the development of a pre-clinical test for ANCA disease. As a whole, this is a comprehensive body of work which determines the complete profile of MPO specific epitopes found in a patient cohort in the southeastern US.

ACKNOWLEDGEMENTS

This dissertation is dedicated to my scientific and clinical mentors, loving family and loyal friends. There have been many people who have aided me on the road to becoming the scientist that I have always wanted to be and I would like to take this time to acknowledge them.

My primary mentor, Dr. Ronald Falk, has taught me so many life lessons that are invaluable. He has instilled in me the ability to be a leader, teacher and mentor. Our close working relationship inspired me to think about the broader aspects of research outside the traditional boundaries to examine questions never previously considered. My clinical mentor, Dr. Patrick Nachman, has opened my mind to examine the larger picture of clinical outcomes and consider aspects of disease from the clinical point of view. I am grateful to have not only made progress on a terrible disease but I have been able to connect with the individuals who are living with ANCA disease.

I would like to thank my dissertation committee: Dr. J. Charles Jennette for being a close mentor and outstanding department chair; Dr. Witold Winnik for his friendship, time and expertise; and Dr. Craig Fletcher for his scientific guidance and professional support. I must wholeheartedly thank my research family at the UNC Kidney Center: Dr. Gloria Preston, Dr. Donna Bunch, Dr. Jia Jian Yang, Anshul Badwhar, Michael Brown, Meghan Free and Elisabeth Berg. My time in the lab would not have been the same without Dr. Gloria Preston who taught me the invaluable skills of scientific writing and more importantly scientific thinking.

I am indebted to the department of pathology and laboratory medicine, my pathology department class of 2009 and our graduate student advisor, Dr. Bill Coleman, who have made my time at UNC Chapel Hill really special.

I cannot thank my family enough for their support and help throughout the highs and lows of graduate school. I especially want to acknowledge my siblings: Rebecca for her kindness, Shoshannah for her advice and guidance, and Jason for all of his optimism. My parents James and

Melanie Roth who deserve a lot of credit for all that I have accomplished in my life. My mother taught me from a very young age that I could achieve everything that I ever dreamed, if it was truly what I wanted.

My friends have played a particularly important role in my life. I want to thank my 'roommates' Jessica Cseter, Maggie Usher, Krysta Hannon and Katie Allen for all of their laughter. Finally, I am forever grateful to my close friends Megan Fleischman and Bradley Macdonald for their encouragement and their constant belief in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

[A]

aa	amino acid
AD	active disease
ANCA	antineutrophil cytoplasmic autoantibodies
Anti-GBM	anti-glomerular basement membrane

[B]

BSA	bovine serum albumin
BVAS	Birmingham vasculitis activity score

[C]

c-ANCA	cytoplasmic-antineutrophil cytoplasmic autoantibodies
CD 4	cluster of differentiation 4
CHO-LDL-D	Chinese hamster ovary O-linked glycosylation deficient cell line
CP	ceruloplasmin
CR	clinical remission
CRC	compact reaction column

[D]

DNA	Deoxyribonucleic acid
DR2 Tg	d-related haplotype transgeneic

[E]

E-GPA	eosinophilic granulomatosis with polyangiitis
ELISA	enzyme-linked immunosorbent assay

[F]

FimH	gram-negative bacterial adhesion protein
FITC	fluoresceine-isothiocyanate
FNGN	focal necrotizing glomerulonephritis
FPLC	fast protein liquid chromatography

FSGS	focal segmental glomerulosclerosis

[G]

GDCN	Glomerular Disease Collaborative Network
GN	glomerular nephritis
GPA	granulomatosis with polyangiitis

[H]

HBSS	Hank's balanced salt solution
HC	healthy control
H&E	hematoxylin and eosin
HEK-293	human embryonic kidney 293 cells
HLA	human leukocyte antigen

[I]

IFA	indirect immunofluorescence assay
Ig	immunoglobulin
IgG	immunoglobulin class G

[K]

kD	kilodalton

[L]

LAMP-2	lysosomal membrane protein 2

[M]

MALDI-TOF/TOF	matrix-assisted laser desorption ionization time of flight
MCD	minimal change disease
MHC	major histocompatibility complex
MPA	microscopic polyangiitis
MPO	myeloperoxidase
MS	mass spectrometry
MS	multiple sclerosis

[N]

NAA	natural autoantibodies
NCBI	National Center for Biotechnology Information
NOD	non-obese diabetic

[O]

OD	optical density

[P]

p-ANCA	perinuclear- antineutrophil cytoplasmic autoantibodies
PAS	periodic acid-schiff
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PDB	protein data bank
PF	pemphigus foliaceus
PMA	phorbol myristate acetate
PNPP	para-nitrophenyl phosphate
PR3	proteinase 3
PyMOL	open-source, user-sponsored, molecular visualization system

[R]

rLAMP-2	recombinant lysosomal membrane protein 2
RT	room temperature

[S]

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism

[T]

TFA	trifluoroacetic acid
TPCK	L-1- tosylamido-2-phenylethyl chloromethyl ketone

[U]

UNC	University of North Carolina at Chapel Hill

UTI	urinary tract infection
UV	ultraviolet

[W]

WKY	wistar kyoto

[Y]

YAG	yttrium-aluminum garnet

PROLOGUE: ANTIBODY MEDIATED AUTOIMMUNE DISEASE

The existence of autoantibodies in autoimmune disease has been known for decades whereas the pathogenesis of autoantibodies in many autoimmune diseases has been debated. There is evidence of pathogenecity both *in vitro* and *in vivo*[1-5] in autoimmune diseases including: anti-GBM, systemic lupus erythematosus (SLE), type 1diabetes and antineutrophil cytoplasmic autoantibodies (ANCA) but the controversy of their role in pathogenicity persists. One disease where the pathogenic role of autoantibodies has been largely accepted is anti-GBM disease (Goodpasture's syndrome) where autoantibodies to the glomerular basement membrane were found in 1967 by *Lerner et. al*. At that time, purified autoantibodies were transferred from a patient with anti-GBM disease to recipient monkeys and induced glomerulonephritis[1]. Over the next 44 years, more and more evidence has supported this initial result and the pathogenesis of these autoantibodies has become central to the diagnosis and treatment of anti-GBM disease.

In contrast, the discovery of anti-neutrophil cytoplasmic autoantibodies (ANCA) which are found in the circulation of patients with pauci-immune necrotizing and crescentic glomerulonephritis, less progress has been made. The most striking experiments have shown that ANCA are pathogenic in an MPO mouse model[5]. A mouse model was developed by Xiao and colleagues using murine myeloperoxidase transferred into a myeloperoxidase knockout mouse and subsequent ANCA IgG derived from the knockout mouse are then transferred to an immune competent mouse. After six days of the initial intravenous injection, all mice develop necrotizing glomerulonephritis which is strikingly similar to human disease[5]. This MPO-ANCA mouse model strongly suggests the

pathogenic role of ANCA in mice. Substantial evidence supports that MPO-ANCA and PR3-ANCA cause granulomatosis with polyangiitis, however specific epitopes are unknown and it is only somewhat accepted that a restricted number of ANCA epitopes have pathogenic potential[4-6]. Little is known about the specificity of disease-causing ANCA and moreover, whether the specificity of ANCA changes over the course of disease. This question has not been answered in GPA, or in many other antibody-induced autoimmune diseases.

Autoantigens in ANCA disease

There are two well characterized autoantigens ANCA target, myeloperoxidase (MPO) and proteinase 3(PR3). In 2008, another autoantigen, lysosomal membrane protein 2 (LAMP-2), was implicated in GPA[7]. All three proteins reside in human neutrophils and monocytes.

In 1988, Carrlsson *et. al.* isolated and characterized h-LAMP-1 and h-LAMP-2 from chronic myelogenous leukemia cells. LAMP-2 is a sialoglycoprotein and polylactosaminoglycan carrier protein which makes up 0.1-0.2% of total cell proteins[8]. Polylactosaminoglycan are high molecular weight saccharides that are known to chaperone potentially antigenic molecules including, blood group antigens and tumor-associated antigens. The sialoglycoprotein carriers of these saccharides are important to characterize because of their unique function. LAMP-2 is a heavily glycosylated protein with both N and O-linked polysaccharides which in an aberrant form is thought to contribute to possible antigenicity in GPA[9]. Interestingly, a nine amino acid sequence in the extracellular domain of the LAMP-2 protein (HGTVTYNGS) has complete homology to a gram-negative bacterial adhesion protein FimH. FimH is a protein found in fimbriated bacteria such as *E. coli* and *P. aeruginosa*.

Myeloperoxidase was first isolated and characterized as veroperoxidase in 1941 by Agner et. al.[10] MPO is a 140kD heme containing enzyme found in myeloid cells and stored in the azurophilic granules of neutrophils and monocytes[11]. It consists of a light chain and heavy chain comprising an asymmetrical dimer *in vivo* that's main function is catalyzing reactions between hydrogen peroxide

and halides to perform its microbicidal role in the immune system[11, 12]. MPO has one known inhibitor, ceruloplasmin (CP), which is a 150kD plasma protein and functions as an iron and copper transporter and also as an acute phase reactant. MPO was first discovered to be the target of ANCA in 1988 by Falk et. al. in patients with systemic vasculitis and necrotizing and crescentic glomerulonephritis[13]. Since that time autoantibodies targeting MPO have been utilized to diagnose and follow disease activity in patients with GPA.

Lastly, proteinase 3 which is a 29kD serine protease, also found in the granules of neutrophils and monocytes, functions as an enzyme that is responsible for the cleavage of a variety of substrates. PR3 was identified as a target of ANCA in 1990 by Ludemann *et. al.* by immunoprecipitation with antibodies purified from patient's sera[14, 15]. PR3-ANCA is more prevalent in the Southwestern U.S. than MPO-ANCA and this serotype is associated with a higher frequency of relapse which correlates with anti-PR3 autoantibody titers[16]. Williams *et. al.* characterized the presumed pathogenic epitopes of PR3 using linear peptide epitope mapping[17].

Autoantibody (ANCA) and autoantigen interaction

Mapping the autoantigenic determinants of MPO and PR3 have been a much sought after area of research since the discovery of ANCA in an effort to glean information about the pathogenicity of autoantibodies. Epitope specific ANCA have the potential to be precise tools to measure disease activity and pinpoint pathogenic mechanisms[18]. Many groups studying ANCA disease have reported epitope targets of both MPO- and PR3-ANCA that are immunodominant in patients during active disease. Initial efforts of epitope mapping PR3-ANCA were successful using overlapping peptides[17], in stark contrast, epitopes on MPO were determined to be conformational[19] and remained an enigma. Studies using overlapping MPO peptides that span the whole molecule have been either inconclusive or present conflicting results[20-24]. To rectify a conformational conundrum, human/mouse chimeric molecules were used to preserve structure with broad unconvincing results[22]. The most recent report on ANCA epitope specificity came from Bruner *et. al.*

who present seven immunodominant epitopes for consideration and suggest their probable influence on disease expression[20]. The goal of epitope mapping MPO-ANCA is clear and the clinical and scientific benefits are far-reaching because of their potential role in pathogenesis.

Presence of ANCA before clinical manifestation of autoimmune disease

Understanding epitope specificity of natural autoantibodies in healthy subjects is equally important as disease specific epitopes. Olson *et. al.* present an intriguing article which describes the presence of asymptomatic autoantibodies (anti-GBM, PR3 and MPO) in healthy individuals years before onset of anti-GBM disease[25]. Interestingly, the existence of autoantibodies found in healthy individuals is not a unique event. This phenomenon has been well documented in several autoimmune diseases including SLE, ANCA glomerulonephritis, anti-GBM and type 1 diabetes[26-29]. As early as 1990, studies demonstrated the presence of islet cell autoantibodies in healthy first-degree relatives of type I diabetes patients[30]. Autoantibodies associated with type I diabetes are highly sensitive markers providing the ability to predict disease onset[27]. More recently, in the case of SLE, an extensive article by Arbuckle et. al. described that 88 percent of patients studied who developed SLE had autoantibodies to at least one autoantigen while still clinically asymptomatic[26]. Further, the autoantibodies were measurable in multiple samples, a mean of 3.3 years before diagnosis; with a significant accumulation of diverse autoantibodies leading up to clinical presentation of SLE[26]. While asymptomatic autoantibodies are reported to be present and detectable in ANCA disease in patients before diagnosis; the final trigger for clinical manifestation of disease is unclear[28].

The most intriguing question in light of the presence of asymptomatic autoantibodies is: how does clinical manifestation of disease occur? The nature of the glomerular basement membrane as an autoantigen gives clues to possible mechanisms. Pedchenko *et. al*. characterized the delicacies of autoantibodies and the nature of the autoantigen recognized during active anti-GBM disease[31]. There is an abundance of data demonstrating that anti-GBM disease autoantibodies are pathogenic and

specific to the autoantigenic region, but the autoantigen itself does not readily present the epitope. A conformation-dependant epitope is known to be the primary pathogenic epitope which is exposed by disassociation of the endogenous hexamer structure[31]. Understanding the development of how autoantibodies may develop in anti-GBM disease may provide clues for ANCA disease. Since patients with GPA carry asymptomatic autoantibodies which are not pathogenic, their specificity for the autoantigen maybe the key to understanding the development of ANCA disease.

Epitope mapping ANCA

In a 2009 review on ANCA epitope specificity, the author relents that there is little known about MPO-ANCA and what is known about PR3-ANCA is limited [32]. What we do know is that MPO-ANCA are most likely directed towards conformational epitopes, favoring a cryptic epitope theory [33]. This is one of a number of theories of autoimmunity to explain the origins of GPA and possibly the reason for the ongoing cycle of relapse and remission characteristic of GPA. Previous efforts to study ANCA and establish immunodominant MPO-ANCA epitopes provided minimal useful information, even when using chimeric molecules to preserve native structure [22]. It may be that natural or asymptomatic autoantibodies, present in the general population, play an active role in the development of pathogenic autoantibodies by disrupting autoantigen conformation. Moreover, no epitope mapping study in ANCA disease has followed patients over time through remission and flares and neither MPO-ANCA titers or isotype class have proven to be good markers of relapse or remission [34-37]. This body of work addresses the issues present in the field today including: conformationally sensitive epitopes, natural and asymptomatic epitopes, patient epitope profiles over disease course, and clinical relevance. It is essential to determine which ANCA have pathogenic potential and if an epitope specific autoantibody is required for disease onset.

Central Hypothesis

To summarize, ANCA interactions with myeloperoxidase, proteinase 3 and LAMP-2 are important in understanding GPA not just for pathogenesis but for diagnosis and treatment. The

central hypothesis of this body of work is ANCA bind many sites on target autoantigens and that these target epitopes are the same at disease onset and relapse while absent during remission. In addition to the central hypothesis it encompasses several sub-hypotheses including 1) LAMP-2 is an autoantigen involved in GPA 2) Disease associated ANCA have pathogenic potential *in vivo* and *in vitro* 3) ANCA negative patients have autoantibodies to neutrophil proteins.

In the following three chapters, this central hypothesis and subsequent sub-hypotheses will be addressed. Chapter 1 describes our efforts to test the putative autoantigen LAMP-2, as a contributing factor in GPA. A recombinant LAMP-2 protein and a synthetic LAMP-2 peptide were produced and used as substrates to determine if anti-LAMP-2 autoantibodies are prevalent in a Southeastern USA cohort of patients with PR3- and MPO-ANCA. Detailed in Chapter 2, is the in depth method of epitope excision using mass spectrometry to test whether anti-MPO autoantibodies are restricted to a limited number of epitopes on MPO and whether circulating autoantibodies target the same epitopes during relapse. Finally, in Chapter 3, we examine the question of ANCA negative glomerulonephritis and what autoantigen(s) and subsequent epitope is targeted in that population of patients.

Chapter 1

ANTI-LAMP-2 ANTIBODIES ARE NOT PREVALENT IN PATIENTS WITH ANTINEUTROPHIL CYTOPLASMIC AUTOANTIBODY GLOMERULONEPHRITIS

This chapter consists of material from a manuscript reprinted with permission from *J Am Soc Neph* 2011[1].

In 2008, Kain et.al. reported a novel autoantigen found in ANCA disease. This autoantigen was lysosomal membrane protein 2 or LAMP-2 and was putatively found in 93% of sera from patients with pauci-immune focal necrotizing glomerulonephritis in Vienna, Austria. This chapter focuses on the investigation of anti-LAMP-2 autoantibodies in sera from the Glomerular Disease Collaborative Network at UNC Chapel Hill.

---

[1]Aleeza J Roth, *Michael C Brown, Rex Neal Smith, MD, PhD, Anshul K Badhwar, Oscar Parente, Hyun chul Chung, Donna O Bunch, PhD, JulieAnne G McGregor, MD, Susan L Hogan, PhD, Yichun Hu, Jia Jin Yang, MD, Elisabeth A Berg, John Niles, MD, J. Charles Jennette, MD, Gloria A Preston, PhD and Ronald J Falk, MD, FASN

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 278 sera samples from patients with ANCA glomerulonephritis (n=103); those with fimbriated, gram–negative *Escherichia coli* urinary tract infection (n=104); disease controls (n=19); and healthy volunteers (n=52). With levels in healthy controls used to define a reference range, anti-LAMP-2 reactivity was present in 21% of ANCA sera; reactivity was also present in 16% of the control group with urinary tract infection. Western blotting and immunofluorescence microscopy did not verify positivity. 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.

Introduction

In 1995, Kain and coworkers reported that lysosomal membrane protein 2 (LAMP-2) is a target of anti-neutrophil cytoplasmic autoantibodies (ANCA) in addition to proteinase 3 (PR3) and myeloperoxidase (MPO).[7] Recently, Kain and coauthors further characterized LAMP-2 autoantibodies and concluded that more than 90% of patients with active pauci-immune glomerulonephritis had circulating anti-LAMP-2 autoantibodies.[9] The majority of these patients also had MPO-ANCA and PR3-ANCA.[15, 38-42] These findings have had a significant impact on both the clinical and research communities.[43-45] Intriguingly, anti-LAMP-2 antibody epitope analysis indicated the antibodies recognized a nine-amino acid peptide in a bacterial adhesion protein (FimH) carried by fimbriated, gram-negative bacteria, including *Escherichia coli*.[9] An immunological

response triggered by bacterial infection led to production of autoantibodies to the human LAMP-2 protein.[9] The resulting anti-LAMP-2 autoantibodies were proposed to be pathogenic and able to cause glomerulonephritis in rats. Rats immunized with FimH peptide developed pauci-immune glomerulonephritis and antibodies to human LAMP-2.[9] 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 a routine screen for anti-LAMP-2 autoantibodies should be initiated for all ANCA-disease patients. Before such steps are taken, their prevalence and their relationship with disease activity should be established in independent cohorts. To establish 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. We present data generated at the UNC-Kidney Center, NC, USA. LAMP-2 antibodies are not prevalent in patients with MPO-ANCA, PR3-ANCA and ANCA negative glomerulonephritis.

Materials and Methods

To test for the presence of LAMP-2 autoantibodies in patients with pauci-immune glomerulonephritis we chose a cohort of 103 patients with biopsy-proven disease comprised of 53 females and 50 males; 48 MPO, 53 PR3, 2 both MPO and PR3 patients with a median age of 53 ± 18.6 years. Birmingham Vasculitis Activity Score or BVAS, which is the measurement of disease activity in vasculitis patients, was used upon chart review to cumulatively define disease status of the patient cohort. As determined by BVAS, 45 of these patients had active disease (BVAS >0) while 57 were in remission (BVAS =0). For healthy control populations we tested 52 healthy individuals as well as 104 patients with urinary tract infections.

Protein, Peptides, and Antibodies

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 FPLC. 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).

ELISAs and Westerns

For the recombinant protein ELISA LAMP-2 was coated on a Costar 96-well high binding EIA/RIA plate (Corning, Lowell, MA) at 4°C overnight (10µg/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 anti-human 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 crosslinked on themselves to enhance plate binding using 10% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS (Invitrogen) at a concentration of 5mg/mL for two days at room temperature. Nunc Polysorp plates (ThermoFisher Scientific) were then irradiated for 20 minutes in a UV Stratalinker (Stratagene, La Jolla, CA) and coated with crosslinked peptide overnight at 4°C (50µg/mL). The proceeding steps of the protocol were carried out the same as in the rLAMP-2 ELISA above. Westerns were used to confirm positives on the recombinant ELISA by probing with serum in 1% blotto at 1:100 overnight (4°C) on 10µg of rLAMP-2 antigen and MPO (Elastin Products Company, Owensville, MO) or recombinant PR3, depending on the patient's diagnosis.

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 anti-human 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's F12 medium (Invitrogen) supplemented with 5% FBS (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen). For fully glycosylated LAMP-2, 20µM galactose and 200µM N-acetylgalactosamine (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% alpha-MEM, and 3% dialyzed FBS (invitrogen)(personal communication). CHO cells were grown on 8-well CC-2 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 was used to detect bound IgG. The INOVA diagnostics ANCA immunofluorescence kit was used to stain human neutrophils with a polyclonal rabbit anti-human LAMP-2 antibody (Abnova) at 1:100. The primary antibody was the only reagent not found in the kit, the method from the kit was used exactly. Dr. J. Charles Jennette reviewed all immunofluorescence slides using an Olympus BX41 fluorescence microscope.

***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 a FPLC. WKY rats were obtained from Harlan Sprague Dawley age and weight matched at about 80g. 10mg anti-LAMP-2 high titer total IgG or normal rabbit IgG was transferred by tail vein injection into rats (5 rats per group). Rats were sacrificed after five days and kidneys were harvested for histological analysis, fixed in formalin, embedded in paraffin, sectioned and stained with H&E and PAS stains. To ensure successful transfer of rabbit IgG, serum was obtained 24 hours after

transfer and coated on a Costar 96 well high-binding EIA/RIA plate overnight at 4°C and blocked with 3% BSA for 1 hour. Bound IgG was detected using alkaline-phosphatase conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Urines were collected on days 0,1,3 and 5 and urinalysis performed with chemstrip 10 MD urinetest 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's complete adjuvant; subsequent boosts were in incomplete Freund's adjuvant. A Rat-ANCA test was performed on a rat total leukocyte preparation from healthy rats. Total leukocytes were put 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, pre-immune and post immunization with LAMP-2 peptide (HGTVTYNGS) was purified from sera with sepharose protein A/G beads (Santa Cruz Biotechnologies) and used at 3mg/mL followed by a goat anti-rabbit FITC conjugated secondary (Jackson Immunology) at 1:200. All slides were reviewed by J. Charles Jennette.

Results

Comparison of LAMP-2 protein substrates used for antibody detection

LAMP-2 (lysosomal-associated membrane protein 2) is normally produced in all cell types. It contains oligosaccharide chains, some of which are polylactosaminoglycans, which are species specific, complex. These are dispersed on two domains of the protein separated by a hinge-like structure containing O-linked oligosaccharides (Fig.1.1A).[46]

A recombinant LAMP-2 protein consisting of the entire extracellular domain[(aa 1-350)] was utilized 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 (Fig.1.1B). Recombinant protein was expressed in Human Embryonic Kidney Cells (HEK) to make possible human-specific protein glycosylation. Affinity purified protein was of high quality as determined by SDS-PAGE (Fig. 1.1C) and was recognized by a commercial, polyclonal anti-LAMP-2 antibody by western analysis (Fig. 1.1D). In

addition a synthetic peptide was synthesized locally which contained the amino acids identified as the FimH-like epitope (Fig. 1.1E). Purity of FPLC-eluted peptide was indicated by a single peak (Fig. 1.1F) and peptide composition confirmed by mass spectrometry (Fig. 1.1G).

Figure 1.1 Substrates used to detect anti-LAMP-2 antibodies in human sera

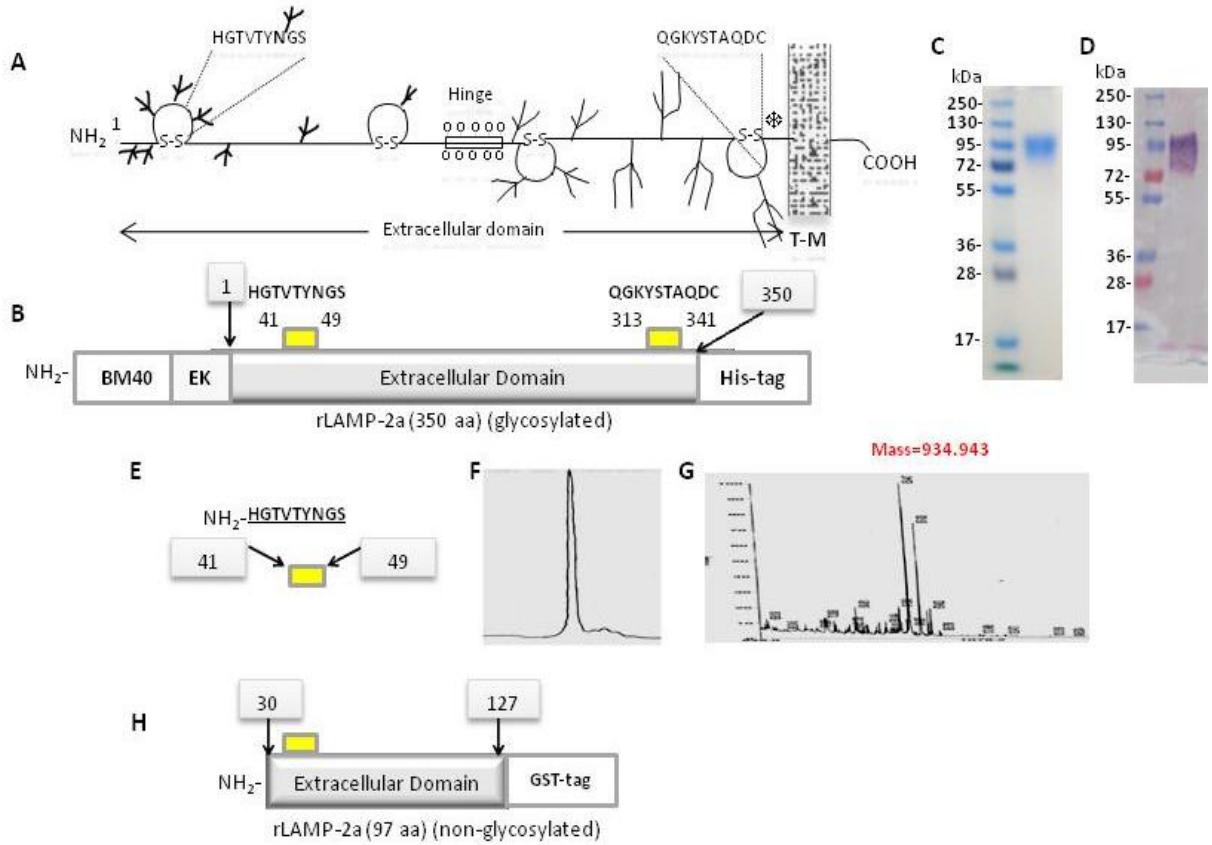


Figure 1.1 LAMP-2 protein and peptide substrates used to screen for LAMP-2 reactivity in sera. (A) Schematic of full length human LAMP-2a protein denoting O-linked hinge region and N-glycosylation sites (adapted from Fukuda et al, 1988). (B) Sites of putative pathogenic epitopes are designated in yellow boxes (HGTVTYNGS) (QGKYSTAQDC). For studies at UNC-Kidney Center, LAMP-2a cDNA was subcloned omitting the C-terminal transmembrane domain (T-M) and cytoplasmic tail. (C) Analysis of recombinant protein produced in HEK cells indicated high purity as assessed by SDS-PAGE . (D) Recombinant protein was recognized by purchased hLAMP polyclonal antibody, assessed by western blot . (E) Peptide utilized in studies at UNC-Kidney Center. (F) Purity of FPLC-eluted HGTVTYNGS peptide was indicated by a single peak. (G) Confirmation of peptide composition by mass spectrometry indicated correct mass of 934.943. (H) Schematic of LAMP-2 protein utilized in Mass. General studies produced in wheat germ extract system.

Table 1.1 Description of sera samples

Source	Number	Samples Analyzed		Description of Sample
Local North Carolina Community	52	Healthy controls		
UNC Kidney Center	103	*ANCA		
		MPO-ANCA	(n=48)	Pauci-immune focal necrotizing glomerulonephritis from GDCN consortium
		PR3-ANCA	(n=53)	
		MPO and PR3 positive	(n=2)	
		† Active	(n=45)	(BVAS > 0)
		Remission	(n=57)	(BVAS =0)
		New Onset	(n=15)	Samples collected at disease onset prior to medication
		Never frozen	(n=10)	Serum obtained and immediately assayed, untreated and treated active disease patients.
UNC Hospitals Mclendon Clinical Laboratories	104	Urinary Tract Infection		Otherwise healthy individuals clinically diagnosed with a FimH positive bacterial infection
UNC Kidney Center	10	SLE disease control		from GDCN consortium
Medical University of Vienna	9	FNGN		Pauci-immune focal necrotizing glomerulophritis provided by
		Positive per Kain et al.	(n=4)	Kain et al.
		Negative per Kain et al.	(n=5)	

*ANCA samples are divided into MPO, PR3 or dual positive; active or remission. New Onset and Never frozen samples are included into the total number of samples.

† *One patient from the test cohort had insufficient data to determine disease activity*.

A recombinant LAMP-2 protein commercially-produced in a Wheat Germ Cell free system was utilized as substrate in studies conducted at Massachusetts General Hospital. Protein that translated this system is non-glycosylated and thus the LAMP-2 substrate can be likened to the one "bacterially" produced and utilized by Kain et al.[9] The amino acid sequence is only a portion of the extracellular domain[(aa 30 -127)], but does contain the FimH-like epitope (Fig. 1.1H). An all-inclusive listing of sera samples analyzed in these studies is provided in Table 1.1. 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 rLAMP-2 protein produced in HEK cells

Kain and coworkers from the Medical University of Vienna graciously provided the UNC-Kidney Center with sera samples (n=9) consisting of four known positives for LAMP-2 antibodies in their assay system and five known negatives. In our assay system, four were positive and five negative against HEK-expressed rLAMP-2 (Fig. 1.2A), providing confidence that the systems were comparable. We found that, of the UNC-ANCA disease patient group (n=103), 21.1% were deemed positive for reactivity against rLAMP-2 in this assay (>mean plus 2SD of healthy controls) (p=0.004) by (Fig. 1.2A). Sera from otherwise healthy individuals with active urinary tract infections (UTI) (n=104), and producing antibodies against the gram-negative bacteria, contained antibodies reactive against rLAMP-2 at a frequency similar to that found in the ANCA disease group (p=0.097). Samples from patients considered to have "new-onset" disease were obtained either prior to therapy (n=7) or immediately after the first dose of glucocorticoids (n=9). Open boxes in the ANCA disease cohort signify patients during active disease with a BVAS>0 (Fig. 1.2A). Sera from SLE patients were negative for rLAMP-2 reactivity (Fig. 1.2A). rLAMP-2 reactivity was not significantly associated with disease onset nor with fresh sera (tested the same day is was drawn - never frozen).

As with most clinical testing, a second assay was employed 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 (lane

1- Fig. 1.2B), meanwhile they 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 samples provided by Kain et al were also negative for rLAMP-2 reactivity by western analysis (data not shown).

Figure 1.2 Sera reactivity against recombinant LAMP-2 protein by ELISA and Western blot

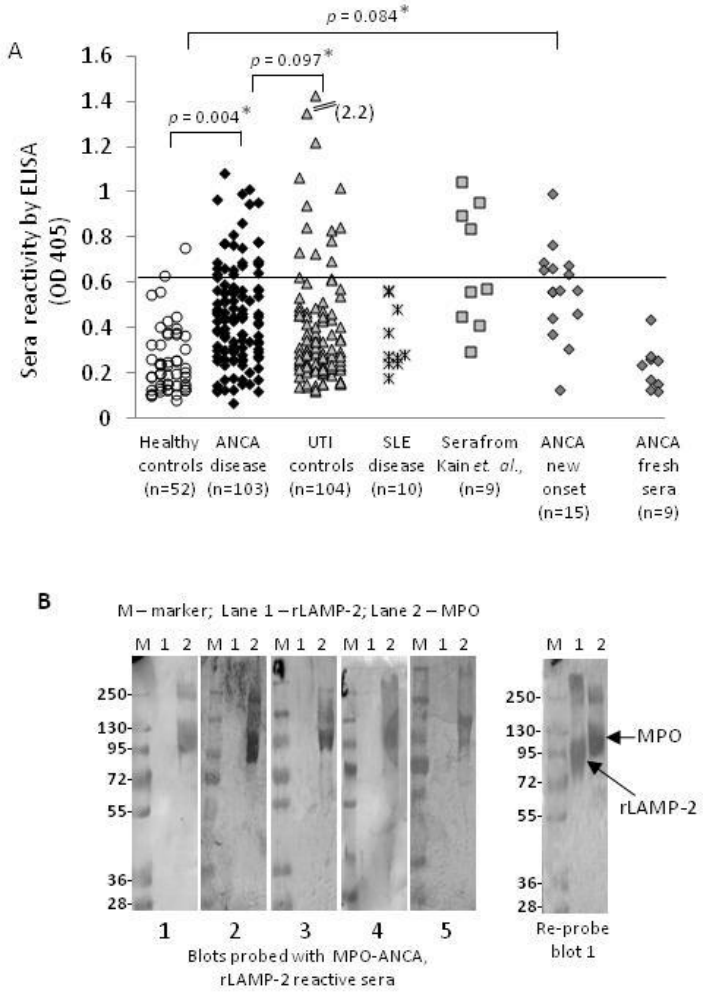


Figure 1.2 Seroreactivity against recombinant LAMP-2 protein produced in HEK293 cells. (A) ELISA results indicate a subgroup of ANCA patients' sera samples were reactive against rLAMP-2 protein, compared to healthy controls, UTI, SLE, ANCA sera from Kain et. al. new onset and never frozen sera (solid bar indicate 2 SD above the mean of healthy controls). In the ANCA disease group open squares indicated "Active" disease and 'filled diamonds' are patients in remission; in the new-onset group 'asterisks' indicate samples collected prior to steroid treatment (B) Evaluation of serum reactivity by western blot analysis (M –size marker; Lane1 – 1ug purified rLAMP-2; 1ug native MPO). MPO-ANCA –positive samples that were also reactive with rLAMP-2 by ELISA did not react with rLAMP-2 (Lane1) but did react with MPO protein (Lane 2). Blot 1 was re-probed with a commercial polyclonal anti-LAMP-2 antibody to verify rLAMP-2 protein loading on the gel.

Reactivity against rLAMP-2 by indirect immunofluorescence microscopy

A third assay was employed (IFA) in effort to validate positive rLAMP-2 reactivity. rLAMP-2 protein, over-expressed in HEK cells, stains with a polyclonal anti-LAMP-2 antibody to produce a cytoplasmic staining pattern consistent with preferential staining of lysosomes (Fig. 1.3A right panel). Low levels of endogenous LAMP-2 protein were detected in non-transfected cells (Fig. 1.3A left panel). None of the healthy controls (n=52) or ANCA disease sera (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. Representative samples are shown in Figure 1.3B of two healthy controls (one with non-specific staining), two disease controls showing nonspecific reactivity (SLE) and four high-titer ANCA disease sera. One MPO-ANCA shows an irregular punctuate staining similar to control samples and differing from the polyclonal anti-LAMP-2 control. Many SLE samples had varying patterns of nuclear staining apparently caused by anti-nuclear antibodies, but none had staining resembling the anti-LAMP-2 positive control.

It was reported that heavy glycosylation of rLAMP-2 produced in HEK cells could alter antigenicity.[9] To address this issue, we acquired the same cell line utilized in studies by Kain et al, which is O-linked glycosylation deficient (CHO-LDL-D cells) and performed IFA on rLAMP-2 overexpressing CHO-LDL-D cells. We could not detect reactivity to this substrate (Fig. 1.3C). Further, cells were grown in varying conditions to alter the glycosylation pattern of the over-expressed rLAMP-2 protein. O-linked glycosylation deficient cells were grown in regular culture medium (alpha-MEM) without additives to produce protein without hinge region O-linked glycosylation. Manipulation of rLAMP-2 produce in CHO-LDL-D did not produce seropositivity (data not shown). Three sera reacted to the non-transfected CHO-LDL-D cells (Fig. 1.3D).

Figure 1.3 Sera reactivity against recombinant LAMP-2 protein by indirect immunofluorescence (IF)

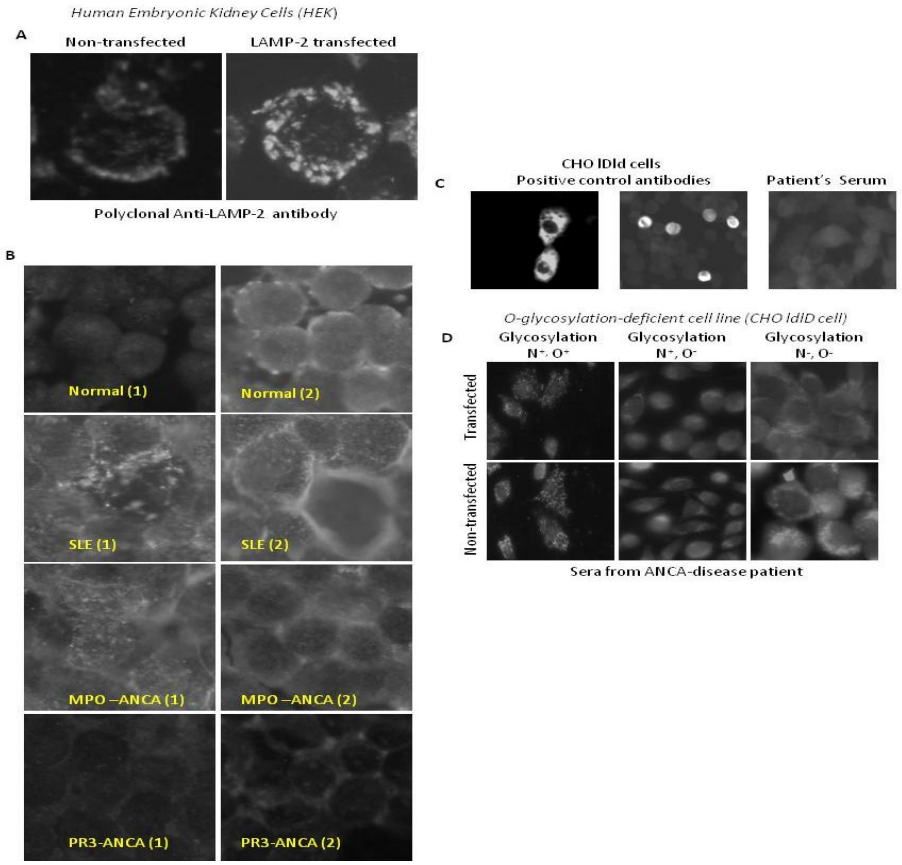


Figure 1.3 Immunofluorescence assays (IFA) using cell lines transfected with rLAMP-2. (A) Polyclonal anti-LAMP-2 antibody (but not negative control antibody) produced low level staining in nontransfected human embryonic kidney cells (HEK) consistent with staining of low level endogenous LAMP-2 protein (left panel) whereas HEK cells transfected with LAMP-2 produced intense cytoplasmic staining (right panel). (B) None of the healthy controls (n=52) or ANCA disease sera (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 pattern, including nuclear staining consistent with anti-nuclear antibodies, but none stained with a pattern similar to the positive control. Representative staining patterns, including low intensity staining that did not correspond to the positive control pattern are shown for two healthy controls (Normal), two SLE controls (SLE) and four high-titer ANCA disease sera. Note that one MPO-ANCA shows an irregular punctuate staining similar to control samples, but no samples resembled the positive control. (C) IFA using rLAMP-2 overexpressing O-linked glycosylation deficient cells (CHO-LDL-D cells) generously provided by Kain et al. Positive control anti-LAMP-2 antibodies produced intense cytoplasmic staining (left two panels) but ANCA-positive patient sera produced no staining (right panel). (D) IFA was performed on CHO-LDL-D cells grown in varying conditions to alter the glycosylation pattern of over-expressed rLAMP-2 protein. Of the 103 samples, 3 reacted to the transfected and non-transfected CHO-LDL-D cells, and the pattern did not resemble the positive control.

Reactivity against LAMP-2 synthetic peptide

We produced a synthetic peptide of the pathogenic epitope (HGTVTYNGS) identified by Kain et al. (Fig. 1.4). Sera were tested for reactivity by peptide-ELISAs. Sera from regional healthy controls were highly reactive raising the threshold for positivity to an OD value of 1.03 (mean plus 2SD of healthy control). Only 4% of ANCA disease samples had results >1.03, which was not statistically significant (Fig. 1.4). UTI, SLE and nine samples from Kain et al were not significantly different from healthy controls. Positivity was defined as two standard deviations above the mean of the healthy controls (1.04). The four positive samples in the total ANCA disease group were all new onset.

Injection of high titer rabbit anti-hLAMP-2 antibodies did not cause glomerulonephritis in WKY 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 glomerulonephritis in WKY rats.[9] We attempted to reproduce these results. Total IgG from a LAMP-2-peptide (HGTVTYNGS) immunized rabbit was highly reactive with rLAMP-2 protein, LAMP-2 peptide and cross-reactive with FimH peptide (Fig. 1.5A). IgG from the immunized rabbit was reactive with rat leukocytes by immunoflourescence 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). Post-injection (24hrs) circulating rabbit-specific IgG was detected in the five rats immunized with anti-LAMP-2 IgG (Fig. 1.5B). Urines were examined days one, three and five (Fig. 1.5C) and none of the rats developed hematuria, proteinuria or leukocyturia. Histological examination of tissues (by JCJ) revealed no histologic abnormalities, including no glomerulonephritis.

Figure 1.4 Sera reactivity against LAMP-2 synthetic peptide

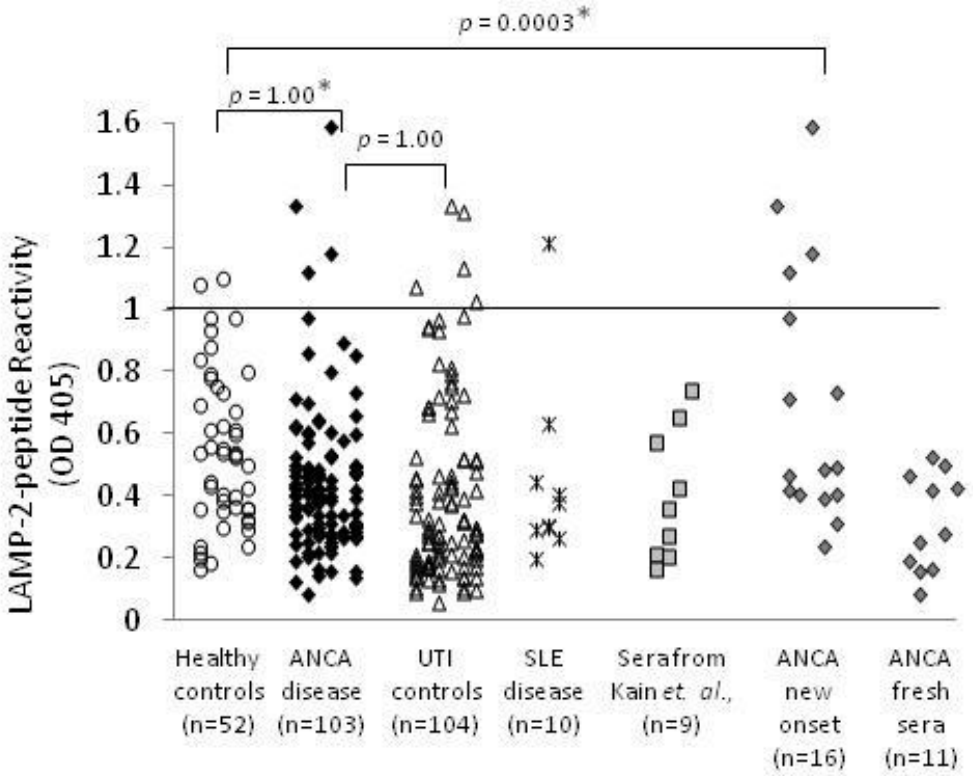
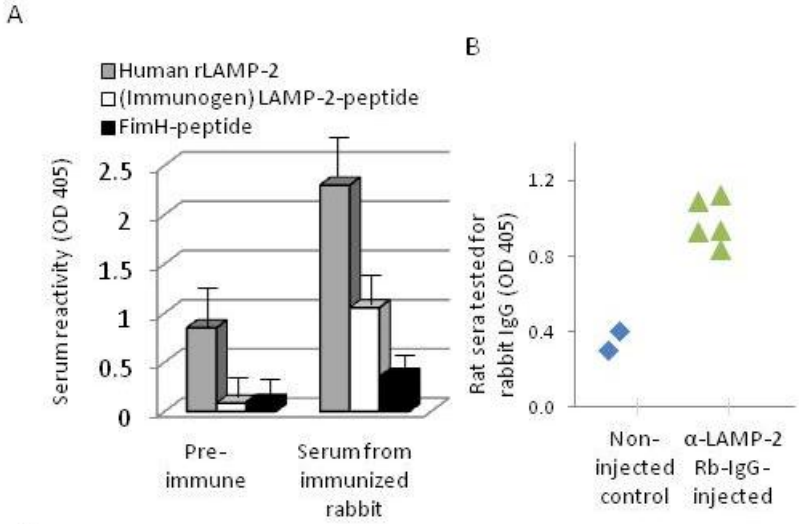


Figure 1.4 Sera reactivity against LAMP-2 peptide (HGTVTYNGS) by ELISA. Positivity was defined as two standard deviations above the mean of the healthy controls (1.04). The four positive samples in the total ANCA disease group were all new onset.

Figure 1.5 Transfer of anti-LAMP-2 peptide antibodies to WKY rats



C

Urine analysis 5 days post-injection

Immunogen	Animal	Blood	Protein	Leukocytes
Normal IgG	1	neg	trace	neg
	2	neg	trace	neg
	3	neg	trace	neg
	4	neg	+1	trace
	5	neg	trace	trace
Anti-LAMP-2 IgG	6	neg	trace	neg
	7	neg	trace	neg
	8	neg	trace	neg
	9	neg	trace	neg
	10	neg	trace	trace

Figure 1.5 Transfer of anti-LAMP-2-peptide antibodies into rats did not cause glomerulonephritis. (A) Characterization of anti-LAMP-2 antibodies generated in a rabbit for transfer into WKY rats. Antibody strongly reacts with LAMP-2 peptide with some cross-reactivity to FimH peptide. (B) Circulating rabbit IgG detected in rats one day post-injection. (C) Five days post-injection there was no detectible hematuria or proteinuria.

Figure 1.6 Staining patterns of anti-LAMP-2 antibodies on human neutrophils

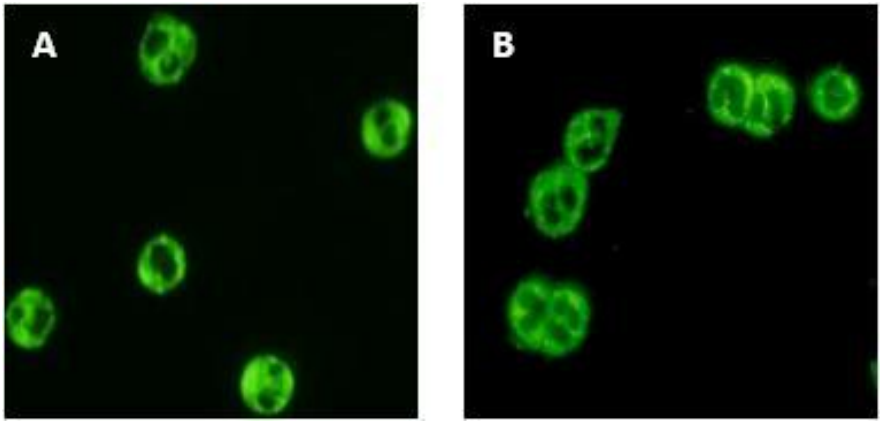


Figure 1.6 LAMP-2 stain human neutrophils with a cytoplasmic pattern (A) Normal Human Neutrophils stained with PR3-ANCA patient's serum (B) Normal Human Neutrophils stained with monoclonal anti-LAMP-2 antibody.

Comparison LAMP-2 titers with PR3-ANCA and MPO-ANCA titers in dual positive sera staining pattern of anit-LAMP-2 antibodies on human neutrophils

If anti-LAMP-2 antibodies coexist in patients' sera with PR3- and MPO-ANCA as reported,[9] how would they affect the results of a routine clinical Immunofluorescence assay? anti-LAMP-2 monoclonal antibody stained human neutrophils with a cytoplasmic pattern (Fig. 1.6A) while normal human neutrophils stained with PR3-ANCA patient's serum (Fig. 1.6B) showed a similar cytoplasmic staining pattern.

Discussion

To examine the frequency of anti-LAMP-2 antibodies associated with ANCA glomerulonephritis in a USA cohort, sera was obtained from patients at the UNC-Kidney Center, North Carolina.

Approximately 22.8% of PR3-or MPO-ANCA positive sera were also reactive with LAMP-2 by ELISA. However, by western blotting and immunofluorescence staining of HEK cells, LAMP-2 positivity could not be validated. Our cohort consisted of about half with active disease and half in remission and there was no significant difference in the incident of LAMP-2 seroreactivity between the groups.

It was reported that antibodies against bacterial protein FimH were cross-reactive with human LAMP-2 protein and that, through molecular mimicry, bacterial infections could contribute to the development of ANCA disease. We tested sera from individuals with gram-negative bacteria urinary tract infections, who were otherwise healthy and negative for PR3- or MPO-ANCA, and found that approximately 16% were reactive with LAMP-2 protein. Dr. Kain and colleagues reported that 9/13 patients with pauci-immune glomerulonephritis had a diagnosis of infection with FimH-expressing bacteria prior to presentation, and that these patients had antibodies that bound the region of LAMP-2 that contained 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.

Recombinant LAMP-2 proteins were utilized as substrates for analyses presented here: one produced in a human embryonic kidney cell line, which post-translationally adds glycosylation moieties to the protein. To address the possibility that the LAMP-2 recombinant proteins utilized in our experiments were not optimal in detecting LAMP-2 reactivity, we obtained the glycosylation-deficient Chinese hamster cell line, utilized in studies by Dr. Kain and colleagues, and screened for sera reactivity to overexpressed human LAMP-2 protein. By manipulating the culturing conditions, we could assess various glycosylation states and their effect on sera reactivity to LAMP-2 by immunofluorescence staining. All samples were negative under all conditions when compared to the staining pattern of a commercially available LAMP-2 antibody.

To explore the pathogenic potential of anti-LAMP-2 antibodies, WKY 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 factors such as the supplier of the particular strain of WKY rats, housing and food in the UNC animal facility may have influenced the susceptibility.

Table 1.2 Characteristics of patient cohort with LAMP-2 reactivity

Categories compared	Reactivity against recombinant LAMP-2		Reactivity against LAMP-2 peptide (P_{41-49})	
	Percent Positive (%)	P value*	Percent Positive (%)	P value*
BVAS vs LAMP-2 reactive		0.1465		1.0000
Active	28.9		4.7	
Remission	15.8		3.5	
ANCA Titer vs LAMP-2 reactive‡				
PR3 ANCA	22.0	0.2323		
MPO ANCA	24.4	0.4129		
ANCA phenotype† vs LAMP-2 reactive		1.0000		1.0000
c- ANCA seropositive	23.1		3.8	
p- ANCA seropositive	21.7		2.3	
Disease type vs LAMP-2 reactive		0.4680		0.13
Renal Limited	13.3		0.0	
Microscopic polyangiitis	22.7		7.5	
Granulomatosis	20.0		2.6	
Others§	50.0		0.0	
Disease Status vs LAMP-2 reactive		0.0842		0.0003
New Onset	40.0		36.0	
Ongoing Disease	18.2		0.0	
Male vs. Female for number of positives				
Positive ANCA patients	10.2 vs 31.5	0.0148	6.0 vs 2.1	0.6193
Positive UTI patients	15.0 vs 11.8	0.7097	0.0 vs 21.0	0.0008

% Positive = percent of individuals with OD values 2SD above the mean of healthy controls

P values were calculated by Fisher's Exact Test

‡ P value was calculated using Wilcoxon two sample test

† Three patients from the test cohort were excluded for this analysis due to insufficient serotype data.

§ Four patients were placed in this category due to insufficient clinical data.

Similar to Dr. Kain and colleagues,[9] 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 glomerulonephritis group that was described previously. There are a number of reasons to explain the differences. The most obvious disparity is geographical differences in the patient cohorts. A second explanation is that our reagents and methodologies are not the same as those of Kain et al even though every effort possible 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 in Dr. Kain's group and the overall results were similar. There was some discordance on sample to sample comparisons, but this did not exceed what would be expected when 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 find very low titers of anti-LAMP-2 antibodies in human sera, although there was no difference 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 demographics (Table 1.2). We conclude that anti-LAMP-2 antibodies are identifiable, low titer, natural or induced antibodies occasionally found in the population.

Chapter 2

AUTOANTIGENIC DETERMINANT PROFILING IN MYELOPEROXIDASE-ANCA DISEASE

Granulomatosis with polyangiitis (GPA) is the most common form of aggressive glomerular disease and is associated in most cases with a systemic necrotizing vasculitis. ANCA have been found to be markers of disease since 1985 and more recently have been found to be pathogenic *in vivo*[5]. Patients with ANCA disease have a polyclonal response to ANCA autoantigens myeloperoxidase (MPO) and proteinase 3 (PR3). ANCA titers correlate with disease activity in some patients (primarily in PR3-ANCA), while the majority of MPO positive ANCA patients exhibit a poor correlation of ANCA titer with disease activity. In this study we analyze MPO-ANCA samples from 66 unique patients to determine the diverse epitope landscape of ANCA disease. We utilized matrix assisted laser desorption/ionization time of flight mass spectrometry (MADLI-TOF/TOF MS) to identify a region of where antibodies bind to antigens which is inherently the antigenic epitope. Further, enzyme-linked immunosorbent assays were used to verify seroreactivity to identify linear epitopes. Healthy subjects and ANCA disease patients share a handful of anti-MPO specific autoantibodies; these autoantibodies do not have pathogenic potential. In patients with MPO-ANCA disease, two disease associated epitopes were found to be linear and correlate to disease activity. Since MPO-ANCA manifests in such a diverse clinical course; genetics, environment but also specificity of autoantibodies likely contribute to the pathogenicity of disease.

Introduction

Protein-protein interactions have primarily been studied in depth using mass spectrometry and x-ray crystallography, more recently these techniques have been applied to human pathology and immunology. Several proteomics approaches have been developed in search of disease biomarkers and therapeutic targets with varying success[47]. Interactions between autoantibodies and autoantigens

are in essence protein-protein interactions, which are highly sensitive to the microenvironment of the human body particularly in autoimmune disease. Methods utilizing proteomics strategies take into consideration: 3-dimensional conformation, microenvironment, kinetics and fluidity[48]. In many autoimmune clinical assays, sheer quantity of autoantibodies may not be specific enough to determine disease activity. In MPO-ANCA disease a focus on individual autoantibody epitope specificity could be a more accurate measure of disease activity.

Patients diagnosed with MPO-ANCA are known to have a polyclonocal response to MPO and their titers are currently followed throughout their disease course to glean information about disease activity[49, 50]. Total MPO-ANCA titer does not correlate to disease. A subset of MPO-ANCA patients have consistently high titers through active disease and remission, while other's total autoantibody titers give no indication of a future relapse. These inconsistencies underline the need to determine the epitope profile of individuals diagnosed with MPO-ANCA disease. Efforts have been made to narrow MPO epitopes using over lapping peptides[20, 21] and deletion mutants[24] which are extremely limiting because of the assumption that anti-MPO autoantibodies will recognize linear epitopes. In order to rectify the issues of conformation, human/mouse chimeric MPO molecules were produced; the limitations of these studies were based on the assumption that human anti-MPO would not cross react with mouse[22]. In addition, chimeric molecules only presume to keep conformation static and do not enable fine epitope mapping because of the large segments of the protein assayed. In an attempt to remedy the limitations presented when using peptides and chimeras for epitope mapping, we utilized a proteomics technique which allows the use of uncompromised native human MPO purified from human leukocytes. In the following investigation of anti-MPO ANCA epitopes a proteomics based approach referred to as epitope excision was adapted from Parker *et. al.*[51] Our hypothesis is that epitope specific anti-MPO autoantibodies will correlate to disease activity more accurately than total anti-MPO titer.

Materials and Methods

Patient Population and Diversity of MPO-ANCA disease

In this study, 88 sera samples from 66 anti-MPO-positive patients with biopsy proven ANCA were collected for analysis. The majority of the cohort were Caucasian (80.3%) and 54.5% were women. The cohort consisted of 32/66 (48.5%) diagnosed with Microscopic polyangiitis (MPA), 24.2% with renal limited disease, 21.2% with granulomatosis with polyangiitis (GPA), 4.5% eosinophilic granulomatosis with polyangiitis (E-GPA) and 1.5% with autoimmune disease overlap of SLE and MPO-ANCA vasculitis. Also included were sera from 10 healthy volunteers for analysis by mass spectrometry with a mean age of 31.5 years (60% female, 70% Caucasian). Healthy volunteers were screened for autoimmune diseases, hypertension and inflammatory diseases. Informed consent was obtained prior to all blood collections in accordance to UNC's Institutional Review Board.

Epitope Excision

Total IgG was purified from sera using protein A/G PLUS-Agarose Reagent according to commercial protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Total IgG was bound to 0.2g CNBr-activated (1mM HCl) Sepharose 4B (GE Healthcare, Sweden) in compact reaction columns (CRC, USB Corporation, Cleveland, OH) for 2 hours at room temperature on an orbital rotator. Excess binding sites were blocked with Tris-HCl 0.1M pH 8.0 for 1 hour on an orbital rotator. CRCs are then washed with alternating wash buffer (0.1M NaAcetate; 0.5M NaCl pH 4.0) and Tris-HCl 0.1M pH 8.0 at least 10 times. Columns are then equilibrated 5 times with PBS. Protein of interest, reconstituted in PBS, is bound to sepharose-IgG complexes (MPO, Elastin Products Co, Inc, Owensville, Missouri) for 3 hours on an orbital rotator. Excess protein is removed by washing with PBS and the column is equilibrated with 50mM Nh4HCO3 pH 7.8 at least 10 times. At this time sepharose-IgG-MPO complex is then incubated with sequencing grade TPCK treated trypsin (Worthington, Lakewood, NJ) at 37°C on a shaker at 105rpm for 2 hours. Excess protein digested by trypsin and no longer bound to antibody complex is removed by washing 10 times with PBS. To elute remaining peptides bound by IgG, incubate with 0.1% trifluoroacetic acid (TFA) for 20 minutes

on an orbital rotator at RT. Peptides are eluted twice in 150uL of o.1% TFA. The sample is then desalted, concentrated and buffer exchanged into 50% ACN; 0.1% TFA using ZipTip$_{c18}$ Pipette Tips (Millipore Corp., Billerica, MA).

Determination of linear epitopes

The same epitope excision method was used as stated above with the exception of protein binding. Myeloperoxidase (80ug per sample) was predigested with 4:1 immobilized trypsin (Promega) overnight at 37°C. Protein sample was centrifuged in a desktop centrifuge for 30 seconds and the supernatant containing the digested protein was used for protein binding.

Heavy Oxygen labeling for low titer antibodies

Heavy oxygen labeling was done on all remission samples (n=36) and healthy controls (n=10). The above epitope excision protocol was followed until the trypsin digestion step. The sample columns were washed with trypsin buffer made with $H_2{}^{18}O$ (Cambridge Isotope Laboratories, Andover, Massachusetts) before digestion. Trypsin was also reconstituted in $H_2{}^{18}O$ and samples were digested for 2 hours at 37°C with agitation. Proteins are labeled on the carboxy terminal with $H_2{}^{18}O$ in a spontaneous reaction during the trypsin digestion of the protein. After digestion, sample columns were washed 7 times with $H_2{}^{18}O$ trypsin buffer. Once $H_2{}^{18}O$ isotope is introduced all buffers (including ziptip and elution) must be used with this isotope to prevent reversion to $H_2{}^{16}O$.

Determination of DR2 Tg mice MPO epitope profile

Total Ig was purified from sera from DR2 Tg mice immunized with either a control albumin peptide or a putative CD4+ T cell MPO epitope aa409-428 (PRWNGEKLYQEARKIVGAMV). The above epitope excision protocol in combination with MALDITOF TOF MS/M was utilized to discover antibody specificity. The protocol was followed as outlined above with the exception of the protein of interest, recombinant mouse MPO protein was used as substrate (R&D systems).

MALDI-TOF-TOF MS

Samples were analyzed on a 4800Plus Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MADLI TOF/TOF MS/MS) in conjunction with ProteinPilot software (AB SCIEX, Foster City, CA). The samples were spotted on a stainless-steel target with α-cyano-4-

hydroxycinnamic acid matrix (Sigma). The instrument has a YAG laser with λ=355nm and the potential difference between the source acceleration voltage and the collision cell was set at 2kV. Calibration was done internally with self digested TPCK treated trypsin. Peak absorbances in MS spectra are not indicative of the abundance of peptide species because of the differences in individual peptides ability to ionize.

All analysis was done with Protein Pilot using an NCBI Mascot search. All MPO peptide samples were analyzed using the same search method: one missed cleavage, Homo sapiens origin, trypsin enzyme cut, 200ppm, 0.5 tolerance, +1 peptide charge, and variable modification of oxidation (M). MPO epitope modeling was done using PyMOL Molecular Graphics System and MPO crystal structure PDB 3F9P.

Enzyme-linked immunosorbent assay (ELISA)

All peptides, peptide/beads and peptide columns were made at the UNC-Chapel Hill peptide synthesis center by Dr. David Klapper. Costar ELISA plates (Corning, Inc., Corning, NY) were coated with approximately 1-2ug of peptide diluted in 100uL of bicarbonate binding buffer (pH 8.3) per well for 2h at RT. Excess binding sites were blocked using SuperBlock PBS (Thermo Scientific, Rockford, IL). Total IgG purified from sera using protein A/G PLUS-Agarose Reagent diluted 1:500 were incubated overnight at 4°C. Plates were washed 5x with PBST and incubated with goat anti-human alkaline phosphate-conjugated secondary antibody (Millipore, Temecula, CA) for 1h on rocker. The plate was then washed 10x and 1-Step PNPP substrate (Thermo Scientific Rockford, IL) was applied for detected of antibody reactivity and read on a microplate reader after 30min at λ=405nm.

Indirect immunofluorescence assay

Indirect immunofluorescence (IFA) was assayed using INOVA Diagnostics, Inc. (San Diego, CA, 708290) with provided protocol. One drop of positive and negative controls were incubated alongside 15ug patient IgG diluted 1:100 in PBS per slide in a moist dark chamber for 25-35 minutes. The wells were washed with 1x PBS in such a manner so as to eliminate cross contamination 5x.

Samples were then incubated with fluorescent conjugate for 25-35 minutes in a dark moist chamber. Repeat wash with 1x PBS, slides were mounted with a coverslip before being observed on an Olympus BX61 microscope (40x oil lens) using a Hamamatsu C10600 (ORCA-R2) Digital Camera. All indirect immunofluorescence slides were analyzed by J.C. Jennette.

Neutrophil activation

Human neutrophils from healthy donors were purified using 1 part HetaCep (Sigma-Aldrich) to 5 parts whole blood. Samples were centrifuged for 6min at 100 x g at RT with break off. Cell separation occurs after 15min at RT and plasma and lymphocyte layers are removed. Cells were layered over 9mL of Histopaque (Sigma-Aldrich) and spun at 1200rpm for 15min and then 1100rpm for 15min at 4°C. The pellet was resuspended in 10mL sterile 0.5x PBS (Cellgro) for 20sec, then immediately 3.3ml of 0.5x PBS was added and mixed well. Cell were spun at 1100rpm for 9min and the subsequent pellet was resuspended in HBSS+/+ at a cell concentration of $1x10^7$. For neutrophil activation, 1ul/mL of cytochalasin B was added to the isolated neutrophils and placed on ice for 15min. 75uL of treated cells were used per well of a 96 well plate and incubated with: 20uL +/-SOD 10uL ferricytochrome C, 10uL PMA, 10uL of affinity purified IgG and HBSS +/+. Duplicate wells were used without SOD as controls. Plate was kept at 37°C and read in a microplate reader at 37°C 550nm every 10min for 60min.

Statistical analysis

P values were calculated by Wilcoxon two sample tests for two samples comparisons, Kruskal-Wallis Test for three groups comparisons and Signed Rank Test for paired groups comparisons. The Bonferroni correction, $\alpha=0.05/3=0.167$ should be used for multiple comparisons.

Results

Identification of Natural Myeloperoxidase Specific Epitopes in Healthy Controls

To test our hypothesis that epitope specificity of anti-MPO autoantibodies are linked with a pathogenic event, we first established a profile of antigenic determinants, or epitopes, of naturally occurring anti-MPO in healthy individuals using the epitope excision method illustrated in Figure 2.1.

Figure 2.1 Epitope Excision with MS

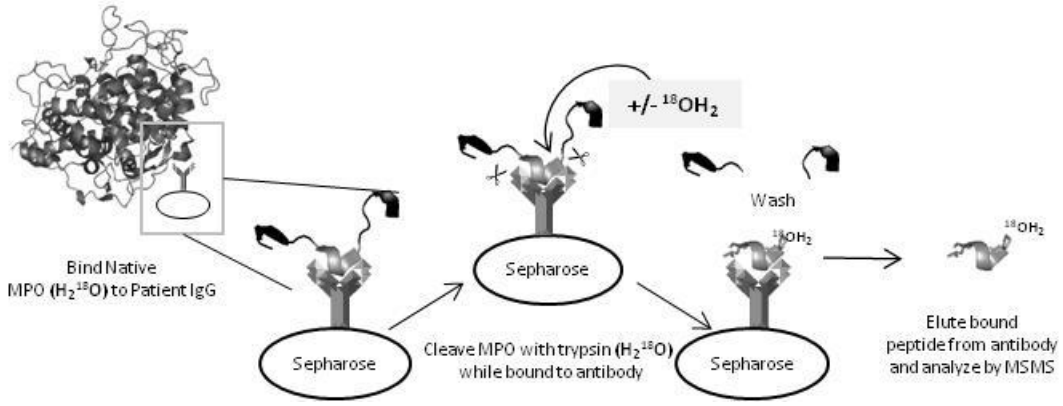


Figure 2.1 Epitope excision method for conformational epitope mapping of MPO. Immobilized antibodies purified from patient sera are bound to native MPO and the epitope is excised by digestion with trypsin (with or without the addition of $^{18}OH_2$) to determine the specificity of autoantibodies to MPO while retaining conformation.

Because these 'natural' autoantibodies exist at very low titers, heavy oxygen ($^{18}OH_2$) labeling was incorporated into the protocol in order to increase sensitivity and specificity. Heavy oxygen ($^{18}OH_2$) labeling is a technique used in proteomics, in place of radioactive isotope labeling, in order to have physical confirmation of amino acid presence identified by a known shift in mass number[52]. Natural autoantibodies found in healthy controls are at such a low titer that heavy oxygen labeling was essential. To the best of our knowledge, this work represents the first report of application of the ^{16}O-to-^{18}O exchange technique to epitope mapping. The details of the labeling reaction are shown in Figure 2.2.

Figure 2.2 Labeling with heavy oxygen ($^{18}OH_2$)

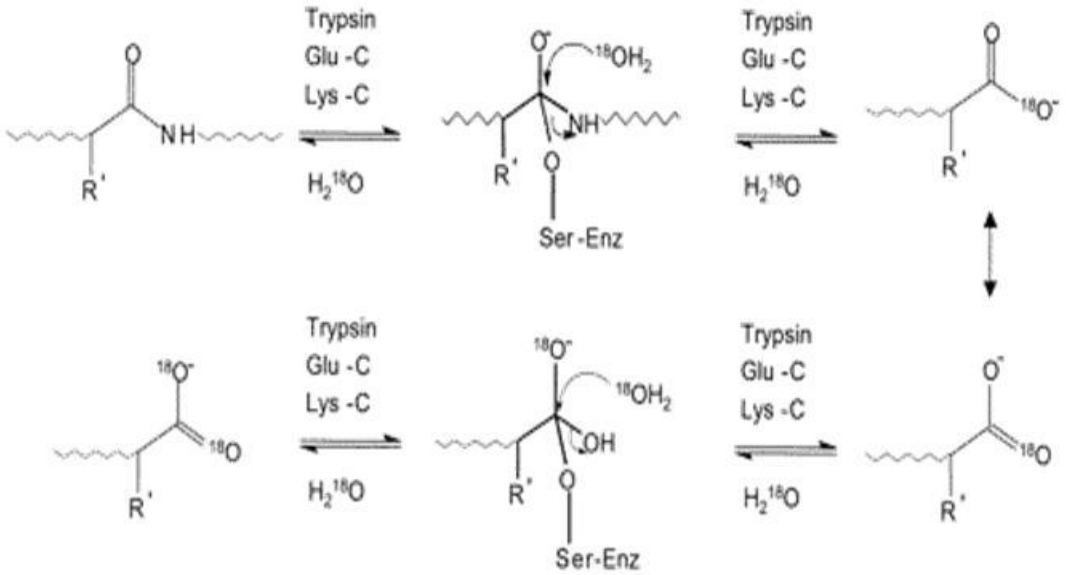


Figure 2.2 An approach with greater sensitivity was required for epitope excision for epitope mapping antibodies from healthy individuals. Using trypsin digestion as the catalyst to the reaction in the exclusive presence of $^{18}OH_2$ isotope, the carboxy end of the cleaved protein adds 18O- and raises the mass unit of the cleaved peptide 4 mass units. The shift in mass is used to identify the physical presence of the MPO peptide.

A total of seven epitopes were identified from control sera and were considered to be the targets of naturally occurring autoantibodies. Epitopes were divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the autoantibody's variable region. A conformational epitope is composed of discontinuous sections of the antigen's amino acid sequence or is dependent on tertiary structure or a particular microenvironment for antibody binding. An epitope was determined to be linear if autoantibody binding was not disrupted by incubation with pre-digested protein. Seven epitopes were found in healthy subjects. Most epitopes were determined to be conformational; four of seven epitopes identified were determined to be conformational and three of the seven were linear.

Epitope profiling of anti-MPO antibodies from patients with MPO-ANCA glomerulonephritis

After uncovering the epitopes targeted by naturally occurring autoantibodies we wanted to examine the epitope repertoire of patients with MPO-ANCA glomerulonephritis and if the same naturally occurring autoantibodies could be observed. To determine the epitope profiles of patients diagnosed with MPO positive glomerulonephritis the same epitope excision with MS analysis (Fig. 2.1) was used. These methods were performed on 88 sera samples from 66 unique individuals. Of the aforementioned sera samples 52 samples were from patients during active disease and 36 samples from patients in clinical remission. The distribution and frequency of the total data collected is shown in Table 2.1. Of the clinical remission sera samples, 26/36 were negative for MPO specific epitopes when using MS with heavy oxygen labeling. Of the remaining 10 samples in which MPO-ANCA epitopes were detectable, the epitope specificity aligned with naturally occurring autoantibodies (Table 2.1). The lack of MPO epitopes found in samples during disease remission is possibly a consequence of immunosuppressive therapies.

Table 2.1 MPO-ANCA epitope profiles

		Epitopes associated with disease																			Epitopes not associated with disease						
		537-548	490-499	328-351	220-228	198-219	448-459	715-725	369-374	442-447	605-622	657-664	184-193	516-524	560-571	692-701	474-480	437-441	396-405	579-590	237-244	461-473	530-536	593-603	572-578	678-691
Active ANCA patients	1	+																			+	+				
	2													+						+	+					
	3			+		+																	+			
	4		+				+																+			
	5	+				+														+						
	6		+			+														+		+				
	7					+														+	+	+				
	8		+	+		+																+				
	9	+	+											+						+			+			
	10		+																	+	+	+		+		
	11		+	+		+																+		+		
	12	+		+			+													+	+					
	13	+	+		+	+										+										
	14	+					+													+	+	+	+			
	15	+	+											+						+	+	+	+			
	16	+	+	+			+													+	+	+	+			
	17	+		+			+									+	+			+	+		+	+		
	18	+			+				+					+	+	+	+			+	+				+	
	19	+	+				+								+	+	+			+	+		+	+		
	20	+	+	+	+	+	+							+						+		+	+			
	21		+	+		+								+						+	+	+	+	+	+	
	22	+	+	+	+	+									+					+	+	+	+	+		
	23	+	+	+										+	+	+				+	+	+	+	+		
	24	+	+	+	+	+								+	+					+	+	+	+	+		
	25	+	+	+	+	+								+						+	+	+	+	+	+	
	26	+	+	+	+	+								+	+					+	+	+	+	+		
	27	+	+	+	+		+							+	+	+				+	+	+	+	+		
	28	+	+	+	+	+									+	+				+	+	+	+	+	+	
	29	+	+				+					+		+	+	+	+			+	+	+	+	+		
	30	+	+	+	+	+								+	+	+				+		+	+	+	+	
	31	+	+	+	+	+									+	+				+	+	+	+	+	+	
	32	+	+		+		+	+						+	+	+				+	+	+	+	+	+	
	33	+	+	+	+	+		+						+	+	+				+	+	+	+	+		
	34	+	+	+	+		+							+	+	+				+	+	+	+	+		+
	35	+	+	+	+	+								+	+	+				+	+	+	+	+	+	
	36	+	+	+	+	+								+	+	+				+	+	+	+	+	+	
	37	+	+	+	+	+	+							+	+	+				+	+	+	+	+		
	38	+	+		+		+		+					+	+	+	+			+	+	+	+	+	+	
	39	+	+	+	+	+	+	+							+	+	+			+	+	+	+	+		
	40	+	+	+	+	+	+							+	+	+				+	+	+	+	+	+	
	41	+	+	+	+	+	+							+	+	+				+	+	+	+	+	+	
	42	+	+	+	+	+	+							+	+	+				+	+	+	+	+	+	
	43	+	+	+	+		+	+						+	+	+	+			+	+	+	+	+	+	
	44	+		+	+	+	+		+					+	+	+	+			+	+	+	+	+	+	
	45	+	+	+	+	+			+					+	+	+	+			+	+	+	+	+	+	
	46	+	+	+	+	+	+							+	+	+	+			+	+	+	+	+	+	
	47	+	+	+	+	+	+							+	+	+	+			+	+	+	+	+	+	
	48	+	+	+	+		+						+	+	+	+	+			+	+	+	+	+	+	
	49	+	+	+	+	+	+	+						+	+	+		+		+	+	+	+	+	+	
	50	+	+	+	+	+	+	+	+					+	+	+	+			+	+	+	+	+	+	
	51	+	+	+	+		+	+	+					+	+	+	+			+	+	+	+	+	+	+
	52	+	+	+	+	+	+	+		+	+			+	+	+	+	+		+	+	+	+	+	+	
Clinical Remission	53-78	26 Clinical Remission Samples Have Zero Epitopes...																								
	78																							⊕		
	79																						⊕			
	80																	+								
	81																		+							
	82														+										⊕	
	83														+							⊕				
	84																						⊕			⊕
	85																			⊕		⊕	⊕	⊕		
	86															+				⊕		⊕	⊕	⊕		
	87													+		+	+			⊕		⊕	⊕	⊕		⊕
Healthy	A																									
	B																									
	C																						⊕			
	D																							⊕		
	E																			⊕		⊕				
	F																					⊕	⊕	⊕		
	G																			⊕		⊕	⊕			
	H													⊕							⊕				⊕	
	I																			⊕		⊕	⊕			⊕
	J																			⊕		⊕	⊕	⊕		⊕

A summary of mass spectrometry epitope excision data is depicted graphically in Figure 2.3. The epitope profiles from all 88 samples divided into two distinct categories: autoantibodies associated with disease or not associated with disease. Disease associated autoantibodies and their corresponding epitopes were defined by appearance in samples taken during active disease only, per physician's review of patient medical records. Common epitopes (n=7) existed in both disease and controls groups and these were determined to be non-pathogenic. Naturally occurring autoantibodies were present in MPO-ANCA positive sera, in conjunction with an additional 18 unique disease-restricted epitopes. One hundred percent (52/52) of MPO-ANCA patients with active disease had at least one disease restricted epitope, i.e. not present in healthy individuals.

Figure 2.3 MPO-ANCA epitope summary

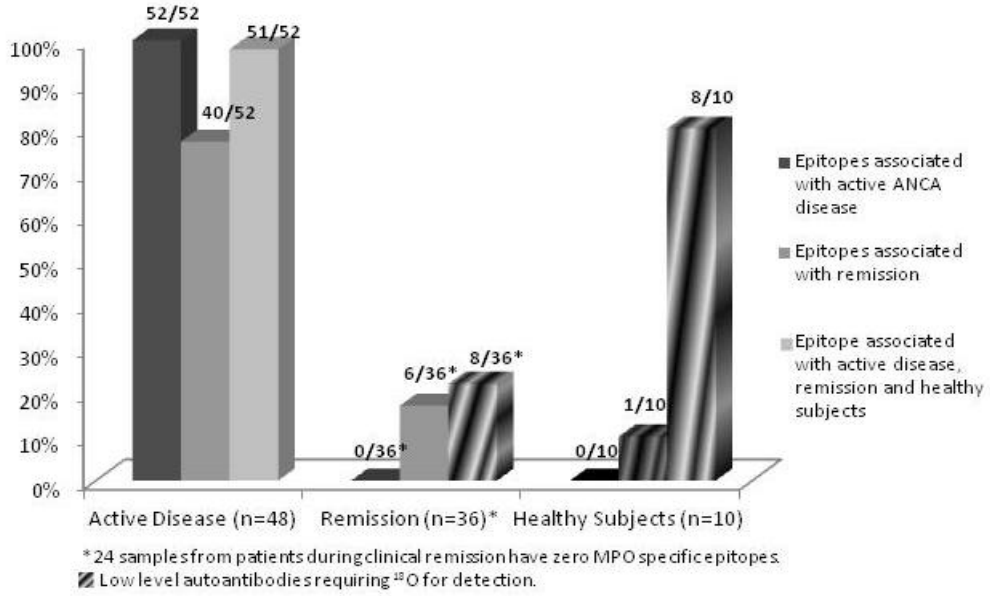


Figure 2.3 MPO epitopes found in patients and controls are distributed into categories of disease association. All 52 patients analyzed during active disease had autoantibodies specific to epitopes associated with active disease only and remission/ healthy subjects. anti-MPO specific autoantibodies were found in healthy subjects and samples taken from ANCA patients during clinical remission in extremely low levels . A more sensitive method was used by the addition of $^{18}OH_2$ labeling to detect these epitopes. This method was not needed to detect these same epitopes in MPO-ANCA patient samples during active disease.

Characterization of conformational and linear epitopes

An epitope was confirmed as conformationally dependent if binding was disrupted by incubation with pre-digested protein and this characterization was confirmed by peptide ELISA. Peptides of three predicted conformational epitopes (aa490-499, aa461-473 and aa593-603) were confirmed to be structurally dependant when assayed against MPO-ANCA patient sera samples (n=40) (Fig. 2.4). All sera samples did not react to the conformational epitopes tested.

Figure 2.4 Conformation dependent epitopes do not react by peptide ELISA

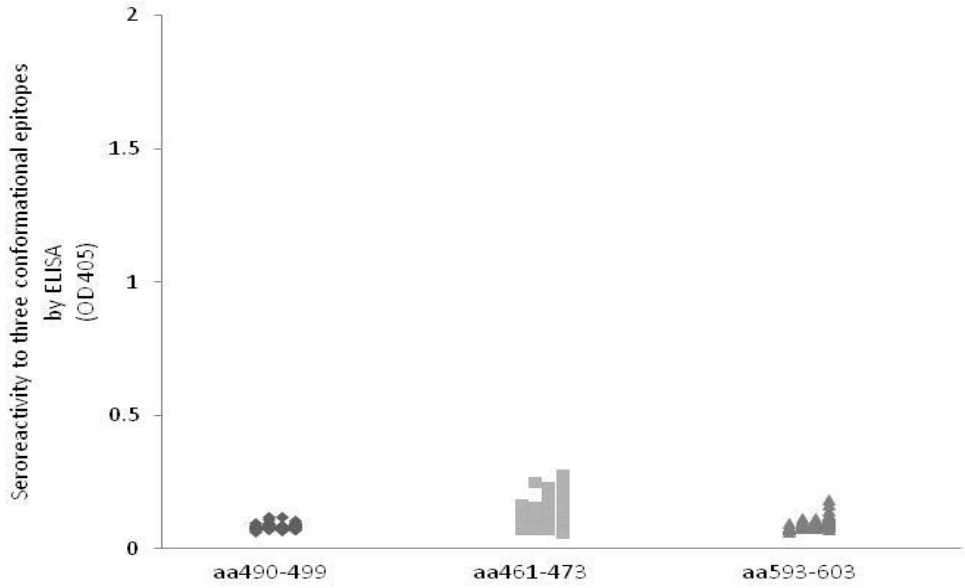


Figure 2.4 Three epitopes which were determined to be conformational were confirmed by peptide ELISA. MPO-ANCA active patient sera (n=40) were all unreactive to epitopes aa490-499, aa461-473 and aa593-603 by peptide ELISA which was predicted using epitope excision with pre-digested MPO.

Linear epitopes not associated with disease (n=3) were also verified for reactivity by peptide ELISA (Fig. 2.5). Autoantibodies specific to linear epitopes (3/7) were indentified in sera from 10 healthy subjects and two more linear epitopes were identified in MPO-ANCA patient samples. Reactivity was present in sera of healthy subjects, patients with active ANCA disease and disease remission when assayed against linear epitopes aa579-590, aa237-248, aa530-536; suggesting that these autoantibodies are not disease related. These naturally occurring autoantibodies trend towards exhibiting higher reactivity in MPO-ANCA patient samples.

Figure 2.5 Linear MPO epitopes not associated with disease are reactive by peptide ELISA

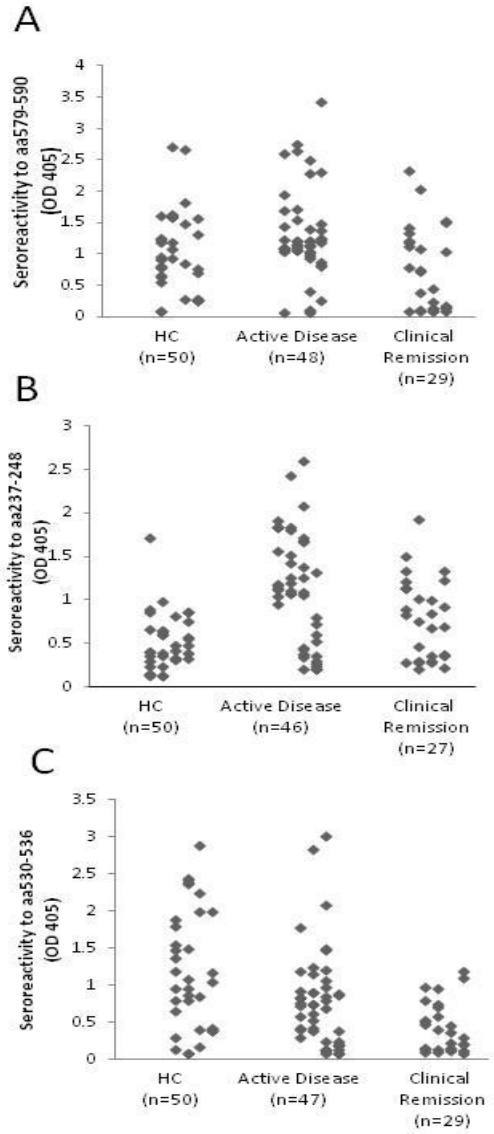


Figure 2.5 Sera from healthy controls (HC) and MPO-ANCA patients during active disease and clinical remission were assayed by ELISA to verify reactivity to linear epitopes not associated with disease. Panel A-C epitopes: aa579-590, aa237-248, aa530-536 respectively which were found in healthy subjects using heavy oxygen labeling and confirmed by peptide ELISA.

Strictly disease associated MPO epitopes included two linear sequences aa516-524 (YQPMEPNER) and aa448-459 (RKIVGAMVQIITY). These were also confirmed for reactivity by peptide ELISA. Approximately, 50% of MPO-ANCA patient samples tested were positive for an autoantibody specific for aa516-524 by both epitope excision and ELISA. At first evaluation (Fig. 2.6A) the level of reactivity was similar in sera during active disease and remission. However, analysis of paired samples from the same patients over the course of disease indicated this autoantibody was more prevalent during active disease (Fig. 2.6C).

The second disease-associated linear epitope aa448-459 (RKIVGAMVQIITY), when tested by ELISA, gave conflicting results: reactive by MS and unreactive by ELISA. A major difference between the two analyses was that whole sera was used for ELISAs but purified immunoglobulin (Ig) in MS. When the ELISA was repeated substituting Ig for sera, the expected reactivity was observed (Fig2.6B). Reactivity to MPO epitope aa448-459 was not observed in any disease remission samples tested by either assay. Further, when reactivity is assayed in paired samples from the same individual, first at active disease and second during clinical remission, a more dramatic picture of disease association is identified (Fig. 2.6D). This specific autoantibody targeting MPO epitope aa448-459 has potential to correlate with disease activity. The discrepancy of the use of sera or Ig for reactivity to MPO epitope aa448-459 will be discussed in depth in Chapter 3.

Figure 2.6 Disease associated linear epitopes are reactive by peptide ELISA

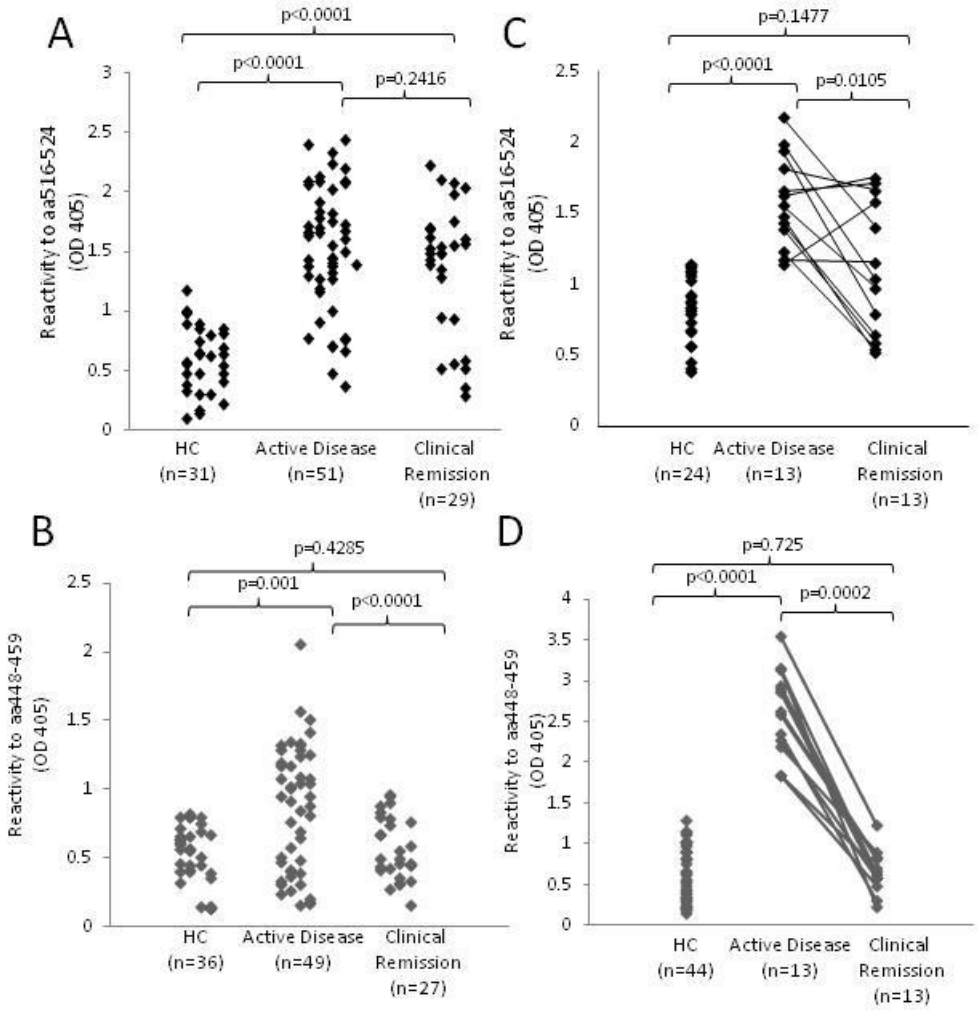


Figure 2.6 Verification of seroreactivity to predicted linear disease associated MPO epitopes found by epitope excision. Disease associated epitope aa516-524 (Panel A) shows specificity to ANCA disease but is also present during disease remission (Panel B). A second linear disease associated epitope aa448-459 was found to be unreactive by ELISA using sera. Total IgG was then used in place of serum to study reactivity to epitope aa448-459 by peptide ELISA which in Panel C and D shows a correlation to disease activity.

Anti-MPO aa448-459 autoantibody titers correlate with disease activity

A subset of patients with MPO-ANCA have consistently positive (above 20) autoantibody titers by antigen specific ELISAs even during long term remission. The following compilation of data shows that autoantibodies in the makeup of clinical titer include autoantibodies not associated with disease or natural autoantibodies. Figure 2.7 shows that disease associated MPO epitope aa448-459 has a more accurate link to disease activity than overall ANCA titer. In this particular case study anti- aa448-459 autoantibody titer (shown in dotted line) rises immediately preceding a relapse and is more consistently negative during periods of remission (shaded areas) compared to whole anti-MPO titer (solid line).

Figure 2.7 Case study of MPO-ANCA patient with consistently high titer anti-MPO

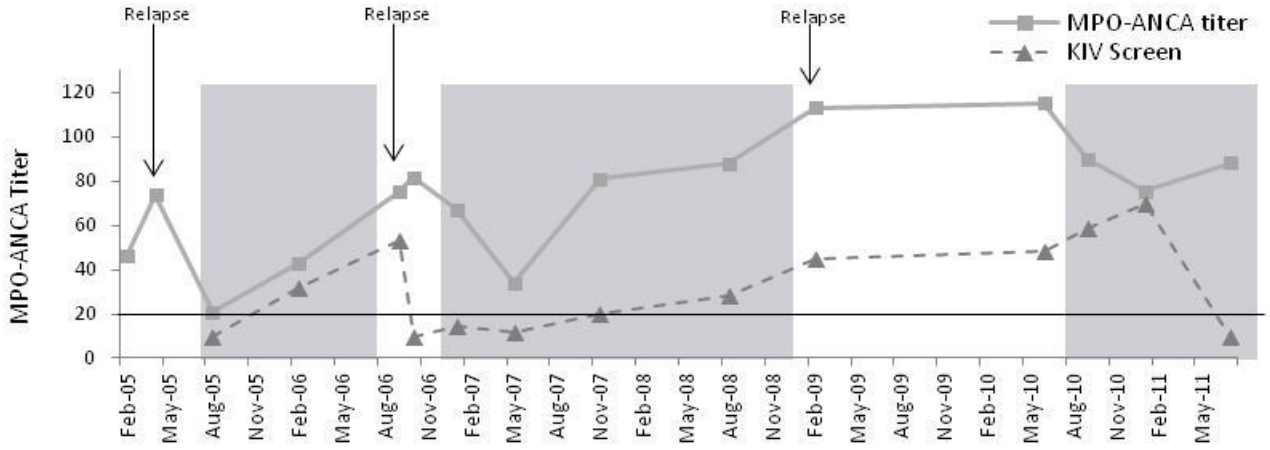


Figure 2.7 A case study of an individual with MPO-ANCA shows a consistently positive anti-MPO titer (above 20) from February 2005 to May of 2011. Relapses are marked by arrows contrasting shades of gray which represent possible times of disease remission. The dotted line shows the titer of disease associated autoantibody anti-aa448-459 which more closely follows disease activity in this particular patient over the course of disease.

To further study this population, seven patients whose autoantibody titers are consistently between 40-100 (regardless of disease activity) were assayed for reactivity to MPO epitope aa448-459. The results shown in Figure 2.8 reveal an association with disease activity much more readily than whole anti-MPO titer. Importantly, during disease remission the whole ANCA titer, which is high in this cohort of patients, is made up of epitopes not associated with disease. It is important to note that there may be disease associated conformational epitopes that also correlate with disease activity that cannot be studied by ELISA.

Figure 2.8 Anti-aa448-459 titer correlates with disease activity

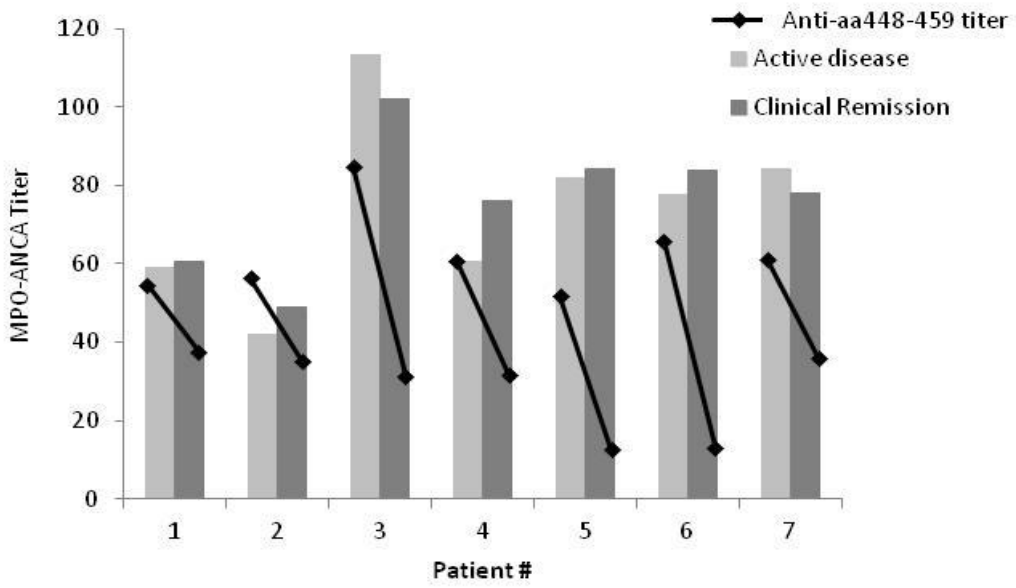


Figure 2.8 Disease associated autoantibody anti-aa448-459 consistently correlates with disease activity. Seven MPO-ANCA patients whose total anti-MPO titer does not decrease to normal levels during remission were assayed for the specific anti-aa448-459 antibody. Anti-448-459 antibody levels (solid line) decrease during remission in every case.

Epitope profiles are consistent within individuals upon relapse of disease

MPO-positive GPA patients relapse infrequently but when a relapse occurs it is important to examine if the same B cell clones are returning to produce similar autoantibodies or does a new set of clones emerge which target different MPO epitopes. Within our cohort of MPO-positive patients, there were five patients who had multiple active samples taken over the course of their disease; consisting of either onset of disease/relapse or relapse/remission/relapse. Epitope profiles were analyzed to determine changes in B cell clonal restriction (Table 2.2). The data indicate that the antibody specificity of a patient remains constant throughout the course of their disease.

Table 2.2 The same B cell clones return during disease relapse

Patient Number	Number of Epitopes at Disease Onset/Relapse	Number of Epitopes at Relapse	Proportion of homologous epitopes that return
1	3	15	**3/3**
2	13	5	**5/5**
3	13	15	**10/13**
4	15	13	**13/15**
5	14	8	**8/8**

Disease associated MPO specific epitopes have pathogenic potential.

It is well known that ANCA target MPO on the surface of neutrophils *in vivo* and cause them to degranulate and damage endothelium resulting in small vessel vasculitis. We can replicate this phenomenon in an *in vitro* assay using cytoclasin B primed neutrophils from healthy donors. Affinity purified autoantibodies were tested for their ability to activate neutrophils using an established neutrophil activation assay[4]. Autoantibodies that targeted linear epitopes were affinity purified and specificity was confirmed by ELISA (Fig. 2.9). First, specificity was determined by assaying a specific autoantibody against varying epitopes and second, reactivity and purity were assayed against each MPO peptide using all affinity purified autoantibodies. Characterization of eluted antibodies verified that the aa448-459 affinity purified autoantibodies reacted specifically aa448-459 peptide by ELISA and was negative for five non-specific peptides (Fig. 2.9A and C). Specificity was further validated when other anti-MPO antibodies purified by chromatography did not react with peptide aa448-459 (Fig. 2.9B and D). Once specificity and purity were examined by ELISA affinity purified autoantibodies were utilized for indirect immunofluorescence on fixed neutrophils and assayed for their ability to activate neutrophils from healthy donors.

Figure 2.9 Reactivity and specificity of affinity purified anti-MPO by ELISA

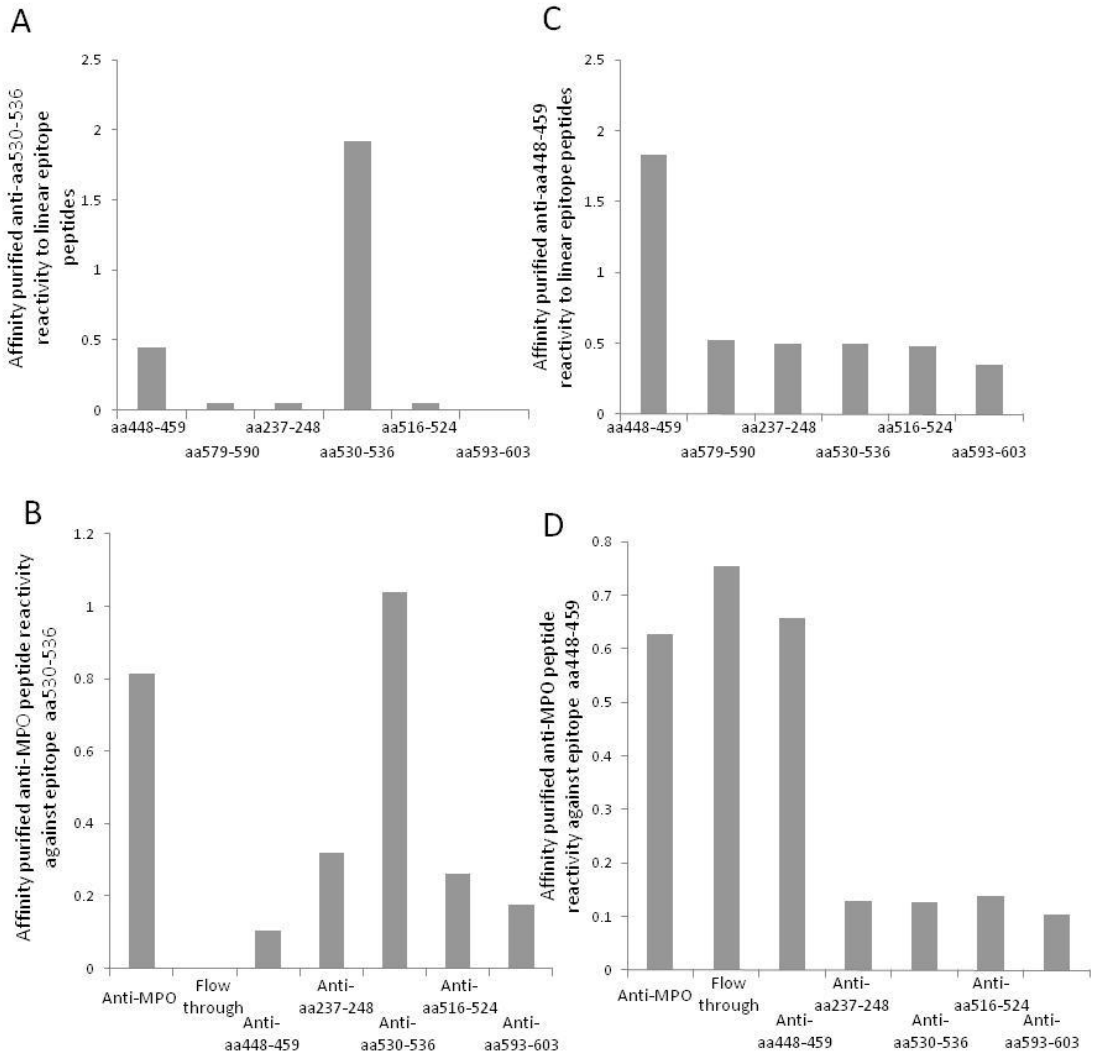


Figure 2.9 Peptide ELISAs utilizing affinity purified epitope specific autoantibodies from patient sample were assayed for reactivity and specificity to their corresponding peptides. Reactivity and purity was assayed against peptide aa530-536 (Panel B) and aa448-459 (Panel D) coated plates and reactivity of their corresponding antibodies in addition to other affinity purified autoantibodies. Specificity was determined by the reactivity of the affinity purified antibodies to a variety of MPO peptide substrates including native MPO as a positive control (Panel A and C).

Figure 2.10 shows the result of neutrophil activation by release of oxygen radicals utilizing neutrophils from four healthy donors. Total anti-MPO, anti-aa448-459, anti-aa516-524 and autoantibodies specific for conformational epitopes all activated healthy neutrophils (Fig. 2.10A). Autoantibodies found in healthy controls and during remission (not associated with disease) did not cause neutrophil degranulation (Fig. 2.10B). These results correlate to the individual autoantibody's prediction of disease association from the MS epitope excision.

Figure 2.10 *in vitro* pathogenic potential of affinity purified anti-MPO

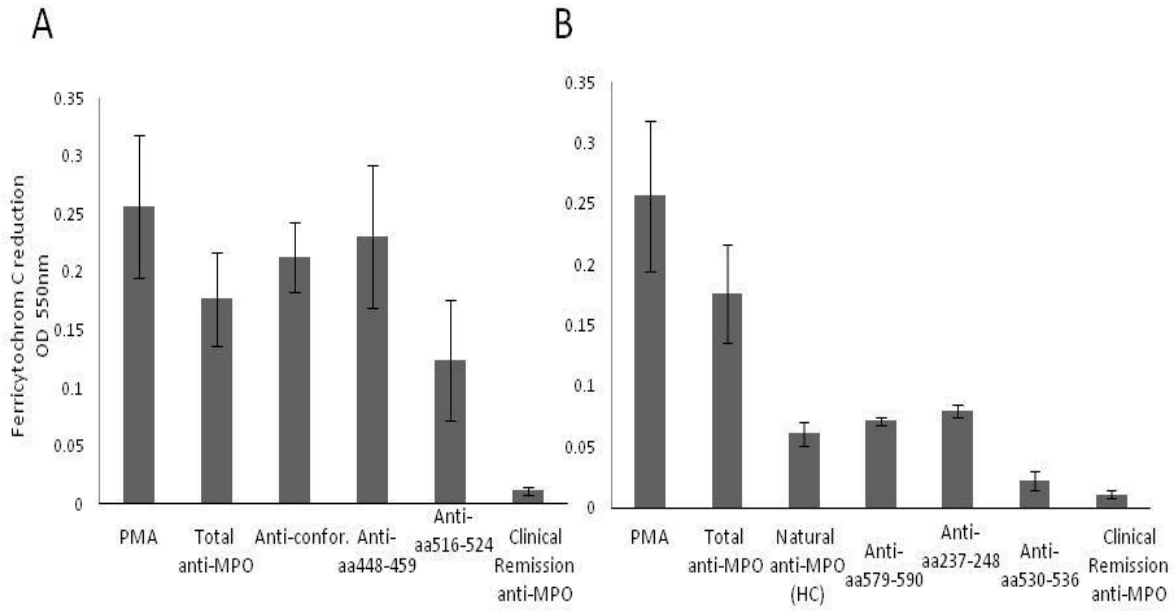


Figure 2.10 Comparison of MPO specific autoantibodies with and without association with disease by pathogenic potential. Neutrophil activation assays were done on neutrophils from 4 healthy individuals. Panel A displays the ability of disease associated autoantibodies to activate primed healthy neutrophils. In contrast, Panel B shows the result of neutrophil activation assays using autoantibodies from healthy individuals and patients which are not associated with disease.

Affinity purified antibodies were assayed for staining patterns on fixed human neutrophils by indirect immunofluorescence using the INOVA-ANCA IFA used by UNC hospitals for clinical diagnostic testing. Figure 2.10 shows the resulting staining patterns A) anti-MPO from a healthy individual B) total anti-MPO from an MPO positive patient C) affinity purified anti-aa530-536 D) anti-aa579-590 E) anti-aa516-524 F) anti-conformational epitope antibody pool. MPO positive stain exhibits a peri-nuclear pattern which can be seen on Fig 2.11B, E and F; while A, C and D are negative. Negative stains resulted from anti-MPO autoantibodies purified from healthy subjects (A) and autoantibodies purified from patients that target epitopes not associated with disease (C and D). Disease associated epitope aa448-459 does not stain healthy neutrophils and will be discussed further in Chapter 3.

Figure 2.11 Indirect immunofluorescence assay of affinity purified anti-MPO on human neutrophils

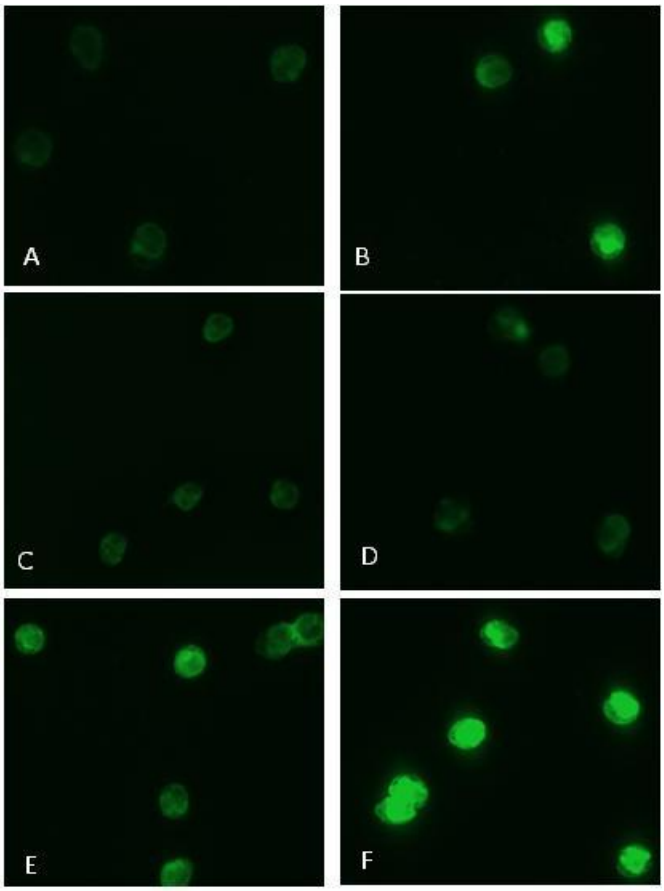


Figure 2.11 Reactivity of affinity purified autoantibodies to fixed human neutrophils using the INOVA-IFA clinical assay. Panel A shows affinity purified anti-MPO from a healthy individual in contrast to anti-MPO from a patient sera sample (Panel B). Panel C and D show a negative stain from autoantibodies not associated with disease anti-aa530-536 and anti-aa579-590 respectively. A perinuclear pattern is shown in Panel E by reactivity with linear disease associated epitope anti-aa516-524. An even more intense staining pattern is seen in Panel F by staining with a pool of autoantibodies to all conformational MPO specific epitopes.

Verification of reactivity to linear MPO epitopes in an independent patient cohort

The independent cohort from Groningen, The Netherlands consisted of 26 active samples with 26 longitudinal remission samples from 26 patients with MPO-ANCA, nine healthy controls and five Ig samples from patients with PR3-ANCA. Epitope profiles were determined from 20 active Ig patient samples included in the previously described cohort (Table 2.3).

Table 2.3 The Netherlands patient cohort; MPO epitope profiles

ID#	537-548	490-499	328-351	220-228	198-219	448-459	715-725	369-374	442-447	605-622	657-664	184-193	516-524	560-571	692-701	474-480	437-441	396-405	579-590	237-248	460-473	530-536	593-603	572-578	TOTAL
296															X										1
302	X																			X		X	X		4
206	X		X																X	X		X			5
260	X	X													X				X			X			5
266	X														X				X		X	X			5
314	X	X												X					X			X			5
218			X		X	X									X				X		X	X			7
230	X		X	X		X								X	X				X						7
242	X					X								X	X				X	X			X		7
236	X	X		X		X													X	X		X	X		8
224		X		X								X	X		X	X			X			X	X		9
278	X	X		X										X	X				X	X	X	X	X		10
320	X	X				X							X	X					X	X	X	X	X		10
248	X	X	X	X		X									X				X	X	X	X	X		11
308	X	X		X		X								X	X				X	X	X	X	X	X	12
252	X	X	X	X		X								X	X				X	X	X	X	X		13
326	X	X		X		X							X	X	X				X	X	X	X	X		13
332	X	X		X		X							X	X	X				X	X	X	X	X		13
254	X	X	X	X		X							X	X	X				X	X	X	X	X		14
200	X	X	X	X	X	X					X			X	X				X	X	X	X	X		15
Total	**17**	**13**	**7**	**11**	**2**	**12**	**0**	**0**	**0**	**0**	**1**	**1**	**5**	**11**	**15**	**1**	**0**	**0**	**18**	**13**	**11**	**17**	**13**	**1**	

The most prevalent disease associated epitope aa537-548 in our US cohort was also the most prevalent in the NL cohort but the distribution was not similar. The epitopes displayed in Table 2.3 are ordered by prevalence in the US cohort and do not correlate with the prevalence in the NL cohort indicated by the totals at the bottom of the table. There are also six epitopes that are not represented in the NL patient cohort. In addition to epitope profiling, disease associated anti-MPO$^{448-459}$ and anti-MPO$^{516-524}$ autoantibodies were assayed for reactivity and specificity by ELISA. The results, which are consistent with the MS data, are shown in Figure 2.12.

Figure 2.12 Reactivity to critical epitopes in an NL cohort by ELISA

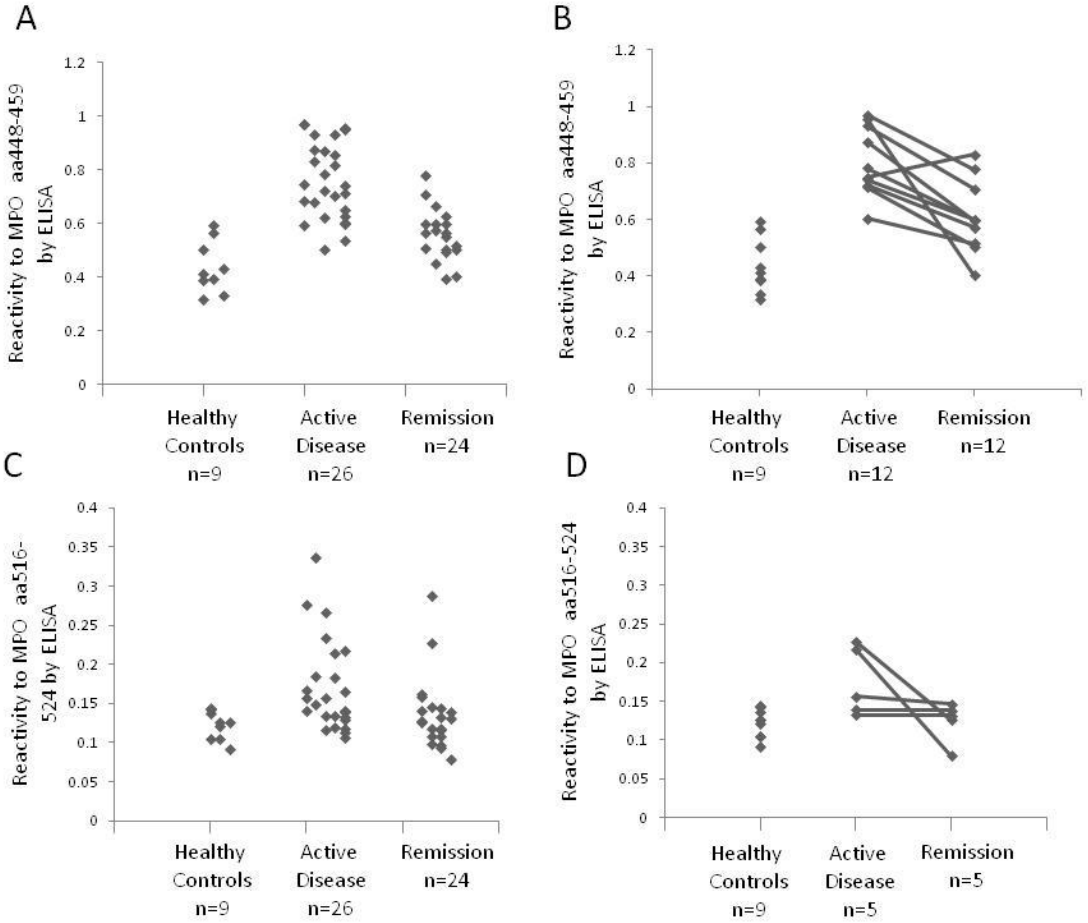


Figure 2.12 Purified Ig was assayed against two disease associated epitopes aa448-459 and aa516-524. Panel A shows the overall reactivity to MPO epitope aa4484-459 and Panel B shows the 12 samples positive for epitope aa448-459 by MS assayed by ELISA with longitudinal samples from the same individual. Panels C and D show the reactivity of Ig against MPO epitope aa516-524 using the NL cohort and longitudinal samples from the same individual positive for the epitope by MS respectively.

***In vivo* immunization of a CD4+ T cell epitope induces glomerulonephritis in DR2 Tg mice**

ANCA are implicated in the pathogenesis of vasculitis and FNGN [5, 13, 53], but although there is a rationale for autoreactive $CD4^+$ cells, there is less evidence of a role for cell-mediated effectors. We hypothesize that MPO-specific effector $CD4^+$ cells are important in disease by localizing to glomeruli and inducing a DTH-like lesion. The existence of autoantigen specific T cells, together with the presence of effector $CD4^+$ cells, macrophages and fibrin in patients' glomeruli provide the foundation for our hypothesis. MPO within the glomerulus exists both within neutrophils and extracellularly [54]. In a murine model of anti-MPO FNGN, where autoimmunity to MPO is induced and glomerulonephritis is triggered by injection of sheep anti-mouse glomerular basement membrane (GBM) antibody, $CD4^+$ T cell depletion during the effector phase attenuated disease [55].

To study the potential immunopathogenecity of MPO epitope aa448-459 *in* vivo, our colleagues at the Monash Medical Center in Victoria Australia were able to immunize mice (human DR2) with an overlapping T cell epitope. We were provided sera from their MPO mouse model. They have used this epitope to test the hypothesis that antigen specific $CD4^+$ T cells recognize this epitope and MPO itself, in glomeruli and induce FNGN. In addition to $CD4^+$ T cells recognition of the MPO epitope, we tested if the sera to determine the MPO epitope profile produced when mice were immunized with just one epitope. The immunodominant CD4+ T cell epitope was identified in mice immunized with 20 amino acid overlapping peptides spanning the murine MPO molecule. Immunization with MPO aa409-428 (PRWNGEKLYQEA**RKIVGAMV**) in combination with Fruend's complete adjuvant was confirmed to induce glomerulonephritis by injection at the base of the tail of DR2 Tg mice. This T cell epitope overlaps with our disease associated B ell epitope aa448-459 (**RKIVGAMV**QIITY). DR2 Tg mice have a human DR2 and immunization caused nephritic autoimmunity and an autoantibody B cell response. Mass spectrometry analysis using the previously described method was performed on the immunoglobulin fraction from sera of four MPO aa409-428 immunized DR2 Tg mice against murine MPO. The epitope profile of the humanized mice is

compared with human epitopes found using the same method in Table 2.3. The majority of the antibodies target MPO epitopes in regions of homology between murine and human MPO. As seen in Table 2.3, the epitope profiles from the DR2 Tg mice not only developed antibodies targeting the initial peptide immunogen but also initiated a polyclonocal response to the entire MPO molecule which targeted similar epitopes as an MPO-ANCA patient profile.

When this *in vivo* data is considered in addition with published data on the pathological role of ANCA in disease, our studies demonstrate that tissue injury in microscopic polyangiitis is mediated by a series of events. These events include distinct roles for both MPO-ANCA activated neutrophils, autoreactive effector $CD4^+$ T cells that provide further evidence to support the immunopathogenecity of not only ANCA but a specific anti-aa448-459 autoantibody.

Table 2.4 Comparison of human and murine MPO epitope profiles

Epitope AA Sequence Number	Epitopes found in Humans	Epitopes found immunized mice	Found in samples: Healthy control, Active disease, or Remission (HC, A, R)	Epitope Structure
490-499	IANVFTNAFR	IANVFTNAFR	A	Conformational
537-548	VVLEGGIDPILR	VVLEGGIDPILR	A	Conformational
328-351	NQINALTSFVDASMV		A	Conformational
220-228	NGFPVALAR		A	Conformational
198-219	WLPAEYEDGFSLPYG		A	Conformational
448-459	**RKIVGAMVQIITY**	**RKIVGAMVQIITY**	**A**	**Linear**
369-374	FQDNGR		A	Conformational
184-193	RSPTLGASNR	RSPTLGASNR	A	Conformational
605-622	FCGLPQPETVGQLGT		A	Conformational
442-447	LYQEAR		A	Conformational
715-725	NNIFMSNTYPR	NNIFMSNTYPR	A	Conformational
657-664	VGPLLACI		A	Conformational
560-571	QNQIAVDEIR		A, R	Conformational
692-701	QALAQISLPR		A, R	Conformational
474-480	KYLPTYR		A, R	Conformational
437-441	WDGER		A, R	Conformational
396-405	IPCFLAGDTR	IPCFLAGDMR	R	Conformational
579-590	IGLDLPALNMQR	IGLDLPALNMQR	A, R, HC	Linear
530-536	VFFASWR	VFFASWR	A, R, HC	Linear
237-248	FPTDQLTPDQER		A, R, HC	Linear
460-473	RDYLPLVLGPTAMR		A, R, HC	Conformational
593-603	DHGLPGYNAWR	DHGLPGYNAWR	A, R, HC	Conformational
516-524	YQPMEPNPR		A, R, HC	Linear
572-578	LFEQVMR	LFEQVMR	A, R, HC	Conformational
678-691	FWWENEGVFSMQQR		A, R, HC	Conformational

Discussion

In this chapter, anti-MPO epitope specificity was identified using an epitope excision proteomics approach with patient and healthy donor sera samples. A total of 25 MPO epitopes were found including: 12 associated with active ANCA disease, six associated with ANCA disease (including remission), and seven not associated with disease. By pre-digestion of MPO, 5/25 epitopes were determined to be linear, with the remaining 20 epitopes which were conformationally dependant. This finding is consistent with two initial reports identifying that MPO-ANCA recognize mostly conformational epitopes[19] and patients have a polyclonocal response[56].

Immunodominant MPO epitopes have been identified in patient cohorts around the world with little to no consensus. The initial report of anti-MPO specific epitopes by Pedrollo *et. al.* incriminate the denatured light chain of a bacterially expressed recombinant MPO protein. All patients tested (n=20) were reactive to whole native protein, 11 were reactive to the urea denatured light chain and zero were reactive to the denatured heavy chain. They concluded that most epitopes were conformationally dependent while the light chain held linear epitopes that were readily recognized by MPO-ANCA sera samples[57]. We have been able to confirm these initial results showing that there is a linear epitope found in the light chain (aa237-248) along with 3 other conformational epitopes.

In 1995, Chang *et. al.* studied the reactivity of five MPO-ANCA patients sera samples against overlapping peptides spanning the entire MPO molecule. They found four epitopes that were significantly reactive to patient Ig but Ig from healthy controls were reactive to the same four epitopes[21]. The epitopes that were reported in this study have overlap with our identified epitopes associated with disease only. These epitopes are not linear and would not be accessible using overlapping peptides. Chang and colleagues concluded that the reactivity seen in their assays was nonspecific.

A group in Japan published a series of manuscripts studying immunodominant MPO epitopes using recombinant MPO fragments[24, 58, 59]. There has been no characterization of the structure of the recombinant fragments or whether they are similar to native MPO. The initial report in 1998 presents a single region where reactivity was seen in all five patients tested. This large region of the heavy chain, which includes 141 amino acids, overlaps with three disease associated conformational epitopes we have found by epitope excision. In 2000, the same recombinant fragments were used to assay 20 MPO-ANCA patient sera samples. Using a larger cohort produced two narrower regions of reactivity (aa279-341 and 341-410) which were within the range of the initial finding and one additional region of 148 amino acids at aa598-745. It should be noted that the narrowing of the initial range did not exclude any epitopes we have found in that same region. The additional range overlaps with two epitopes not associated with disease and three disease associated epitopes which were all found to be conformationally dependent for antibody interaction. Their most recent paper published in 2007, again used their recombinant MPO fragments on an even larger population of MPO-ANCA patients (n=74) in order to study the link with disease severity. They were able to further narrow their epitope ranges to two sequences (aa279-341 and aa474-512) which in our studies overlaps to three disease associated epitopes which are conformationally dependent.

In an effort to rectify the gathering evidence of conformational dependent MPO epitopes and to preserve molecular architecture, Erdbrugger *et. al.* constructed human/mouse chimeras[22]. Their advantage was the close homology (90%) between murine and human MPO which in theory would equate to a similar structure to native MPO. The MPO molecule was divided into five segments: light chain, and 4 segments of the heavy chain (A-D). They found the most reactivity (n=14 patients with n=43 samples) in segments C (aa517-667) and D (aa668-745) which in combination is a total of 249 amino acids. The results presented in this chapter confirm that 12 epitopes fall within these two segments. Erdbrugger *et. al.* also found that 6/14 patients in at least one sample reacted with their segment B (aa387-516) which overlaps with an additional seven epitopes found by epitope excision.

While using chimeras yielded many more results than overlapping peptides, the regions of reactivity were too large to predict any specific immunodominant epitopes.

The most recent report is from Bruner *et. al.* in 2011, where they implicate seven immunodominant epitopes by utilizing an overlapping peptide ELISA[20]. We were able to confirm one of their immunodominant epitopes aa511-522 (RLDNRYQPMEPN) which overlapped with our linear disease associated epitope aa516-524 (YQPMEPNPR). Five other epitopes which we identified by MS epitope excision also overlapped with their linear epitopes but we determined that they were conformational. In this recent study, their cohort was limited to 14 individuals with MPO-ANCA which could have lead to the discrepancy in results.

Reviewing the literature describing potential MPO epitopes there is little evidence that points to a consistent ideally sized (3-5 amino acids) immunodominant epitope. While there is consensus a that the anti-MPO autoantibody response is polyclonal, the heavy chain holds the majority of disease associated epitopes and the majority of epitopes recognized by human anti-MPO autoantibodies are conformational. The idea of an immunodominant MPO epitope may only be relevant on an individual patient basis. Table 2.4 summarizes these findings and illustrates the overlap with the 25 epitopes presented determined by MS.

Table 2.5 Comparison of reported MPO epitopes

Manuscript (Author, year)	Protein substrate	Patient cohort	Epitope[#]	Overlap	My pathogenic prediction*
Pedrollo E., 1993	Bacterially expressed recombinant	n=20	Denatured Light chain aa166-278	aa184-193	DA
				aa198-219	DA
				aa220-228	DA
				aa237-248	NDA
Chang L., 1995	Overlapping peptides	n=5	aa119-130	aa442-447	DA
			aa179-180	aa437-441	DA
			aa439-450		
			aa649-670	aa657-664	DA
Tomizawa K., 1998	Recombinant protein fragments	n=4	aa269-409	aa328-351	DA
				aa369-405	DA
				aa396-405	DA
Fujii A., 2000	Recombinant protein fragments	n=20	aa279-341	aa328-351	DA
				aa369-374	DA
				aa396-405	DA
			aa341-410	aa593-603	NDA
			aa598-745	aa605-622	DA
				aa678-691	NDA
				aa692-701	DA
				aa715-725	DA
Erdbrugger U., 2005	Human/mouse chimeric recombinant	n=14	aa517-667	aa516-524	DA
				aa530-536	NDA
				aa537-548	DA
			aa668-745	aa560-571	DA
				aa572-578	NDA
				aa579-590	NDA
				aa593-603	NDA

				aa605-622	DA
				aa657-664	DA
				aa678-691	NDA
				aa692-701	DA
				aa715-725	DA
Suzuki K., 2007	Recombinant protein fragments	n=74	aa279-341	aa328-351	DA
			aa474-512	aa474-480	DA
				aa490-499	DA
Bruner B.F., 2011	Overlapping peptides	N=12	aa91-100	Aa220-228	DA
			aa213-222	Aa396-405	DA
			aa393-402	Aa442-447	DA
			aa437-446	Aa474-480	DA
			aa479-488	Aa516-524	DA
			aa511-522		
			aa717-726	Aa715-725	DA

 # amino acid sequence based off of MPO sequence gi 34719

***DA = disease associated epitope**

NDA = not disease associated

The search for an immunodominant epitope has obvious implications for the pathogenesis of disease but the development of these autoantibodies is another area of interest. Natural autoantibodies (NAA) which are defined as antigen (MPO and PR3) specific autoantibodies found in circulation in blood samples from healthy donors were first identified and isolated in an effort to understand the mechanism of ANCA production[28]. Purification of natural autoantibodies was done in 2010 by Cui *et. al.* from IgG fractions from healthy donors (n=20)[28]. Autoantigen specificity was proven by an indirect immunofluorescence assay (IFA) where both anti-PR3 and anti-MPO NAA showed a cytoplasmic pattern consistent with a positive PR3-ANCA IFA. This is not consistent with our results where autoantibodies not associated with disease do not stain human neutrophils by IFA. This discrepancy could be due to the fact that we were only able to test the 3 NAA that were specific to linear epitopes. Interestingly, Cui *et. al.* could not be detect NAA in serum or IgG fractions which is indicative of low titer antibodies, this finding correlates with our observations.

In 2011 Xu *et. al.* compared the characteristics of NAA found in healthy individuals to patients with ANCA disease. Plasma samples from five healthy blood donors and 10 patients with MPA were used to purify autoantibodies to MPO. Autoantibodies were assayed for reactivity by ELISA, western blot, for titer, avidity, IgG subclass, neutrophil activation and MPO/CP binding. They found that anti-MPO NAA were lower titer, lower avidity, had a lower inhibitory effect on MPO/CP binding, a lower ability to activate primed neutrophils and were comprised of different IgG subclasses than MPO-ANCA from patients. This report gives a full picture of the characteristics of the NAA which is in agreement with our results using epitope excision. In order to further the knowledge of NAA in ANCA disease, we have identified the sequence of 7 epitopes on MPO that are the targets of NAA; 3 of which are linear. Both reports admit that the role of NAA are still unclear but suggest that their role could be either protective or potentially pathogenic.

We hypothesize that the presence of NAA, autoantigens and autoantibody specificity are just some factors in a cascade of pathogenesis (likely variable between individuals) which results in

autoimmune disease[60]. Once we had determined epitope profiles from patient and healthy donor samples, the crystal structure of human MPO (pdb 3F9) was used to determine relationships between disease associated epitopes and non-disease associated epitopes. Disease associated epitopes shown in blue (Fig. 2.13) are in many cases found in conjunction with epitopes found in healthy individuals (green). Fine epitope mapping (shown in red) incriminates specific amino acids which are important for autoantibody binding in these pairs. We propose that individuals who express non-pathogenic MPO autoantibodies[28] are likely to not develop disease; however, these same potentially non-pathogenic autoantibodies in an individual with dysfunctional T cells[61-63] could aberrantly display sequential variations of the epitope. These autoreactive T cells are able to stimulate B cells to produce autoantibodies that are now potentially pathogenic and create active disease associated autoantibodies targeted at previously unrecognized neighboring MPO sequences.

Figure 2.13 Hypothesis: Origins of disease associated epitopes

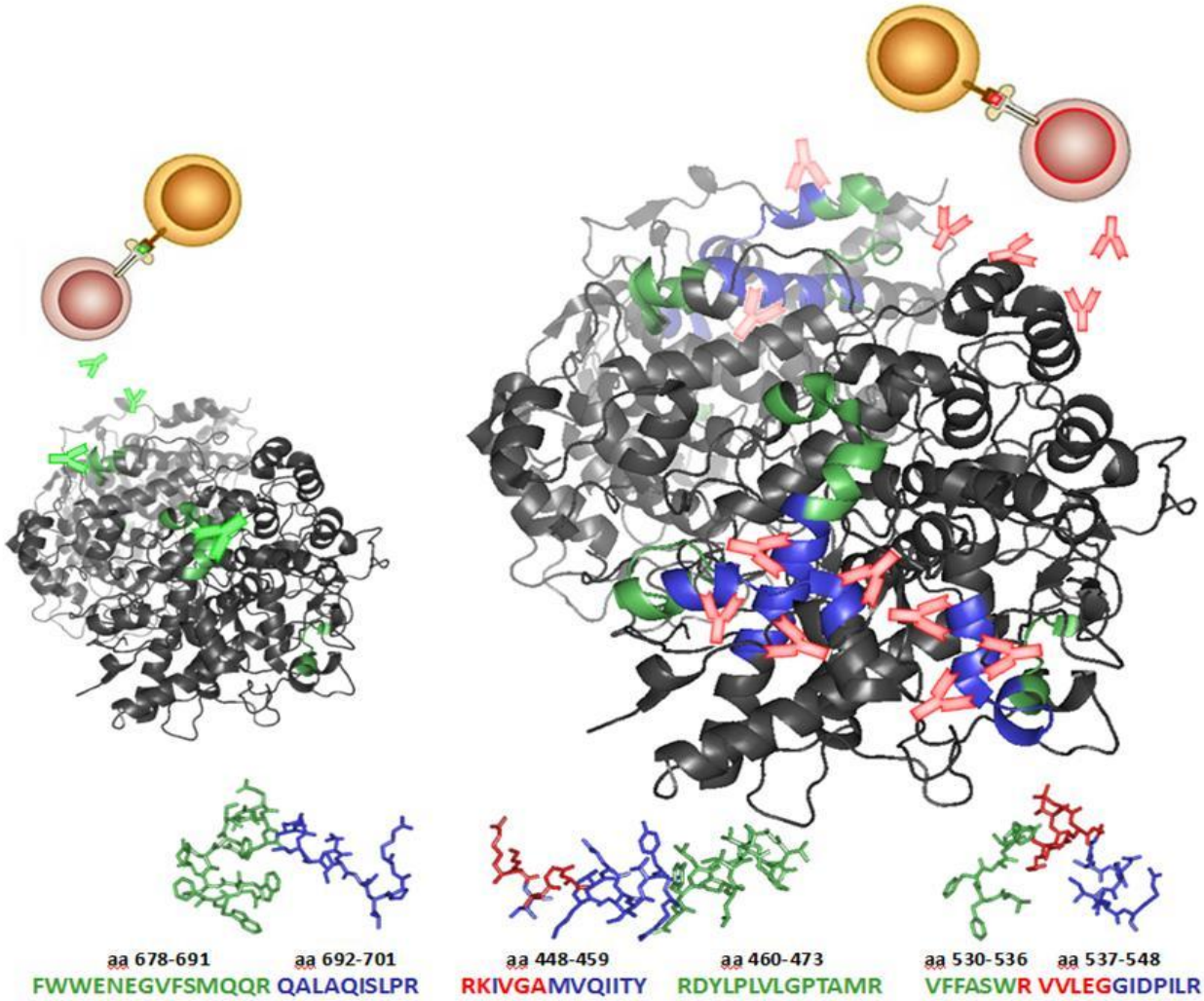


Figure 2.13 Development of active disease associated anti-MPO autoantibodies through aberrant T cell antigen presentation. In healthy individuals T cells present peptides to B cells that produce antibodies (left side) to MPO specific epitopes unrelated to autoimmune disease. Disease associated and non disease associated epitopes were found in pairs and upon fine epitope mapping, critical amino acids (highlighted in red) that correlate with disease activity.

We postulate that epitope specificity of anti-MPO-ANCA may also depend greatly on the genetic background of the individual. MHC classes and HLA subclasses direct the immune response and illicit a wide variety of a clonal B cell response. There is a range of autoantibody production in individuals' which may be dependent on their HLA subclass. The 52 active MPO-ANCA serum samples studied showed a wide range of B cell clonality. When patients were in an active disease state the range of unique autoantibodies found in their sera corresponding to 3-20 epitopes. Also, within the same individual upon a disease relapse, the same profile of anti-MPO autoantibodies reemerge and tended to target the same MPO epitopes as previous active disease states.

The amalgamation of the patient and animal data indicate that a MPO epitope aa448-459 restricted response is sufficient to cause pauci-immune vasculitis, underscored by the restricted response found in ANCA-negative glomerulonephritis patients. The murine $MPO_{409-428}$ CD4+ T cell epitope can also induce MPO-ANCA in DR2 Tg mice. While it is not necessary for autoreactive T and B cell epitopes to be from similar parts of the autoantigen, the identification of overlapping T and B cell epitopes in a similar region of the heavy chain of MPO with substantial cross reactivity strengthens the case for the relevance of this region of the MPO molecule in MPO-ANCA associated vasculitis. Targeted antigen-specific therapy is a long term aim in treating autoimmune disease. The identification of an important epitope within MPO provides a platform for further work aimed at developing antigen specific therapies, given the apparently relatively restricted range of autoantigens in microscopic polyangiitis with MPO-ANCA.

When considered with the published data on the pathological role of ANCA in disease, our studies demonstrate that tissue injury in microscopic polyangiitis is mediated by a series of events. These events include distinct roles for both MPO-ANCA activated neutrophils, as well as autoreactive effector $CD4^+$ T cells that recognize MPO planted in glomeruli by ANCA-activated neutrophils.

MPO-ANCA titers have not proven to be good markers of disease activity and their targets have been elusive until now[35, 64]. Our data shows that the majority (18/20) of disease associated anti-MPO-autoantibodies depend on a conformational structure for successful binding. Our current methods of testing autoantibody titers as a predictor of disease activity may be insufficient. The technique used in this study is drastically more sensitive than current clinical testing and has uncovered extremely low titer anti-MPO NAA found in healthy subjects as well as specific autoantibodies that correlate with disease activity.

Chapter 3

MYELOPEROXIDASE SPECIFIC AUTOANTIBODY IN ANCA-NEGATIVE GLOMERULONEPHRITIS

ANCA negative glomerulonephritis

ANCA glomerulonephritis is the most common form of aggressive glomerular disease and is associated with a systemic necrotizing vasculitis including microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA).[14] In general, patients with systemic necrotizing vasculitis have autoantibodies reactive with myeloperoxidase (MPO-ANCA), or proteinase 3 (PR3-ANCA). However, in some cases, patients with systemic necrotizing vasculitis have an ANCA negative serology. In 2009, Chen *et. al.* retrospectively reviewed clinical data collected from patient charts of ANCA glomerulonephritis cases in China and the UK and determined that 10-30% of studied cases were ANCA-negative[62]. The percentage of ANCA negative glomerulonephritis case varies widely and diagnosis is problematic with conflicting and inconsistent results from clinical testing. For example, antigen specific ELISAs are negative while indirect immunofluorescence assays against human neutrophils show a positive staining pattern, or when a patient presents as ANCA negative but as their disease progresses clinical tests indicate a positive ANCA serology. The observance of these clinical discrepancies, when taken as a whole, lead to the following possible hypothesis: 1) ANCA-negative glomerulonephritis is a distinct syndrome; 2) autoantibodies are targeting an autoantigen not included in the clinical test; 3) existing clinical tests lack sensitivity and/or specificity.

One of the MPO epitopes we have found to be exclusively prevalent in patients with active disease appears to be a critical immunodominant epitope found not only in ANCA positive patients,

but also is the sole reactive epitope in some patients with ANCA negative disease. The reason for the inability to detect this anti-MPO antibody in conventional clinical tests is a consequence of its blockade by a fragment of the natural inhibitor of MPO, which is ceruloplasmin. It is the discovery of an immunodominant epitope and corresponding anti-MPO autoantibody in ANCA negative patients hidden from conventional clinical assays that raises the possibility of similar phenomenon in other "seronegative" autoimmune diseases thereby opening a window into the possible experimental approach to exploring other autoimmune disease.

In this chapter, we present experiments that revealed the existence of MPO-ANCA in sera of patients previously diagnosed as "ANCA negative systemic necrotizing vasculitis." We were able to discern a pathogenic epitope on MPO (aa448-459), which was previously identified as a linear epitope associated with disease (Chapter 2). Autoantibodies to MPO epitope aa4484-59 were previously undetected in clinical assays due to a 'blocking' factor resident in serum. Indications are that monitoring for this specific ANCA-epitope is a better indicator of not only MPO-ANCA disease activity but also it is important in the diagnosis of ANCA disease.

Autoantigen epitope profiling in ANCA negative glomerulonephritis

Eight patients diagnosed with ANCA negative glomerulonephritis were selected based upon clinical presentation with small vessel vasculitis with a negative result by indirect immunofluorescence microscopy or antigen-specific enzyme-linked immunosorbent assays (ELISA). Participants were included if they were persistently ANCA negative but had histological confirmation of the disease by a biopsy of the kidney, lung or upper respiratory tract consistent with pauci-immune small vessel vasculitis or glomerulonephritis, with or without granulomatous inflammation. Three patients with persistently negative ELISAs but positive p-ANCA by immunofluorescence were also included. The above patient population was 87.5% female and 62.5% Caucasian with a mean age of 35.6 years.

Sera samples from patients diagnosed with ANCA negative glomerulonephritis (n=8) were assayed for autoantibodies by mass spectrometry using the MS epitope excision protocol (Chapter 2) with the exception that the total leukocyte cell lysate from a healthy donor was used as a non-biased substrate to search for unknown antigens. All patients with ANCA-negative glomerulonephritis displayed a restricted autoantibody response to myeloperoxidase, specifically, to epitope aa448-459 (RKIVGAMVQIITY). Mass Spectrometry spectral analysis identifies the peptide bound to patient Ig is from myeloperoxidase. In Figure 3.1 an MS spectrum from one ANCA-negative glomerulonephritis patient shows the peptide at a mass of 1491.841.

Figure 3.1 Autoantibody specificity in ANCA-negative glomerulonephritis by MS

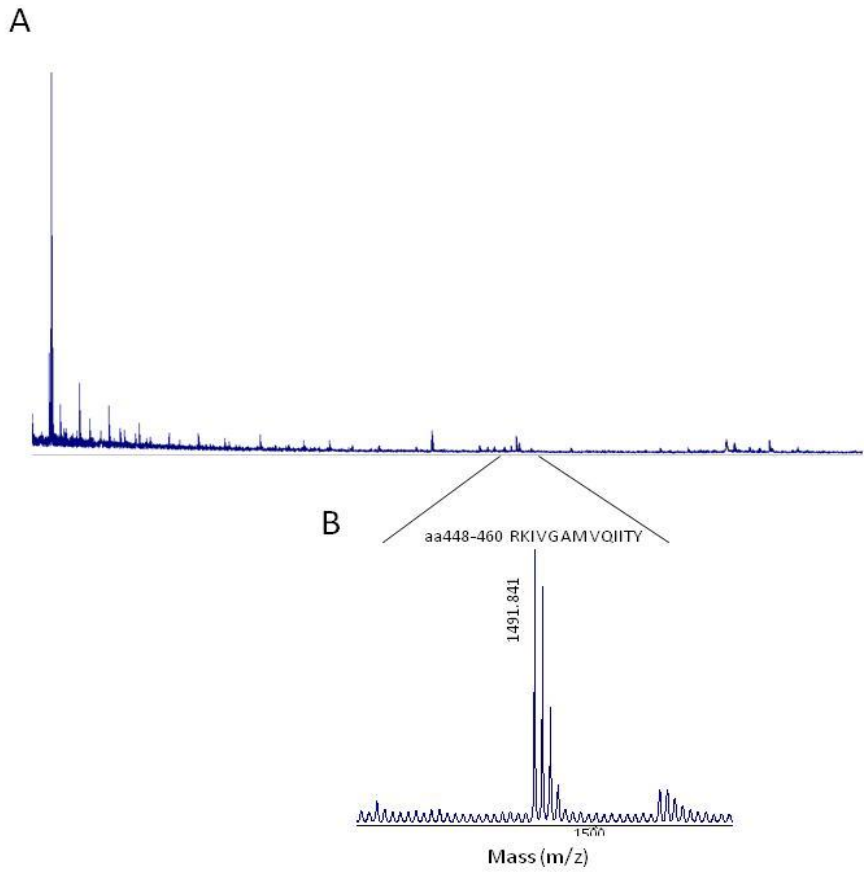


Figure 3.1 Immunoglobulin fractions were purified from patient sera and subjected to epitope excision and MS analysis (Panel A). Patients were found to have a restricted autoantibody response to MPO. MS analysis revealed autoantibody binding to an MPO fragment mass of 1492.14, identifying the epitope as aa448-459(Panel B).

Upon further analysis MPO epitope profiles were determined for all twelve patient samples. Table 3.1 shows the epitope profiles of the 8 patients studied in this cohort (four of which had samples also during disease remission), all active samples identified only one disease associated epitope, MPO epitope aa448-459.

Table 3.1 MPO epitope profiles from patients diagnosed with ANCA-negative glomerulonephritis

	537-548	490-499	328-351	220-228	198-219	448-459	715-725	369-374	442-447	657-664	184-193	516-524	560-571	692-701	474-480	437-441	396-405	579-590	237-244	460-473	530-536	593-603	572-578	678-691
1						+												+	+		+			
2						+																		
3						+													+		+			
4						+													+					
5						+												+	+		+			
6						+												+						
7						+																		
8						+														+				
9-11	zero epitopes observed																							
12																			⊕					
A																					⊕			
B																								
C																					⊕			
D																						⊕		
E																		⊕		⊕				
F																				⊕	⊕	⊕		
G																		⊕		⊕	⊕			
H												⊕							⊕				⊕	
I																		⊕		⊕	⊕			⊕
J																		⊕		⊕	⊕	⊕		⊕

Reactivity was confirmed by ELISA using the native MPO and MPO peptide aa448-459 as substrate (Fig. 3.2). The results of the ELISA show that in comparison to healthy subjects, the Ig fraction is reactive against native MPO (Fig. 3.2A) and epitope aa448-459(Fig. 3.2B). Paired samples from four of the eight patients were also assayed for reactivity by both substrates by ELISA (Figure 3.2C and D). Samples were collected during active disease exhibited reactivity to native MPO, while samples collected during disease remission had significantly decreased reactivity. The data correlate with the initial observation of anti-aa448-459 autoantibodies in active MPO-ANCA disease.

Figure 3.2 Reactivity of anti-aa448-459 in ANCA-negative glomerulonephritis

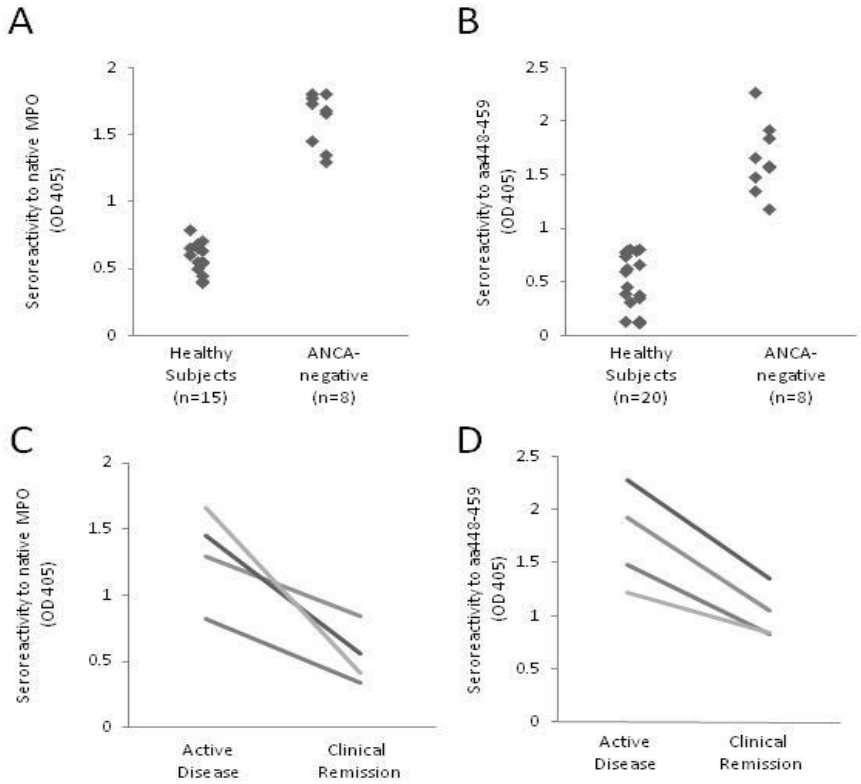


Figure 3.2 Reactivity to MPO epitope aa448-459 and native MPO were assayed by ELISA with samples from ANCA-negative glomerulonephritis patients (n=8) and healthy individuals (n=15, n=20). Panels A and B show the seroreactivity of Ig from ANCA-negative sera against native MPO and the synthetic peptide aa448-459, as compared to healthy controls. Panels C and D display the correlation of anti-MPO reactivity with disease activity using paired samples from the same individual during active disease and clinical remission.

It is important to note that all assays were carried out using the Ig fraction purified from patient sera whereas both ELISA and IF clinical assays used whole sera. We hypothesized that the failure of detecting anti-aa448-459 autoantibodies in the clinical assay was due to masking by a serum component. To test this hypothesis, ELISAs were used to test the reactivity against native MPO and MPO peptide aa448-459 using both sera and Ig fraction from the same sample. Figure 3.3 shows that a positive autoantibody reactivity against native MPO or MPO peptide aa448-459 by ELISA was highly dependent on using purified Ig, while results were negative using the same patient's whole serum (Fig. 3.3A and B). These results are consistent with the prediction of a serum 'epitope-blocking' factor observed in Chapter 2. Further, this observation was confirmed by an inhibition experiment in which increasing amounts of serum were added to reactive Ig fractions and all reactivity was blocked (Fig 3.3C). The addition of sera from a healthy individual or a MPO-ANCA patient yielded a steep reduction of reactivity at a concentration of 1:1000 (Ig : sera). It was determined that the serum component is abundant in sera and is not disease specific.

Figure 3.3 Seroreactivty to MPO using sera and Ig

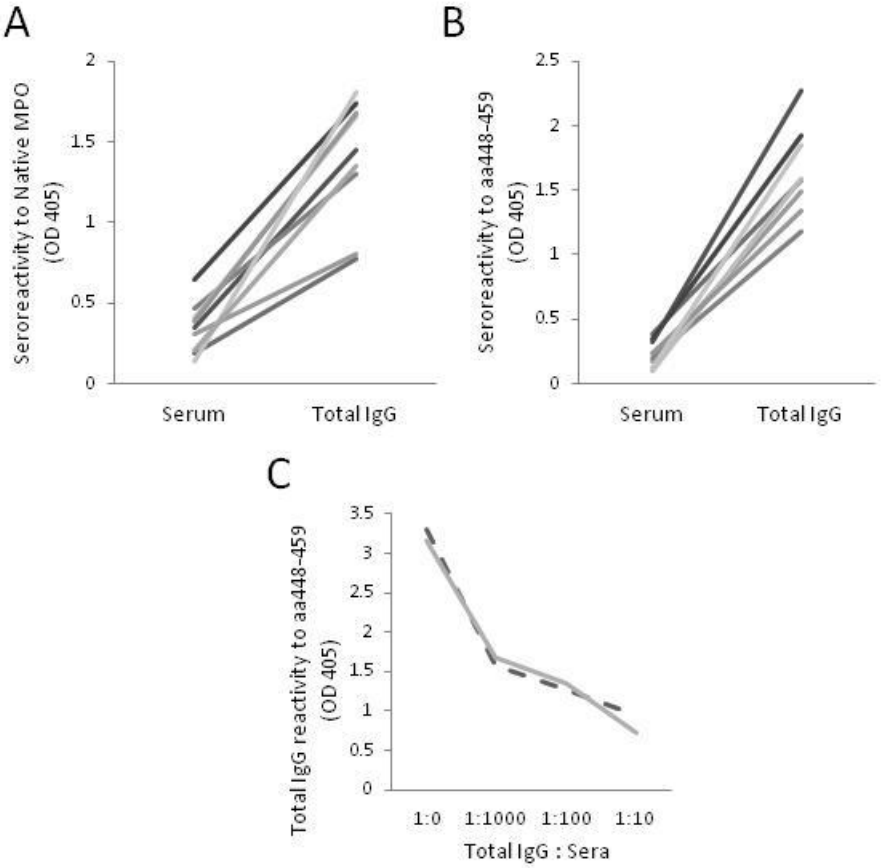


Figure 3.3 Sera from eight ANCA-negative glomerulonephritis patients were assayed against native MPO(Panel A) and a synthetic peptide of epitope aa448-459 (Panel B). All sera were non-reactive in contrast to total Ig. Titration of serum, from both a healthy subject (dashed line) and a patient serum (solid line), into total Ig preparations blocked reactivity to MPO peptide aa448-459 (Panel C).

Ceruloplasmin blocks anti-448-459 autoantibody interactions on native MPO

To identify the 'blocking-factor' present in sera, MPO peptide aa448-459 (irreversibly bound to polystyrene beads) was used to discover potential binding partners. Peptide beads were incubated with sera from patients and healthy individuals. The stained gel of eluted proteins showed a band on all five healthy control and five active MPO-ANCA patients at ~151kD (Fig. 3.4A). There was also a protein band of approximately 50kD in size. Both bands were excised for sequencing by MALDI-MS/MS and were identified as ceruloplasmin (CP). MALDI-MS/MS data was confirmed by western blot using a ceruloplasmin specific polyclonal antibody (Fig. 3.4B). A western blot probed with a polyclonocal anti-CP antibody recognized both sizes of protein one at 151kD (full length CP) and about 50kD (CP fragment). From this data we were able to determine that full length CP and a smaller CP fragment are both able to bind specifically to MPO epitope aa448-459. There are no obvious differences in amount of CP that bound to MPO peptide aa448-459 between healthy subjects and active MPO-ANCA patients by western blot.

Figure 3.4 Identification of serum component bound to aa448-459

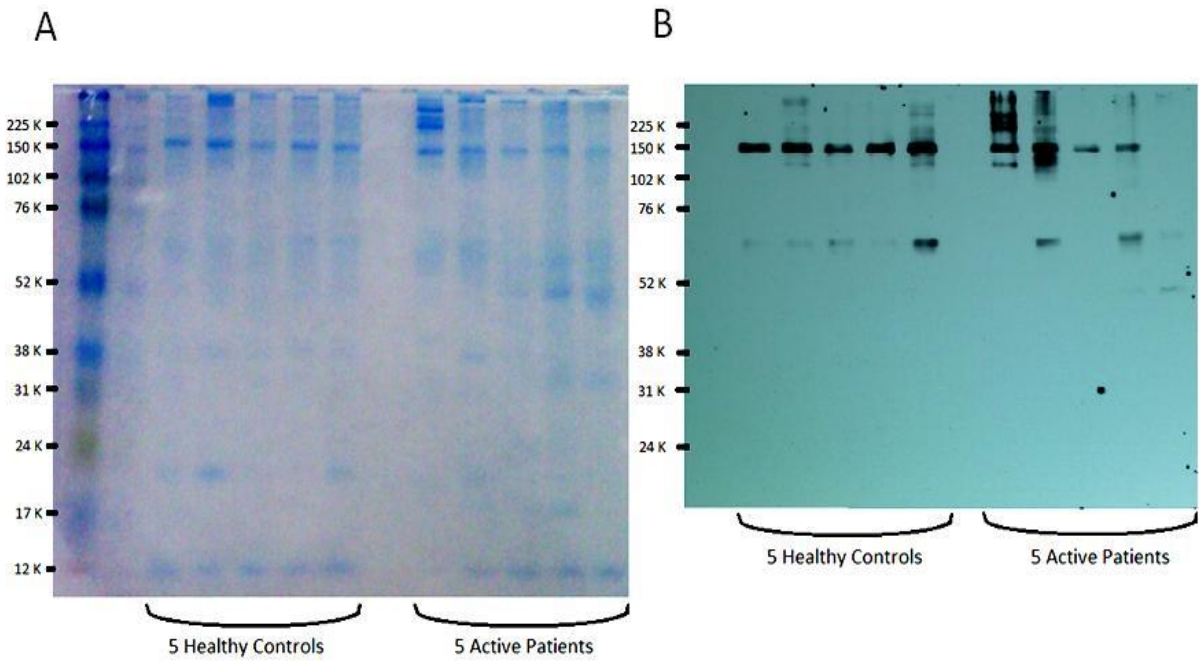


Fig 3.4 Sera 'epitope-blocking' factor was purified using the target MPO epitope aa448-459 and analyzed by gel electrophoresis. Distinct bands at ~150kD can be detected in all 10 samples irrelevant of disease (A). The gel was transferred to nitrocellulose and probed with an anti-ceruloplasmin polyclonal antibody which confirmed that bands at ~150kD and ~50kD were full length ceruloplasmin and a protein fragment, respectively (B).

To study the inhibition of ANCA binding to the MPO epitope aa448-459 *in vitro*, full length CP and also the smaller CP fragment were required. In order to replicate the CP fragment at ~50kD, full length CP was digested by a variety of serum proteases shown in Figure 3.5. Serine proteases chymotrypsin, plasmin, thrombin and urokinase were determined to digest CP into appropriate sized fragments whereas lysozyme digestion did not. Plasmin, thrombin and urokinase were chosen as candidates to cleave ceruloplasmin into a fragment that could potentially bind MPO epitope aa448-459.

Figure 3.5 Full length ceruloplasmin digested by various serine proteases

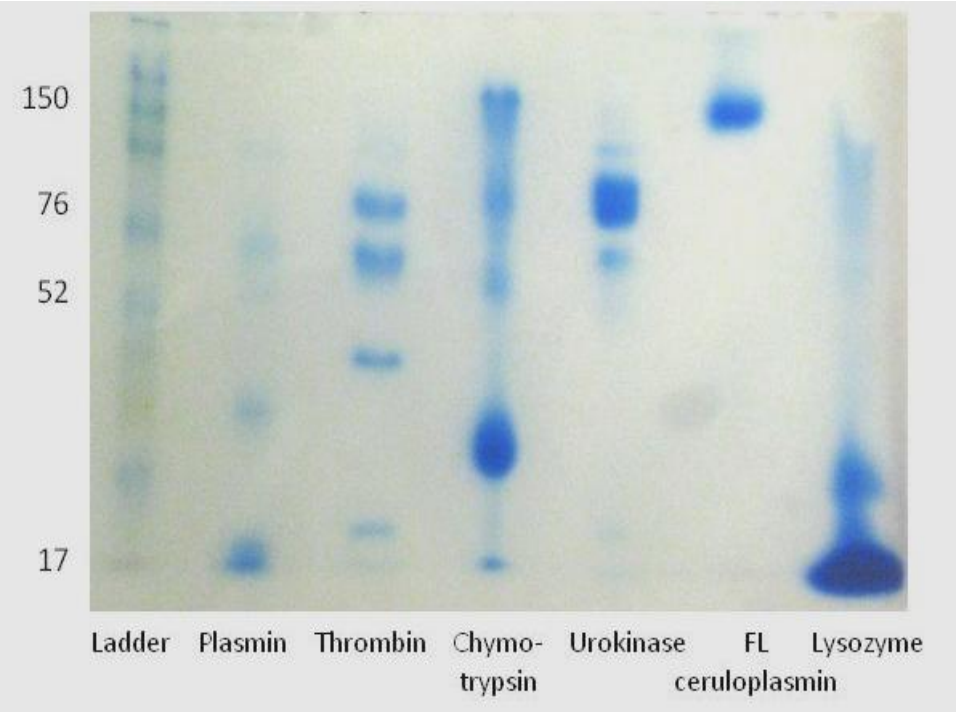


Figure 3.5 Serine protease digestion of full length ceruloplasmin. Ceruloplasmin was digested by plasmin, thrombin, chymotrypsin, urokinase and lysozyme to identify a fragment at ~50kD. Full length ceruloplasmin can be observed in lane 6 at ~150kD. Proteases: plasmin, thrombin and urokinase were chosen for further analysis.

Figure 3.6 shows the specificity of the enzymatic cleavage product of CP to block autoantibody binding. Ig samples from a vasculitis patient diagnosed with ANCA-negative glomerulonephritis (who were identified as reactive for MPO epitope aa448-459) and an MPO-ANCA positive patient sample were assayed to observe the effect of the CP fragment on autoantibody reactivity. Neither full length ceruloplasmin nor ceruloplasmin cleaved by thrombin successfully inhibited the autoantibody to bind MPO epitope aa448-459(Fig. 3.6A and B). In contrast, both fragments of ceruloplasmin cleaved by either urokinase or plasmin preferentially bound epitope aa448-459 and inhibited autoantibody binding (Fig. 3.6C and D). These results indicate the dominate species of ceruloplasmin bound to peptide aa448-459 was a cleavage product of full length ceruloplasmin.

Figure 3.6 Specific ceruloplasmin fragment blocks autoantibody binding on native MPO

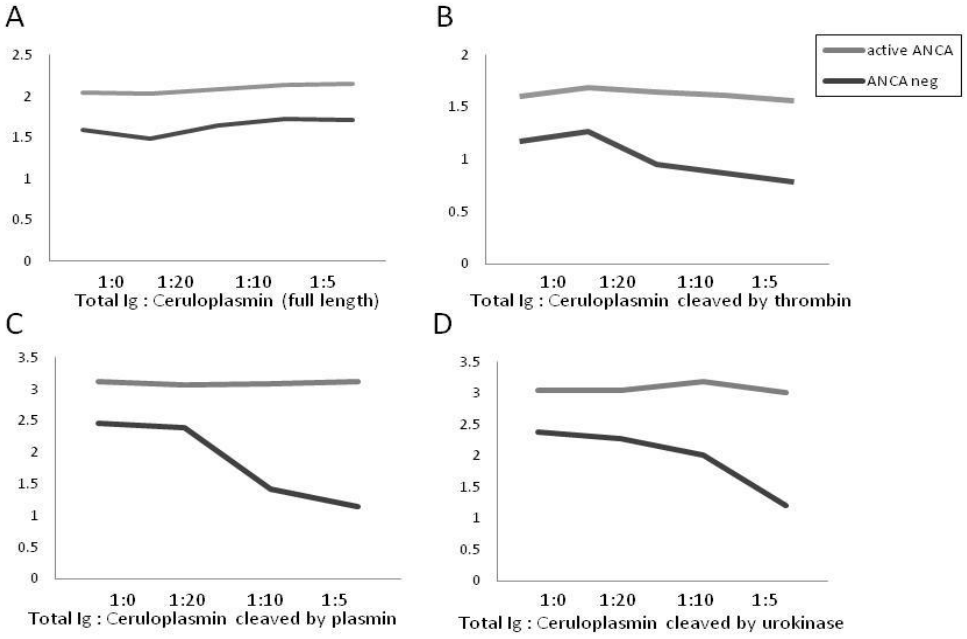


Figure 3.6 Inhibition of autoantibody binding to MPO epitope aa448-459 by addition of Ceruloplasmin. Full length CP (A), CP cleaved by thrombin (B) did not inhibit binding whereas the addition of CP cleaved by plasmin (C) and CP cleaved by urokinase (D) significantly inhibited binding.

We hypothesize that some patients with ANCA-negative glomerulonephritis are unreactive to both clinical tests for ANCA disease (ELISA and IFA) because of a specific proteolytic fragment of ceruloplasmin normally present in sera which is blocking reactivity. These patients have a markedly restricted polyclonal response to MPO. The antigen-specific ELISA used for clinical diagnosis is simulated in Figure 3.7A where the addition of the plasmin cleaved CP fragment blocks reactivity of seven sera samples from patients with ANCA-negative glomerulonephritis against native MPO. The ELISA shows that with the addition of the specific epitope 'blocker' reactivity is inhibited which results in a false negative. This phenomenon is not observed when using Ig from MPO-ANCA patients (n=4), as they have a polyclonal autoantibody response against native MPO. The clinical diagnostic test utilizing indirect immunofluorescence may also provide a negative finding or an irregular staining pattern for patients deemed as ANCA-negative. Could this be due to the presence of ceruloplasmin on the fixed neutrophils? Using a polyclonal anti-ceruloplasmin antibody, an abundance of ceruloplasmin was detected which could be already bound to MPO at aa448-459 and therefore obscuring the autoantibody binding site (Fig. 3.7B).

Figure 3.7 Detection of anti-aa448-459 autoantibodies is obscured in clinical assays by a cleavage product of a common serum protein

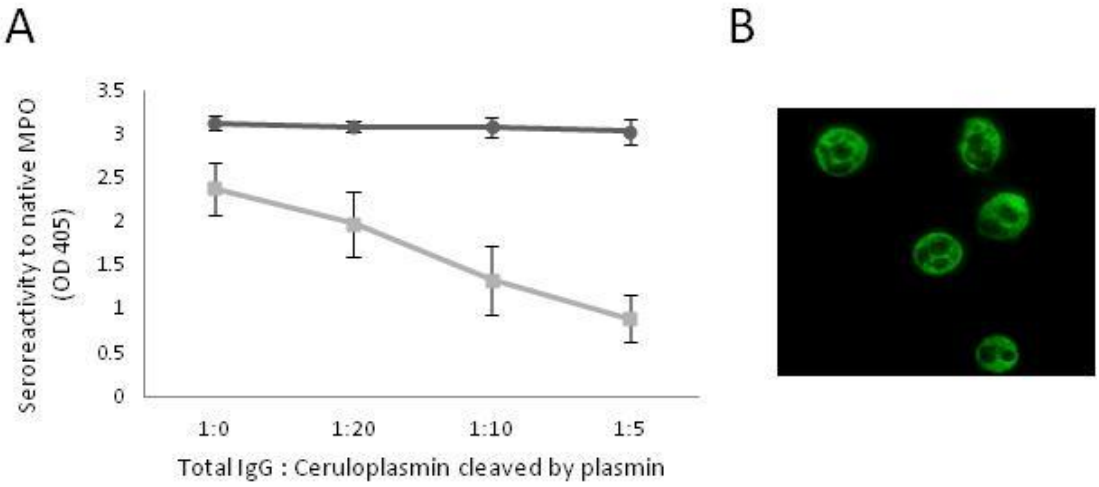


Figure 3.7 Panel A shows by ELISA that detection of anti-MPO 448-459 autoantibodies in total IgG from ANCA-negative patients (n=7) is muted by addition of cleaved ceruloplasmin (squares). Due to the polyclonal nature of MPO-ANCA positive patients, the total Ig (n=4) (circles) reactivity appears unaffected by addition of ceruloplasmin. Indirect Immunofluorescence staining (Panel B) shows that fixed neutrophils, when probed with anti-ceruloplasmin antibody, are positive for ceruloplasmin, explaining the negative IFA test of sera from ANCA-negative glomerulonephritis patients.

The results presented in this chapter confirm that these eight patients diagnosed with ANCA-negative glomerulonephritis are unreactive to one or both clinical tests for ANCA disease (ELISA and IFA) because of a proteolytic serum fragment indentified as ceruloplasmin which is able to specifically bind to native MPO at aa448-459 thereby masking the autoantibody binding site.

Clinical implications and biological significance

The clinical implications of this discovery are far reaching. Current treatment protocols rely on ANCA-positive tests, and therefore a false negative diagnostic test would have a detrimental impact on treatment decisions. Understanding the intricate biology of autoimmune disease may help to further improve clinical testing, diagnosis and treatment. Biologically, a serum component acting as a 'blocking-factor' binding to a known autoantigen raises the question of whether the body is actively making efforts to protect against autoimmunity, once tolerance is broken. In the case of ANCA disease, the biological role of myeloperoxidase and its inhibitor ceruloplasmin (CP) and how they interact may give us clues to how aspects of autoimmunity can be regulated by the human body. Therefore, based on these results we hypothesize that MPO epitope aa448-459 is a pathogenic site on MPO and a specific ceruloplasmin fragment binds this site to protect from autoantibody binding.

The interaction between CP and MPO has been studied in MPO-ANCA glomerulonephritis in relation to disease activity. The initial theory was that patients who were diagnosed with ANCA disease were deficient in ceruloplasmin. A ceruloplasmin deficiency or aceruloplasminemia, is marked by diabetes mellitus and retinal and neurodegeneration caused by a disruption of iron homeostasis[65]. This hypothesis predicted that low levels of ceruloplasmin would be insufficient for the inhibition of MPO to produce hypocholorus acid which in excess would cause severe endothelial damage when neutrophils released copious amounts of MPO after activation by ANCA.

First characterized by Segelmark et. *al.*, CP and MPO were reported to have a physical interaction under physiological conditions and CP was able to inhibit the peroxidase reaction of MPO *in vitro*[12]. Upon the discovery of CP to inhibit MPO, MPO-ANCA weas determined to disrupt the binding of CP and MPO. Griffin *et. al.* reported that ANCA had a greater affinity to MPO than CP and were able to reverse the inhibition of MPO by CP[66]. More evidence of the affect MPO-ANCA has on the CP-MPO complex was reported by Xu *et. al.* to showed that MPO-ANCA were able to influence the oxidative activity of MPO. They reported a correlation between the oxidation activity

of MPO after binding MPO-ANCA which positively correlated with the level of ceruloplasmin binding MPO in the presence of MPO-ANCA[67].

Many studies have tried to correlate the serum levels of ceruloplasmin in patients with ANCA glomerulonephritis with disease activity in order to prove the initial hypothesis that CP levels in these patients would be decreased compared to healthy subjects. A study done in 1999 by Ara *et. al.* assayed sera CP levels in 21 patients with ANCA glomerulonephritis and found that in active disease none of the patients had low CP protein levels and 57% of their cohort had higher than normal levels[68]. A second study done in 2002 by Baskin *et. al.* assayed sera CP levels in 45 patients during both active disease and clinical remission[69]. They reported that samples taken from patients during an active disease state had on average higher than normal levels of CP and in disease remission CP levels were normal. Again zero patients had lower than normal levels of CP in either active or remittent disease. In all of these studies, the CP that was assayed for was full length ceruloplasmin of 130kD or 151kD.

There is no resolved crystal structure which reveals the exact location of where CP binds to MPO but Sokolov *et. al.* characterized the complex of CP and MPO by three separate methods[70]. Using electrophoresis, gel filtration and photon-correlation spectroscopy they determined that the normal plasma stoichiometry of these protein levels was 1MPO:2CP. They hypothesized that two CP molecules bound to the exposed heavy chain of MPO. If CP does indeed bind to the heavy chain of MPO, this would lend more credence to previous reports of MPO-ANCA ability to disrupt this interaction which we now know primarily target the heavy chain.

Our results diverge with these finding because instead of ANCA inhibiting the MPO/CP complex, a proteolytic CP fragment is prohibiting the binding of an anti- aa448-459 autoantibody. Despite the contrast, if MPO epitope aa448-459 is an autoantigenic site it is plausible, in order to protect against ANCA, for the body to produce more CP for the goal of generating more of the CP fragment to block the binding of a pathogenic autoantibody. We do not know what the affect this

would have on the function of MPO. We have uncovered that both full length CP and a CP fragment bind MPO epitope aa448-459 but only the fragment is able to compete for binding in the presence of MPO-ANCA. The binding site, which is the epitope, can be seen on the crystal structure of MPO (Fig. 3.8).

Figure 3.8 Ceruloplasmin fragment binding site of MPO epitope aa448-459

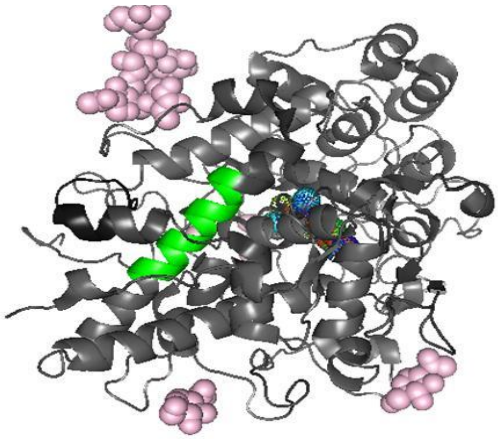


Figure 3.8 Myeloperoxidase crystal structure shown as a monomer. Predicted glycosylation residues are shown in pink spheres with a multi-color heme in the center of the molecule. MPO epitope 448-459 is shown in green.

This observation suggests the existence of a positive feedback loop of an important CP fragment after an unexpected release of MPO to prevent endothelial damage by MPO byproducts, yet we know CP levels do not correlate with decreased disease severity. This begs the question: Do patients with MPO-ANCA have a SNP in ceruloplasmin that makes this fragment non-functional or unable to bind MPO epitope aa448-459? There are numerous SNPs found in CP and MPO that have been validated and result in a change in the amino acid sequence. However, there are no studies connecting these known SNPs to ANCA disease. None of the known SNPs found in MPO results in an amino acid change within the MPO epitope aa448-459. With new data implicating a new role of a ceruloplasmin fragment, it is important to further study the known SNPs in CP and whether they play a role in proteolytic cleavage or MPO binding. Uncovering a deficiency in function of CP or its cleavage enzyme may be an important aspect of the pathogenicity of ANCA. The data presented in this chapter brings to the forefront future directions to examine ceruloplasmin and its function in ANCA disease.

SUMMARY

This body of work focuses on the specificity of autoantibody-antigen interactions and how differences in autoantibody binding sites (epitopes) impact disease. In Chapter 1, while LAMP-2 was not identified as an important autoantigen specific to ANCA disease, we were able to uncover autoantigenic sites on MPO. Little may be gleaned, from the clinical perspective, pertaining to the ability to predict remission and relapse in MPO-ANCA glomerulonephritis using autoantibody titer. Efforts in epitope mapping anti-MPO-autoantibodies using peptide libraries and chimeric molecules have been previously reported, and together have failed to uncover an immunodominant epitope.[20, 22, 24]

Our data in Chapter 2, illustrates that the majority (16/18) of disease associated anti-MPO-autoantibodies are dependent on conformational structures for successful binding. MPO epitopes aa516-524 and aa448-459, which are both found exclusively in ANCA disease, correlate with disease activity. Further, when B6 mice were immunized with a T cell epitope, overlapping with the B cell epitope aa448-459, the mice mounted an autoantibody response similar to an active ANCA patient. Chapter 3 describes how MPO epitope aa448-459 was found to be blocked by a specific cleavage product of ceruloplasmin by a blood clotting factor.[12, 71] Once ceruloplasmin was removed, sera from some patients diagnosed as ANCA-negative were reactive to MPO, specifically epitope aa448-459. The specificity of the autoantibody autoantigen interaction could be responsible for differences in disease expression, and may explain why clinical tests developed for autoantibody detection do not correlate with disease activity.[35, 64]

The mass spectrometry based method presented in this study is drastically more sensitive than current clinical testing and has uncovered extremely low titer MPO epitopes found in healthy

subjects as well as specific epitopes that correlate with disease activity. The utility of a more sensitive method to monitor patient epitope profiles over their disease course would not only enable more accurate diagnostics but also the ability to predict clinical outcomes based on the appearance of pathogenic autoantibodies possibly preventing organ damage.

EPILOGUE

THE ROLE OF ANCA IN GLOMERULONEPHRITIS: DISEASE DIAGNOSIS, PROGNOSIS AND TREATMENT

Insight into the origin of ANCA disease

In the past several years, the theory of molecular mimicry in GPA has resurfaced as a possible resolution to the question of the origin of ANCA. The mechanism whereby infectious agents cause a compromise in immune tolerance leading to generation of self-reactive antibodies remains speculative. Taking into account the many purported theoretical possibilities from numerous research groups, molecular mimicry remains the main postulated mechanism [72-74]. According to this hypothesis, a susceptible host acquires an infection with an agent that is immunologically similar to the host antigens but differs sufficiently to induce an immune response when presented to T cells. As a result, tolerance to autoantigens breaks down, and the pathogen-specific antibody cross-reacts with host structures to cause tissue damage and disease. Although LAMP-2 autoantibodies were not specific for ANCA disease, they were found in individuals with UTI infections which lends credence to the theory of molecular mimicry through cross reaction with a bacterial protein FimH. A similar hypothesis can be made concerning MPO epitopes. Disease associated immunodominant MPO epitopes could mimic the sequence of a foreign antigen, bacterial or viral, and be responsible for triggering disease in susceptible individuals.

Implications in the field of autoimmunity

The data in this dissertation presents a novel utility for proteomics in autoimmune disease. The obvious next step is to determine the disease associated epitopes relevant in PR3-ANCA. We have begun to use the same MS epitope excision approach to map anti-PR3 autoantibody specificity and have encountered many complications. PR3 is an autocatalytic protein which can cleave itself as

well as autoantibodies, and the use of an inhibitor or recombinant PR3 diminishes the accuracy of results compared to the use of native protein. Also, PR3 does not have ideal enzymatic cleavage sites which are essential for MS analysis. Given the numerous caveats in generating studying human PR3-ANCA epitope profiles, further investigation will build the current knowledge base of epitope mapping in PR3-ANCA disease.

The ability to uncover conformationally dependent disease associated epitopes is not only valuable to ANCA disease but to other antibody mediated autoimmune diseases as well. For many years the pathogenic epitopes of a debilitating skin disease, pemphigus foliaceus (PF), have been sought after. PF causes spontaneous cutaneous blistering on the epidermis as the result of IgG targeting the cell surface of keratinocytes, specifically one of the desmosomal cadherins, desmoglein 1[75]. Autoantibodies targeting desmoglein 1 disrupt cell-cell adhesion through an unknown mechanism. Efforts to uncover the pathogenic epitopes of desmoglein utilizing overlapping peptide arrays have been unsuccessful thus requiring immunoblotting to assay reactivity[76]. All epitopes targeted by autoantibodies were determined to be conformationally dependent. PF is an excellent candidate for analysis by MS epitope excision because of the known autoantibody specificity and favorable properties of desmoglein 1.

This technique can also be used to identify autoantigens in diseases long thought to be autoimmune in nature, including two common kidney diseases--minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS). These diseases affect podocyte architecture and thus the ability of the glomerular filter to prevent urinary protein loss. These podocytopathies are believed by many to lie along a continuum, with MCD reversed by immunosuppression, whereas the changes of FSGS develop into irreversible scar. Investigators since the 1970's have believed MCD to be immunologic in origin, yet it's pathogenesis is still poorly understood[77]. Ninety percent of children with idiopathic nephrotic syndrome have MCD. Electron microscopy of kidney biopsies reveals diffuse effacement of podocyte foot processes, the clinical effect of which is dramatic proteinuria. No

immune deposits or cellular infiltrate are seen by light microscopy[77]. Our hypothesis is that MCD is caused by development of autoantibodies against a circulating leukocyte (rather than tissue) which releases toxic cytokines damaging the podocytes. MS epitope excuision is useful to test this hypothesis by 'fishing' for autoantigens in patient's serum and leukocyte lysates. Uncovering autoantigens is essential for defining the pathogenesis of autoimmune diseases, allowing for precise diagnosis and therapy.

Potential for improvement on current therapies

Current treatments for autoimmune diseases center around the same immunosuppression options used in various cancer or anti-organ rejection therapies. Drugs like cyclosporine, steroids, and more recently rituximab have been used to eliminate the immune cells thought to be responsible for autoimmunity. The same risk of secondary infection holds for patients with autoimmune disease who are treated with immunosuppression. While their immune system is disabled, their disease progression arrests but for many patients upon the return of their B and T cells, relapse occurs. In order to manipulate the immune system in patients suffering from autoimmune diseases, Stienman *et. al*. purpose an idea called inverse vaccination in order to eliminate specific B cell and T cell responses and reduce the risk of nonspecific immunosuppression[78]. Researchers have successfully created a vaccine for multiple sclerosis (MS) in mice which targets autoantibody binding sites on myelin. Stienman *et. al* reported that the DNA based vaccines were able to tolerize mice against particular sites on myelin after their first episode of paralysis and reduce or eliminate relapses. These studies have translated to human phase I and II clinical trials with patients diagnosed with MS and have yielded promising results. The identification of pathogenic epitopes on MPO could be used for not only disease profiling but potential therapies not unlike the idea of an inverse vaccine. Peptide or protein treatments could be used to tolerize patients to autoantigenic epitopes. Drugs that target certain B cell clones or better timing of immune therapy may save patients from unnecessary immunosuppression.

Another autoimmune disease that has had significant progress in the development of a vaccine is type 1 diabetes. This is a disease in which autoantibodies target pancreatic islet cells causing the destruction of the only source of insulin in the body. There is currently no cure or prevention of type 1 diabetes, only a life-long treatment of daily injections of recombinant insulin. Antigen specific immunotherapy is thought to be the best course of action to prevent or disrupt the autoimmune response in this disease[79]. Type 1 diabetes usually develops in adolescence and therefore a vaccine which immunizes against the initial autoantigen (which is still under debate) could be enough to prevent the subsequent development of autoimmunity. Vaccination of NOD mice with a single autoantigen or a disease associated epitope have been successful and have been translated to human clinical trials[79]. Antigen specific immunotherapy and inverse vaccination are promising therapies that may enable prevention or someday even a cure for autoimmune diseases which autoantigens and pathogenic epitopes are known.

Future directions

Like any dissertation project there are many future directions to consider. With the knowledge of disease associated MPO epitopes, a more sensitive clinical test can be developed not only for diagnosis but to monitor disease progression. A method must be established to study conformational epitopes to determine the immunodominant epitope across disease populations. Translating human epitope profiles into animal models can also further the understanding of other disease factors such as: complement activation and the innate immune response *in vivo*.

Additional studies to pinpoint the role of a potentially protective ceruloplasmin fragment and how this fragment is functional or nonfunctional may play a role in disease severity. The enzyme responsible for the cleavage of CP requires analysis of its serum levels and functionality in patients during active disease and clinical remission compared to healthy controls. Once an epitope or inhibitor is determined, progress can be made to acquire better treatments or even prevention of ANCA disease.

Conclusions

In the past decade, the field of antibody mediated autoimmune disease has made very slow progress in elucidating the origins of autoimmunity. Equally important to discerning the cause of a break in self tolerance, is uncovering B cell epitope specificity. The knowledge of autoantigenic epitopes is useful not only for diagnosis and treatment but also for the prevention of disease through vaccination. The importance of the site of autoantibody-autoantigen interaction is as essential to understanding disease pathogenesis as the existence of the autoantibodies themselves.

REFERENCES

1. Lerner RA, Glassock RJ, Dixon FJ. The role of anti-glomerular basement membrane antibody in the pathogenesis of human glomerulonephritis. J Exp Med 1967;126:989-1004.

2. Wilson CB, Dixon FJ. Anti-glomerular basement membrane antibody-induced glomerulonephritis. Kidney Int 1973;3:74-89.

3. Bansal PJ, Tobin MC. Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal pulmonary hemorrhage and renal involvement. Ann Allergy Asthma Immunol 2004;93:398-401.

4. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci U S A 1990;87:4115-9.

5. Xiao H, Heeringa P, Hu P, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J Clin Invest 2002;110:955-63.

6. Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. Kidney Int 1992;41:375-83.

7. Kain R, Matsui K, Exner M, et al. A novel class of autoantigens of anti-neutrophil cytoplasmic antibodies in necrotizing and crescentic glomerulonephritis: the lysosomal membrane glycoprotein h-lamp-2 in neutrophil granulocytes and a related membrane protein in glomerular endothelial cells. J Exp Med 1995;181:585-97.

8. Carlsson SR, Roth J, Piller F, Fukuda M. Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan. J Biol Chem 1988;263:18911-9.

9. Kain R, Exner M, Brandes R, et al. Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. Nat Med 2008;14:1088-96.

10. Agner K. Veroperoxidase: a ferment isolated from leukocytes. Acta Physiology Scand 1941;2:1-62.

11. Nauseef WM, Malech HL. Analysis of the peptide subunits of human neutrophil myeloperoxidase. Blood 1986;67:1504-7.

12. Segelmark M, Persson B, Hellmark T, Wieslander J. Binding and inhibition of myeloperoxidase (MPO): a major function of ceruloplasmin? Clin Exp Immunol 1997;108:167-74.

13. Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N Engl J Med 1988;318:1651-7.

14. Ludemann J, Csernok E, Ulmer M, et al. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis: immunodiagnostic value, monoclonal antibodies and characterization of the target antigen. Neth J Med 1990;36:157-62.

15. Ludemann J, Utecht B, Gross WL. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. J Exp Med 1990;171:357-62.

16. Hogan SL, Nachman PH, Wilkman AS, Jennette JC, Falk RJ. Prognostic markers in patients with antineutrophil cytoplasmic autoantibody-associated microscopic polyangiitis and glomerulonephritis. J Am Soc Nephrol 1996;7:23-32.

17. Williams RC, Jr., Staud R, Malone CC, Payabyab J, Byres L, Underwood D. Epitopes on proteinase-3 recognized by antibodies from patients with Wegener's granulomatosis. J Immunol 1994;152:4722-37.

18. van der Geld YM, Stegeman CA, Kallenberg CG. B cell epitope specificity in ANCA-associated vasculitis: does it matter? Clin Exp Immunol 2004;137:451-9.

19. Falk RJ, Becker M, Terrell R, Jennette JC. Anti-myeloperoxidase autoantibodies react with native but not denatured myeloperoxidase. Clin Exp Immunol 1992;89:274-8.

20. Bruner BF, Vista ES, Wynn DM, James JA. Epitope specificity of myeloperoxidase antibodies: identification of candidate human immunodominant epitopes. Clin Exp Immunol;164:330-6.

21. Chang L, Binos S, Savige J. Epitope mapping of anti-proteinase 3 and anti-myeloperoxidase antibodies. Clin Exp Immunol 1995;102:112-9.

22. Erdbrugger U, Hellmark T, Bunch DO, et al. Mapping of myeloperoxidase epitopes recognized by MPO-ANCA using human-mouse MPO chimers. Kidney Int 2006;69:1799-805.

23. Griffith ME, Coulthart A, Pemberton S, George AJ, Pusey CD. Anti-neutrophil cytoplasmic antibodies (ANCA) from patients with systemic vasculitis recognize restricted epitopes of proteinase 3 involving the catalytic site. Clin Exp Immunol 2001;123:170-7.

24. Tomizawa K, Mine E, Fujii A, et al. A panel set for epitope analysis of myeloperoxidase (MPO)-specific antineutrophil cytoplasmic antibody MPO-ANCA using recombinant hexamer histidine-tagged MPO deletion mutants. J Clin Immunol 1998;18:142-52.

25. Olson SW, Arbogast CB, Baker TP, et al. Asymptomatic autoantibodies associate with future anti-glomerular basement membrane disease. J Am Soc Nephrol;22:1946-52.

26. Arbuckle MR, McClain MT, Rubertone MV, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med 2003;349:1526-33.

27. Batstra MR, Aanstoot HJ, Herbrink P. Prediction and diagnosis of type 1 diabetes using beta-cell autoantibodies. Clin Lab 2001;47:497-507.

28. Cui Z, Zhao MH, Segelmark M, Hellmark T. Natural autoantibodies to myeloperoxidase, proteinase 3, and the glomerular basement membrane are present in normal individuals. Kidney Int;78:590-7.

29. Yang R, Cui Z, Hellmark T, Segelmark M, Zhao MH, Wang HY. Natural anti-GBM antibodies from normal human sera recognize alpha3(IV)NC1 restrictively and recognize the same epitopes as anti-GBM antibodies from patients with anti-GBM disease. Clin Immunol 2007;124:207-12.

30. Sandler M. Can we predict and/or prevent type I diabetes? S Afr Med J 1990;78:462-7.

31. Pedchenko V, Bondar O, Fogo AB, et al. Molecular architecture of the Goodpasture autoantigen in anti-GBM nephritis. N Engl J Med;363:343-54.

32. Specks U. Epitope-specific anti-neutrophil cytoplasmic antibodies: do they matter? Can they be detected? APMIS Suppl 2009:63-6.

33. Lanzavecchia A. How can cryptic epitopes trigger autoimmunity? J Exp Med 1995;181:1945-8.

34. Birck R, Schmitt WH, Kaelsch IA, van der Woude FJ. Serial ANCA determinations for monitoring disease activity in patients with ANCA-associated vasculitis: systematic review. Am J Kidney Dis 2006;47:15-23.

35. Hogan SL, Falk RJ, Chin H, et al. Predictors of relapse and treatment resistance in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis. Ann Intern Med 2005;143:621-31.

36. Falk RJ, Jennette JC. ANCA are pathogenic--oh yes they are! J Am Soc Nephrol 2002;13:1977-9.

37. Harper L, Williams JM, Savage CO. The importance of resolution of inflammation in the pathogenesis of ANCA-associated vasculitis. Biochem Soc Trans 2004;32:502-6.

38. Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N Engl J Med 1988;318:1651-7.

39. Jennette JC, Wilkman AS, Falk RJ. Anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and vasculitis. Am J Pathol 1989;135:921-30.

40. Mulder AH, Heeringa P, Brouwer E, Limburg PC, Kallenberg CG. Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process. Clin Exp Immunol 1994;98:270-8.

41. Hagen EC, Ballieux BE, van Es LA, Daha MR, van der Woude FJ. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. Blood 1993;81:1996-2002.

42. Niles JL, Pan GL, Collins AB, et al. Antigen-specific radioimmunoassays for anti-neutrophil cytoplasmic antibodies in the diagnosis of rapidly progressive glomerulonephritis. J Am Soc Nephrol 1991;2:27-36.

43. Salama AD, Pusey CD. Shining a LAMP on pauci-immune focal segmental glomerulonephritis. Kidney Int 2009;76:15-7.

44. Bosch X, Mirapeix E. Vasculitis syndromes: LAMP-2 illuminates pathogenesis of ANCA glomerulonephritis. Nat Rev Nephrol 2009;5:247-9.

45. Willcocks LC, Lyons PA, Rees AJ, Smith KG. The contribution of genetic variation and infection to the pathogenesis of ANCA-associated systemic vasculitis. Arthritis Res Ther;12:202.

46. Fukuda M, Viitala J, Matteson J, Carlsson SR. Cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Comparison of their deduced amino acid sequences. J Biol Chem 1988;263:18920-8.

47. Hanash S. Disease proteomics. Nature 2003;422:226-32.

48. Robinson WH, Steinman L, Utz PJ. Proteomics technologies for the study of autoimmune disease. Arthritis Rheum 2002;46:885-93.

49. De'Oliviera J, Gaskin G, Dash A, Rees AJ, Pusey CD. Relationship between disease activity and anti-neutrophil cytoplasmic antibody concentration in long-term management of systemic vasculitis. Am J Kidney Dis 1995;25:380-9.

50. Tervaert JW, Stegeman CA, Kallenberg CG. Serial ANCA testing is useful in monitoring disease activity of patients with ANCA-associated vasculitides. Sarcoidosis Vasc Diffuse Lung Dis 1996;13:241-5.

51. Parker CE, Tomer KB. MALDI/MS-based epitope mapping of antigens bound to immobilized antibodies. Mol Biotechnol 2002;20:49-62.

52. Mirza SP, Greene AS, Olivier M. 18O labeling over a coffee break: a rapid strategy for quantitative proteomics. J Proteome Res 2008;7:3042-8.

53. Schreiber A, Xiao H, Falk RJ, Jennette JC. Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. J Am Soc Nephrol 2006;17:3355-64.

54. Brouwer E, Huitema MG, Mulder AH, et al. Neutrophil activation in vitro and in vivo in Wegener's granulomatosis. Kidney Int 1994;45:1120-31.

55. Ruth AJ, Kitching AR, Kwan RY, et al. Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. J Am Soc Nephrol 2006;17:1940-9.

56. Audrain MA, Baranger TA, Moguilevski N, et al. Anti-native and recombinant myeloperoxidase monoclonals and human autoantibodies. Clin Exp Immunol 1997;107:127-34.

57. Pedrollo E, Bleil L, Bautz FA, Kalden JR, Bautz EK. Antineutrophil cytoplasmic autoantibodies (ANCA) recognizing a recombinant myeloperoxidase subunit. Adv Exp Med Biol 1993;336:87-92.

58. Fujii A, Tomizawa K, Arimura Y, et al. Epitope analysis of myeloperoxidase (MPO) specific anti-neutrophil cytoplasmic autoantibodies (ANCA) in MPO-ANCA-associated glomerulonephritis. Clin Nephrol 2000;53:242-52.

59. Suzuki K, Kobayashi S, Yamazaki K, et al. Analysis of risk epitopes of anti-neutrophil antibody MPO-ANCA in vasculitis in Japanese population. Microbiol Immunol 2007;51:1215-20.

60. Roth AJ, Falk RJ. Autoantibodies: What's in Their Teeth? J Am Soc Nephrol;22:1783-4.

61. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CG. Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. Arthritis Rheum 2007;56:2080-91.

62. Morgan MD, Day CJ, Piper KP, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. Immunology;130:64-73.

63. Rimbert M, Hamidou M, Braudeau C, et al. Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis. PLoS One;6:e18734.

64. Pagnoux C, Hogan SL, Chin H, et al. Predictors of treatment resistance and relapse in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis: comparison of two independent cohorts. Arthritis Rheum 2008;58:2908-18.

65. Xu X, Pin S, Gathinji M, Fuchs R, Harris ZL. Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. Ann N Y Acad Sci 2004;1012:299-305.

66. Griffin SV, Chapman PT, Lianos EA, Lockwood CM. The inhibition of myeloperoxidase by ceruloplasmin can be reversed by anti-myeloperoxidase antibodies. Kidney Int 1999;55:917-25.

67. Xu PC, Chen M, Cui Z, Zhao MH. Influence of myeloperoxidase by anti-myeloperoxidase antibodies and its association with the disease activity in microscopic polyangiitis. Rheumatology (Oxford);49:2068-75.

68. Ara J, Pascual J, Mirapeix E, Rodriguez R, Abellana R, Darnell A. Ceruloplasmin in small vessel vasculitis. Nephrol Dial Transplant 1999;14:515-7.

69. Baskin E, Bakkaloglu A, Besbas N, et al. Ceruloplasmin levels in antineutrophil cytoplasmic antibody-positive patients. Pediatr Nephrol 2002;17:917-9.

70. Sokolov AV, Ageeva KV, Cherkalina OS, et al. Identification and properties of complexes formed by myeloperoxidase with lipoproteins and ceruloplasmin. Chem Phys Lipids;163:347-55.

71. Sokolov AV, Zakharova ET, Shavlovskii MM, Vasil'ev VB. [Isolation of stable human ceruloplasmin and its interaction with salmon protamine]. Bioorg Khim 2005;31:269-79.

72. Fujinami RS, Oldstone MB, Wroblewska Z, Frankel ME, Koprowski H. Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. Proc Natl Acad Sci U S A 1983;80:2346-50.

73. Levin MC, Lee SM, Kalume F, et al. Autoimmunity due to molecular mimicry as a cause of neurological disease. Nat Med 2002;8:509-13.

74. Kohm AP, Fuller KG, Miller SD. Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. Trends Microbiol 2003;11:101-5.

75. Dasher D, Rubenstein D, Diaz LA. Pemphigus foliaceus. Curr Dir Autoimmun 2008;10:182-94.

76. Chan PT, Ohyama B, Nishifuji K, et al. Immune response towards the amino-terminus of desmoglein 1 prevails across different activity stages in nonendemic pemphigus foliaceus. Br J Dermatol;162:1242-50.

77. Mathieson PW. Minimal change nephropathy and focal segmental glomerulosclerosis. Semin Immunopathol 2007;29:415-26.

78. Steinman L. Inverse vaccination, the opposite of Jenner's concept, for therapy of autoimmunity. J Intern Med;267:441-51.

79. Nicholas D, Odumosu O, Langridge WH. Autoantigen based vaccines for type 1 diabetes. Discov Med;11:293-301.