MYELOPEROXIDASE IN VASCULAR DISEASE AND AUTOIMMUNITY

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ABSTRACT

Joshua Michael Astern: Myeloperoxidase in Vascular Disease and Autoimmunity

(Under the direction of Ronald J. Falk, M.D. and Gloria Preston, Ph.D.)

This dissertation defines mechanisms whereby myeloperoxidase (MPO) can mediate vascular damage when released into the vessel, and explores the pathogenesis of an autoimmune disease targeting MPO. The most abundant neutrophil granule protein, MPO generates powerful oxidants that contribute to innate host defense. However, these same oxidants cause host injury; the release of MPO into the vessel correlates with the impairment of vasoregulatory processes and cellular injury. Herein, we report that cytokeratin 1, an endothelial protein, mediates MPO binding and internalization. Cytokeratin 1 also functions as a scaffolding protein for the vasoregulatory plasma kallikrein-kinin system. This system produces bradykinin, a potent inducer of endothelial nitric oxide synthesis. Our investigations revealed that MPO bound and co-localized with high molecular weight kininogen on endothelial cells, and this interaction interfered with bradykinin cleavage by plasma kallikrein. Further, MPO oxidized and inactivated both kininogen and kallikrein, thus preventing bradykinin release. This work identified cytokeratin 1 as a facilitator of MPO-mediated responses, and provided a new paradigm by which MPO affects vasoregulatory processes during inflammation. One disease characterized by excess intravascular neutrophil degranulation is antineutrophil cytoplasmic autoantibody (ANCA)-mediated vasculitis. One major ANCA specificity is for MPO (MPO-ANCA). The origin of

these pathogenic autoantibodies is unknown, though our group previously published studies implicating proteins complementary in sequence to autoantigens as the inciting elements of autoimmune disease in patients with proteinase 3-specific ANCA. In chapter 2, we demonstrated the presence of anti-complementary MPO antibodies in patients; this implied that the development of MPO-specific antibodies was a result of an anti-idiotypic response against the anti-complementary protein antibody, and in this way normal tolerogenic mechanisms were bypassed. For this dissertation, we tested the hypothesis that complementary proteins could cause disease in an MPO-ANCA mouse model. While results were not as we predicted, the work revealed the importance of identifying a pathogenic epitope. An epitope mapping study was carried out using a mass spectrometry-based technique, a tool that may generate powerful data for us in the near future. While the human data suggests a role for complementary proteins in MPO-ANCA disease, proving causation in an animal model remains an elusive goal.

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This has not been easy, trudging through an intense series of battles with myself: the 6-year war. I think I've won, though I'm still working on why my hands don't do what my brains tells them to do and why my brain doesn't do what it knows its supposed to, but, hey - it's all a work in progress, no? Thankfully, I've had solid coaching along the way. My first thank you goes to my makers, Mom and Dad, who conducted their own little genetics experiment 30 years ago …and here I am. They are as strong a safety net as one could ask for. This work is dedicated to my grandparents – all four! – who send an overwhelming amount of love and good vibes my way. I'm proud to make them proud. Special thanks go the sister as well, an inspiring creature, she, whom has been very supportive to me any time I needed her. I must congratulate her as well, as she is a soon-to-be card-carrying speech pathologist (if the pacemakers don't get in the way, I think my grandparents' hearts will explode with pride this year).

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABCA-1	ATP-binding Cassette Transporter 1
ANCA	Antineutrophil Cytoplasmic Autoantibody
bHK	Biotinylated High Molecular Weight Kininogen
Bis-Q	3,3'-bis-[alpha-(trimethylammonium)methyl]azobenzene
BLAST	Basic Local Aligment Sequence Tool
BSA	Bovine Serum Albumin
cDNA	Complementary Dioxyribonucleic Acid
CDR	Complementary Determining Region
CK1	Cytokeratin 1
CK9	Cytokeratin 9
Cl^-	Chloride
cMPO	Complementary MPO
DNA	Deoxyribonucleic Acid
EAhy.926	Hybridoma of human umbilical vein endothelial cells and a human airway epithelial cell line
ELISA	Enyme-linked Immunosorbant Assay
GBM	Glomerular Basement Membrane
gC1qR	Receptor for the Globular Head of Complement 1q
H_2O_2	Hydrogen Peroxide

HDL	High Density Lipoprotein
HEK 293	Human Embryonic Kidney Cell Line
HK	High Molecular Weight Kininogen
HOCl	Hypochlorous Acid
HUVEC	Human Umbilical Vein Endothelial Cells
IgG	Immunoglobulin G
kD	Kilodalton
KKS	Plasma Kallikrein-Kininogen System
LDL	Low Density lipoprotein
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization – Time of Flight
m-cMPO	Complementary Myeloperoxidase
MG	Myesthenia Gravis
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
NO	Nitric Oxide
NO_2^-	Nitrite
$NO_2^{\cdot}$	Nitrogen Dioxide

NO_2Cl	Nitryl Chloride
PAT	Pathologic Antisense Transcript
PilB	*Neisseria gonnorrhoeae*-derived Methionine Sulfoxide Reductase
PR3	Proteinase 3
RAG-2 -/-	Recombinase Activating Enzyme-2 Knockout mouse
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyle Sulfate Polyacrylimide Gel Electrophoresis
TIMP-1	Tissue Inhibitor of Metalloproteinase 1
TSH	Thyroid Stimulating Hormone
uPAR	Urokinase Plasminogen Activator Receptor

PROLOGUE

MYELOPEROXIDASE, THE ENZYME

In 1941, Agner isolated an iron-containing protein with peroxidase activity from the purulent debris of patients with tuberculous empyema.[1] He named this protein verdoperoxidase due to its intensely green color, but it was renamed myeloperoxidase (MPO) when subsequent studies revealed that its tissue distribution was limited to myeloid cells. MPO is one of the principal enzymes stored in azurophilic granules of neutrophils and peroxidase-positive lysosomes of monocytes, and makes up 5% and 1% of each cell's total protein, respectively.[2]

The expression of the MPO gene, as well as other genes coding for granule proteins, is linked to the differentiation status of the myelocyte.[3] In healthy individuals, MPO gene expression begins at the promyelocyte stage in the bone marrow and ceases after the cells mature and enter the circulation.[4] MPO expression status was demonstrated *in vitro* using human promyeloid leukemic cell lines, such as HL-60 cells that express the MPO gene. Transcription of the MPO gene was inhibited when the cells were induced to differentiate using retinoic acid, dimethyl sulfoxide, or phorbol-12-myristate-13-acetate.[5, 6]

Correlating with gene expression, MPO protein production and packaging is completed in the bone marrow. The primary translation product undergoes proteolytic cleavage to generate an inactive apoproMPO in the endoplasmic reticulum (ER). Subsequent glycosylation and insertion of a heme group in the ER yields an active, 90 kD pro-MPO

molecule; pro-MPO is trafficked through the *trans*-Golgi network and undergoes a series of proteolytic steps to generate a 75 kD protomer consisting of a heavy and light chain.[7-11] The resultant protomers dimerize, linked by a disulfide bond, and the mature enzyme is targeted to the azurophilic granules for storage. Some active proMPO is constitutively secreted into the bone marrow and is detectable in circulation.[9, 12, 13]

MPO plays a major role in innate host defense by participating in the oxygen-dependent killing of invading microorganisms. Responding to chemical signals derived from the infected tissue, circulating neutrophils attach to and penetrate the vascular wall, migrate to the site of injury, accumulate, and phagocytose the invading pathogen. Concomitant with phagocytosis, the activated neutrophil undergoes a respiratory burst producing reactive oxygen species, including copious amounts of superoxide that is rapidly dismutated to hydrogen peroxide (H_2O_2).[14] The granules fuse to the phagosome and release their armory to destroy the microorganism. Granule constituents are also secreted into the extracellular space to combat larger organisms that cannot be phagocytosed, as well as at sites of intense acute inflammation that are not induced by infectious pathogens but nevertheless have extensive neutrophil activation, such as sites of acute inflammation induced by autoimmune disease. MPO makes up approximately 25% of azurophilic granules' protein, and achieves concentrations of about 100 mg/ml in the vacuole (1mM).[15, 16] MPO utilizes H_2O_2 to affect post-translational modifications of target proteins. MPO imparts its anti-microbicidal properties, in part, by catalyzing a unique reaction between hydrogen peroxide and chloride to create the powerful oxidant hypochlorous acid (HOCl), the main chemical component of household bleach (Figure *i*). MPO can generate other hypohalous acids as well (using

bromide, iodide, and thiocyanate), but chloride is the most likely substrate at physiologic concentrations.[17]

HOCl is the major oxidant produced by the neutrophil, accounting for 70% of the oxygen consumed during the respiratory burst.[18] HOCl is a highly reactive, and thus short-lived, species capable of both chlorination and oxidation reactions. HOCl can react with all neighboring thiol and thioether groups (methionine is readily oxidized), as well as iron-sulfur centers, heme groups, and unsaturated fatty acids.[19] Chloramines, the most abundant HOCl breakdown products, are more stable and retain some of the reactant capacity of HOCl.[20] There are many potential effects of HOCl-mediated alterations of bacteria including the interruption of the membrane electron-transport chains, a loss of microbial membrane transport, dissipation of adenylate energy reserves, and suppression of DNA synthesis.[21] Proteins altered by HOCl become more susceptible to proteolysis and more immunogenic, enhancing both the humoral and cytotoxic immune responses.[20] The enzymatic products of myeloperoxidase can also promote acute inflammation in tissue by inactivating protease inhibitors, such as α-1 protease inhibitor and plasminogen activator inhibitor, thus protecting the function of granule-derived proteases at inflammatory foci.[22, 23]

In addition to its use of chloride as a substrate, MPO can generate reactive nitrogen species through the oxidation of nitrite (NO_2^-), a stable end-product of nitric oxide (NO) metabolism (Figure *i*).[24] An H_2O_2-dependent reaction, nitrite is oxidized by MPO to form nitrogen dioxide ($NO_2^{\cdot}$), a species which is capable of nitrating both free and protein-bound tyrosines.[25, 26] MPO-derived HOCl can potentially catalyze an additional reaction with nitrite to form nitryl chloride (NO_2Cl), which is also a reactive nitrogen species. The nitration of tyrosine residues is linked to the altered structure and function of proteins, though

evidence suggests that MPO-generated nitrating species are not a major source of oxidants in the phagosome and their contribution to the MPO-mediated antimicrobial system remains questionable.[27] However, these nitrating reactions likely occur when MPO and H_2O_2 are released into the extracellular space *in vivo*.[28, 29]

MYELOPEROXIDASE IN VASCULAR DISEASE

While promoting host defense, normal inflammatory processes cause host injury that is generally repaired following the resolution of the initial insult. In severe and chronic inflammatory conditions, the excess release of enzymatically active MPO into the circulation and the production of chlorinating and nitrating species (among others) is linked to endothelial dysfunction and the progression of cardiac disease.[30-34]

While exploring the effects of neutrophil granule proteins on endothelial cells *in vitro*, our group discovered that MPO was internalized into cultured cells with a resultant rise in intracellular oxidant production.[35] Other groups confirmed and extended these studies *in vivo,* and found that free MPO can interact with anionic endothelial cell-surface glycosaminoglycans.[29] MPO transcytoses through endothelial cells, independent of neutrophil diapedesis, and settles in the subendothelial matrix where it can remain active. One functional consequence of this interaction is the nitration of fibronectin, which the authors propose could impair vascular matrix function. MPO is also known to bind serum albumin through both sequence and charge-based mechanisms.[36] The MPO-albumin complex can be internalized into caveoli via cell surface albumin binding protein receptors. In this dissertation, we describe a new, specific mechanism for MPO uptake by endothelial cells.

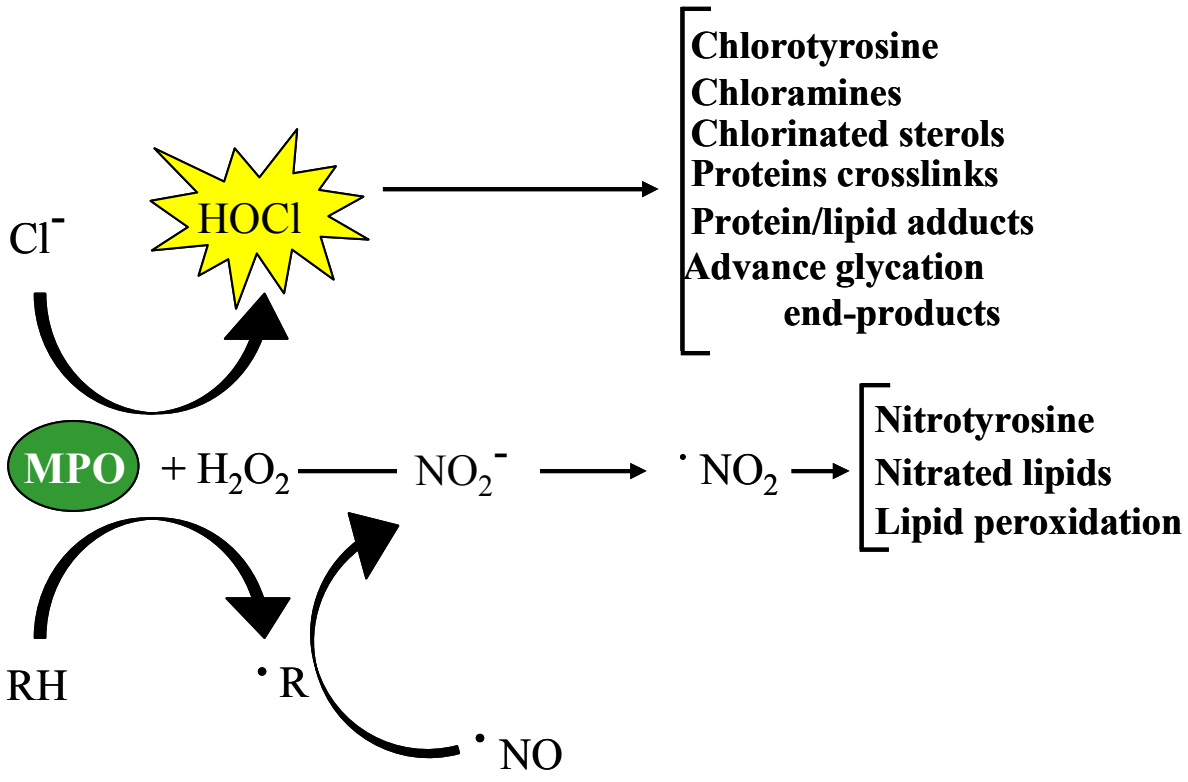

Figure i. Myeloperoxidase generated oxidants and their products. Shown is an addendum of MPO's oxidant capacity. MPO generates hypochlorous acid, a powerful and highly reactive oxidant. Radical products of MPO catalysis (RH = reducing substrate; ·R = oxidized radical product) promote the catalysis of nitric oxide (·NO) to nitrite (NO_2^-). A hydrogen peroxide-dependent reaction, MPO converts nitrite to nitrogen dioxide ($\cdot NO_2$), a reactive nitrogen species.

One form of endothelial dysfunction is the lack of normal dilatory responsivity to appropriate stimuli, for example, because of a decrease in the bioavailablilty of nitric oxide.[37] MPO is now recognized as a nitric oxide oxidase, and can interfere with endothelial NO signaling multiple ways. As MPO localizes at the interface between endothelial and smooth muscle cells, it creates small radical intermediates that catalytically consume NO, oxidizing it to nitrite.[24] Altering the efficiency of endothelial nitric oxide (eNOS), which produces NO, hypochlorous acid can chlorinate L-arginine thereby depleting the substrate for eNOS.[38] Additionally, HOCl can chlorinate lipoproteins, such as HDL and LDL. These chlorinated lipoproteins have been localized to atherosclerotic lesions, and can both provoke the dissociation of eNOS from the cell membrane and destabilize eNOS mRNA, collectively decreasing the amount of NO produced.[39, 40] Herein, we present a new paradigm whereby MPO may contribute to endothelial dysfunction in inflammatory conditions.

The role of MPO in endothelial dysfunction has been investigated *in vivo* in both mouse models and clinical studies. MPO null mice presented with acute inflammatory stimuli had improved vascular function and increased NO availability when compared to wild type mice.[24] Multiple studies reveal that plasma levels of MPO inversely correlate with the endothelial response to NO-liberating acetylcholine in patients undergoing reperfusion from myocardial ischemia and as well as others with documented coronary artery disease[41]. Further, circulating MPO plasma levels independently predict endothelial dysfunction in humans.[33] Taken together, these studies support the clinical significance of the mechanistic link between vascular-associated MPO and NO catabolism.

Endothelial dysfunction is a common condition underlying hypertension, diabetes, aging, and atherosclerosis. There is a growing body of laboratory and clinical evidence linking oxidative stress and MPO to such disorders, especially atherosclerosis. Smooth muscle cells proliferate and migrate into developing atherosclerotic lesions, leading to the enlargement of lipid-laden plaques. A normal role of NO production by endothelial cells is to suppress smooth muscle cells proliferation; this regulatory mechanism could be compromised by MPO's interference with NO signaling.[42] Immunohistochemical studies have demonstrated the co-localization of MPO and HOCl-modified lipoproteins in atherosclerotic plaques.[40, 43] MPO oxidizes HDL and LDL in both atherosclerotic plaques and in the circulation. The lipoproteins isolated from atherosclerotic plaques and the circulation of patients with established coronary disease showed increased levels of nitrotyrosine, and chlorotyrosine modification, as compared to healthy controls.[44-47] These oxidized lipoproteins are preferentially removed from circulation by macrophages, leading to foam cell formation and the progression of atherosclerotic plaques. The oxidation of HDL creates a dysfunctional molecule. HDL is referred to as the "good" cholesterol because it promotes cholesterol efflux from cells in the artery wall by the ATP-binding cassette transporter A1 (ABCA1). When apolipoprotein A1, which accounts for 70% of total protein associated with HDL, is chlorinated by MPO, HDL loses its ability to remove cholesterol from cells.[44, 48, 49] This leads to further buildup of lipids in the artery wall, promoting atherogenesis.

In addition to promoting growth of the plaque, the production of HOCl by MPO can also lead to plaque rupture and an acute cardiac event. The capping of plaques through extracellular matrix deposition plays a key role in stabilizing atherscerotic lesions. Matrix

metalloproteinases (MMP) degrade matrix proteins through their proteolytic activity and are under strict control by endogenous protein inhibitors, including TIMP-1, as well as reactive oxygen and nitrating species. Low levels of HOCl can not only directly activate MMPs, but also repress TIMP-1 activity leading to matrix degradation and plaque instability.[50] HOCl can provoke desquamation and endothelial cell death by either apoptotic or oncotic cell-death pathways, potentially leading to plaque erosion and a thrombotic event.[51]

The growing body of evidence implicating MPO in cardiovascular disease has lead to a number of exciting clinical studies. A study in 2001 identified MPO as marker for coronary artery disease.[34] The researchers found that increased blood-MPO was significantly greater in patients with established coronary artery disease compared to healthy controls. Two independent studies in 2003 revealed that blood levels of MPO could powerfully predict the incidence of major cardiac events following the initial visit of patients presenting with acute coronary syndrome or chest pain.[30, 52] Interestingly, both studies revealed that MPO predicted adverse outcome even when troponin T, a marker of myocardial necrosis commonly used to predict risk, did not. The authors bring to light the importance of the predictive value MPO in the absence of circulating troponins, as this suggests that MPO release actually *preceeds* myocardial injury and identifes patients with unstable plaques before complete microvascular obstruction.

It is becoming increasingly clear that the MPO-H_2O_2-Cl^- system, through vascular neutrophil and monocyte degranulation, contributes to morbidity in several diseases. Increased plasma levels of MPO have been noted in inflammatory diseases such as Behcet's disease, dermatitis herpetiformis, systemic inflammatory response syndrome, and ANCA-mediated glomerulonephritis.[53-56] In addition to being a plasma marker of inflammation,

MPO clearly contributes to the progression of degenerative and immunologic mediated diseases of the kidney and is present in inflamed lung tissue.[36, 57] Because of these deleterious effects, it is important we understand the interaction between MPO and the vasculature. Exploring the physiologic consequences of these interactions may reveal therapeutic targets for intervention as a means of alleviating the inflammatory burden on patients and retarding disease progression.

In Chapter 1 of this dissertation, we explored the relationship between MPO and endothelial cells. We found that endothelial cells internalize MPO, in part, through an interaction with cell-surface cytokeratin 1. Interestingly, cytokeratin 1 was previously described as an endothelial surface scaffolding protein for the plasma kallikrein-kinin system. This system produces the vasoactive peptide bradykinin, the most potent known inducer of endothelial nitric oxide production. Because MPO is recognized to interfere with nitric oxide signaling, we explored the interaction of MPO with the plasma kallikrein-kinin system and found that MPO diminished bradykinin production. The work in Chapter 1 provides a new paradigm by which MPO can modulate vascular function in acute and chronic inflammatory conditions.

MYELOPEROXIDASE, THE AUTOANTIGEN

MPO is a target autoantigen in a humoral autoimmune disease: anti-neutrophil cytoplasmic autoantibody (ANCA)-mediated small vessel vasculitis. Twenty-five years ago, Davies correlated the existence of anti-neutrophil antibodies in the serum of patients with idiopathic, segmental, crescentic, and necrotizing glomerulonephritis.[58] A couple years later, van der Woude and colleagues determined that antineutrophil antibodies strongly

correlated with disease activity in Wegener's granulomatosis, a disease with renal pathology that mirrors the description provided in Davies' report.[59] Our group's own investigations of patients with similar glomerulonephritides revealed that a subset of these antineutrophil autantibodies were specific for MPO.[60] Soon thereafter, proteinase 3 (PR3) was identified as the other major target autoantigen.[61, 62]

ANCA are now associated with three syndromes characterized by small vessel vasculitis: Wegener's granulomatosis, microscopic polyangiitis, and Churg-Strauss Sydrome.[63] While these syndromes all have disease features to distinguish one from another, the common features include the frequent presence of ANCA (of some specificity) and a necrotizing glomerulonephritis with a paucity of immunoglobulin deposits in the glomeruli. ANCA-mediated effects are commonly observed in glomerular and alveolar capillaries; life-threatening injury to these organs often develops quickly but can be controlled by immunosuppressive treatments.[63, 64]

The most accepted model regarding the pathogenesis of ANCA-mediated vasculitis suggests that ANCA activate cytokine-primed neutrophils within the micro-circulation to release noxious granule constituents and toxic oxygen radicals, leading to severe bystander effects on endothelial cells and rapid escalation of inflammation. Our group and others have demonstrated that ANCA activate neutrophils *in vitro*, however a debate raged on as to whether ANCA were merely serological markers or catalysts of disease.[65-68] The controversy has waned recently, as animal models of MPO-ANCA glomerulonephritis and systemic vasculitis revealed that ANCA are in fact pathogenic, and circulating neutrophils are critical to induce disease.[69, 70] Additionally, two separate case reports described a mother with an MPO-ANCA induced microscopic polyangiitis flare during pregnancy and

apparent trans-placental transfer of disease to the newborn. Tests revealed that the newborn had MPO-ANCA titers at birth and marked pulmonary hemorrhage and renal abnormalties.[71, 72] Following therapy and clearance of the ANCA from the neonate, the pulmonary-renal syndrome resolved, thus adding clinical support to the pathogenic potential of ANCA . This case may represent a human "model" demonstrating that MPO-ANCA are pathogenic.

COMPLEMENTARY PROTEINS AND THE ORIGINS OF AUTOIMMUNITY

Why do these autoantibodies develop? The etiology of ANCA, and of *all* autoimmune diseases, remains a mystery in medical science. Many theories exist as to the origins of autoimmunity, all of which have some supporting evidence, but none of which can fully account for all autoimmune diseases. Such theories include: molecular mimicry between foreign and self antigenic determinants that results in production of antibodies that cross-react to self proteins, exposure of a "hidden" alloantigen that elicits an autoimmune response, incomplete clonal deletion of self-reactive T- or B-cells, and a genetic predisposition to autoimmunity. Our group proposed and published a new theory, termed the Theory of Autoantigen Complementarity (Figure *ii*).[73] This theory proposes that the antigen that incites the immunologic cascade leading to autoantibody production is actually a protein complementary to the autoantigen. The immune system mounts an idiotypic response against the complementary protein, and subsequently, anti-idiotypic antibodies are produced that cross-react with the autoantigen leading to disease. The original work conducted on this theory was in the context of PR3-ANCA vasculitis, and it was found that patients harbored antibodies specific to a complementary PR3 peptide, but healthy and disease controls did not.

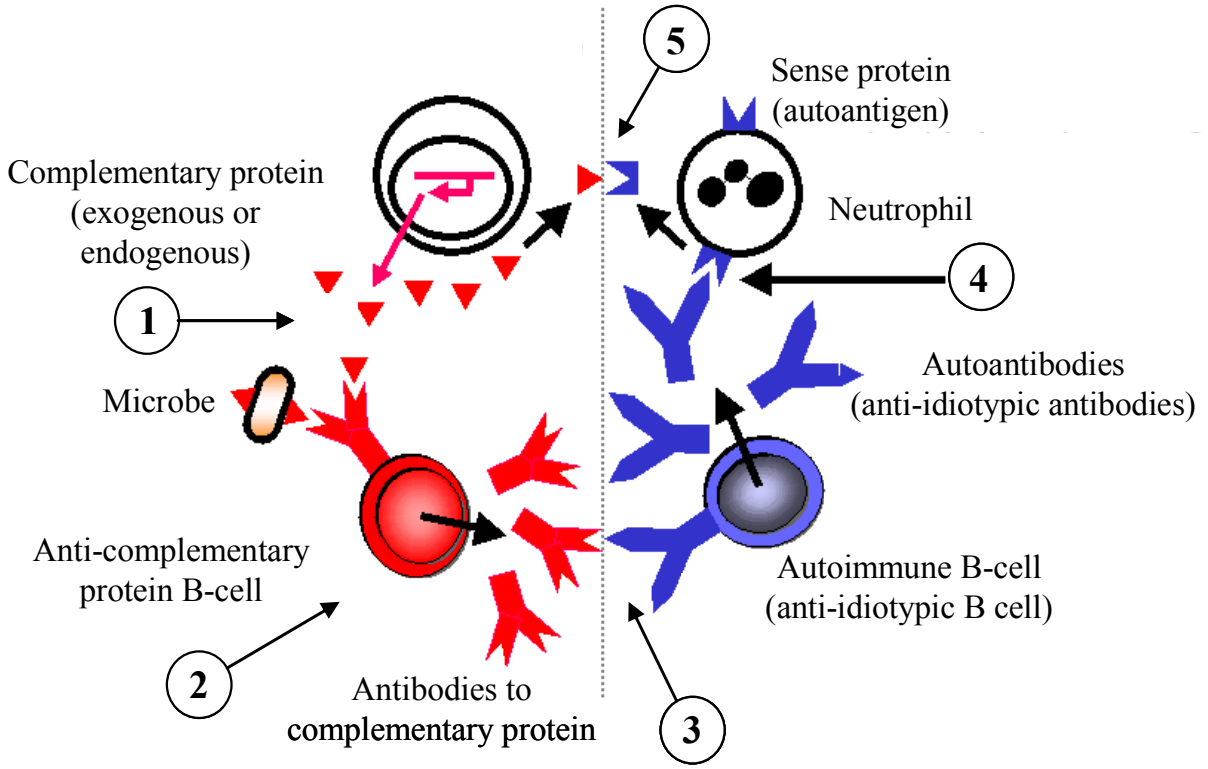

Figure *ii.* Schematic of the theory of autoantigen complementarity, as demonstrated in PR3-ANCA disease. **1)** The host is exposed to a protein complementary in sequence and/or structure to a known autoantigen, derived from either an invading microbe or the translation of an antisense transcript. **2)** An antibody is produced in response to the complementary protein. **3)** Through the idiotypic network, a second antibody is elicited against the first antibody, an anti-idiotypic response. **4)** Autoimmunity: The anti-idiotypic antibody reacts with the autoantigen whose sequence and/or structure is complemntary to the initiating antigen. **5)** Complementary proteins have a natural affinity because the amino acid sequence, surface contour, and/or hydropathy of one is the opposite of the other. Adapted from *Pendergraft et al, Nature Medicine 2004*.

Further, when mice were immunized with a complementary PR3 peptide, they not only developed antibodies against the complementary peptide, but also PR3-specific antibodies (ANCA) as the theory predicts. These observations are in accord with Mekler's ideas of complementary protein interactions, and Jerne's Idotypic Network Theory, both of which remain somewhat controversial though they have been independently confirmed and extended by multiple investigators.

Our work with complementary proteins came about somewhat by chance, in an attempt to map the pathogenic ANCA epitopes of the PR3 protein. Small fragments of PR3 cDNA were subcloned into a bacterial expression vector and screened for reactivity using patient sera. As expected, patients reacted to polypeptides derived from the sense strand of PR3, but a subset were also positive for short polypeptides coded by the antisense strand of the gene; some cDNA had inserted into the vector in the antisense orientation and was translated as such. This discovery led us to fuse the fundamental ideas of Mekler and Jerne into one cohesive explanation of how ANCA, and other autoimmune diseases, can sidestep normal tolerogenic mechanisms by exposure to complementary epitopes and subsequently generate autoantibodies.

In our work, a complementary protein is defined as one translated from the antisense RNA of a particular gene. This definition of complementary peptides arose from Mekler's observations regarding interesting characteristics of the genetic code. He found that translation of a codon in a 5'→3' direction on an antisense strand yielded an amino acid that always has an inverted hyropathic profile compared to its sense counterpart. Blalock and Smith have extended these ideas and found that the hydropathic character of any given codon is determined by the second base; a second base of U generally specifies hydrophobic amino

acids, whereas a second base A specifies hydrophilic amino acids. Because these two bases are complementary, amino acid sequences derived from the antisense strand will yield peptides with exactly opposite hydropathic characteristics. Though the molecular details are still undefined, these complementary proteins have a natural affinity for each other. One proposed mechanism for interaction is attributed to the folding of the peptides based on their hydropathy, as the shape of each respective peptide is proposed to mirror-image one another. If the folding of proteins largely has to do with charged interactions (hydrophobic side chains tend to congregate with other hydrophobic side chains, same for polar side chains), then the complementary sequences (with inverted hydropathy patterns) will produce peptides with an inverted shape and drive their interaction. This is the essence of Blalock's Molecular Recognition Theory. The interaction between a hydrophobic residue and its complementary hydrophilic correlate may seem counterintuitive, but these interactions have been shown experimentally to occur.[74] Brentani and Chaiken have proposed that hydrophobic interactions between side chains of nonpolar residues with the hydrocarbon chains supporting their polar partner residue could account for complementary amino acid interactions.[75, 76] The converse can be true as well, as hydrophilic side chains are often connected to a nonpolar alkyl chain that could interact with a nonpolar side chain.[77]

Not only have researchers been able to design complementary peptides that act as agonists or antagonists of hormone receptors in biological assays (ie. corticotropin or growth hormone-releasing hormone receptors), but there is evidence that complementarity mediates protein-protein interactions in nature.[78, 79] Bost *et al* showed that the alignment of mRNA sequences of interleukin-2, epidermal growth factor, and transferrin and their respective receptors yielded both complementary mRNA and protein sequences in the ligand binding

regions of these receptors.[80] Ruiz-Opazo *et al* screened a cDNA library with antisense oligonucleotide probes for both angiotensin II and vasopressin, expecting to extract clones of their respective receptors.[81] To their surprise, they found a novel, dual angiotenin/vasopressin G-protein coupled receptor; site-directed mutagenesis studies of the angiotensin-binding site demonstrated that sense and complementary sequences were responsible for the receptor-ligand interaction. While much of the work in this field has focused on receptor-ligand interactions, there are also reports that complementarity may mediate HIV binding to CD4 receptor, peptide binding to MHC molecules, and T-cell receptor binding.[82-84] Importantly, and relative to our work, antibody-antigen binding may also be governed by sense/complementary interactions.

As part of their early work, Blalock and colleagues showed that complementary peptides to peptide hormones could mimic the binding site of the hormone receptor, as they represent an "internal image" of the hormone.[85] They proposed that antibodies raised against the complementary peptides should mimic the shape of the hormone and interact with the hormone receptor. This idea was successfully employed to identify an opiate receptor, as antibodies raised to a complementary endorphin peptide bound to the opiate receptor and competed for endorphin binding in a neuroblastoma cell line.[86] This technique has since been used to identify several receptors of peptide hormones or proteins such as luteinising hormone releasing hormone, angiotensin II, fibronectin, arginine vasopressin, and substance P.[87-92]

If complementary peptides have complementary shapes, and the antibodies raised against one peptide reflect the shape of its complement, then it stands to reason that the two pools of antibodies raised against each individual peptide of a complementary pair would

also have complementary shapes, and could thus interact in an anti-idiotypic manner. This idea was put to test by Smith *et al*, and they found that immunoglobulin raised against corticotropin specifically bound immunoglobulin raised against a complementary corticotropin peptide; the same was true for antibodies specific for β-endorphin and its complement.[93] This work was replicated by the same group using arbitrary complementary peptide antigens.[94] In more recent work, Blalock and others have harnessed this system to create peptide vaccines that alleviate autoimmune diseases with defined pathogenic epitopes. Myasthenia gravis (MG) is an autoimmune disease in which patients develop autoantibodies against the acetycholine receoptor that lead to destruction of the neuromuscular junction and progressive weakness. MG can be induced in rats by immunizing the rats with purified acetylcholine receptor. Pre-immunization with a peptide complementary to the acetylcholine receptor induced anti-idiotypic immunity to the pathogenic antibody and alleviated disease symptoms, due in part to the binding and clearance of the pathogenic autoantibodies by their anti-idiotypic counterpart.[95] Additionally, passive transfer of the anti-idiotypic antibodies similarly reduced MG incidence and severity.[96] Similar studies were successful in targeting the T-cell mediated symptoms of multiple sclerosis (experimental autoimmune encephalomyelitis) and Guillain-Barre' syndrome (experimental autoimmune neuritis).[92, 97, 98]

Substantiated by observations that antibodies can interact in a specific, anti-idiotypic manner based on complementary shapes, Blalock proposed that complementarity may form the biochemical basis of Niels Jerne's Idiotypic Network Theory.[93] Jerne hypothesized and provided some experimental evidence that antibodies *themselves* can act as antigens and elicit anti-antibodies, or the aforementioned anti-idiotypes, directed against the

complementarity-determining regions (CDRs), which are the antigen-binding regions of immunoglobulin molecules.[99] In turn, anti-idiotypes can elitic anti-anti-idiotypes and so on. Under normal conditions this network is balanced, but the introduction of an antigen disrupts this balance and the network is induced to restore balance. Jerne's group demonstrated in multiple reports that anti-idiotypic antibodies were formed in rabbits immunized with immunoglobulin.[100] Taking this further, what if a person was exposed to an antigen complementary in sequence and/or shape to a self antigen? The antigen would elicit production of an antibody; this antibody could represent the internal image of the known self-antigen. If Jerne's idiotypic network is induced, an anti-idiotypic antibody would be formed that would in turn cross-react with the self-antigen as the anti-idiotype would mimic the shape of the complementary peptide. The end result is an immune reaction driven against a self antigen, or autoimmunity; this is the foundation of our Theory of Autoantigen Complementarity. Three of Jerne's postulates are very important relative to this theory: 1) antibodies produced by B-cells can recognize foreign or self-epitopes, 2) antibody molecules have immunogenic idiotopes, and 3) antibody idiotypes can mimic foreign or self epitopes.[99, 101]

As a whole, our ideas are not entirely new. Others have made this connection to autoimmune disease, though there has been relatively little direct experimental evidence that complementary proteins can induce autoimmune disease. Certainly, clues were evident in work investigating the idiotypic network by Shechter and collegues. They found that mice immunized with insulin not only made anti-insulin antibodies, but anti-idiotypes that bound to the insulin receptor and produced physiologic effects.[102, 103] This reactivity to the receptor was shown to be specific, as a single residue mutation in the insulin used for

immunization prevented the development of anti-receptor antibodies. Additionally, the development of the antibodies was temporal, as the anti-idiotypic antibody only appeared when the original anti-insulin antibody was cleared. This work suggested that the idiotypic network may be one way in which the immune system is fooled into skirting normal tolerogenic mechanisms.

As with us, serendipitous observations led Erlanger's group to investigate the role of anti-idiotypy in autoimmune disease. They purified antibodies specific to Bis-Q, an acetylcholine receptor agonist, and transferred these antibodies into rabbits in an attempt to induce experimental MG. These antibodies were hypothesized to mimic the acetylcholine receptor, thus the anti-idiotypic antibodies (anti-anti-Bis-Q) generated against them should cross react and bind to the acetylcholine receptor. Four of five animals showed severe-to-mild signs of disease. Additionally, following immunization of mice with the Bis-Q agonist, they made hybridoma cell lines from splenocytes and used the rabbit anti-Bis-Q antibodies to screen for monoclonal antibody-secreting clones that produced anti-idiotypes (anti-anti-Bis-Q).[104] They found multiple clones producing what they termed "auto-anti-idiotypes" that also cross reacted with purified acetylcholine receptor. Further, this same monoclonal strategy was utilized in the setting of Grave's disease, in which patients have anti-thyroid stimulating hormone (anti-TSH) antibodies that induce hyperthyroidism. Anti-idiotypic clones that bound the TSH receptor were derived from animals immunized with TSH.[105] In line with the Theory of Autoantigen Complementarity, they found anti-idiotypic antibodies to one of their anti-TSH monoclonals in one Grave's disease patient's plasmapheresis material.

Along these same lines, animal studies by Schoenfeld *et al* have demonstrated that the idiotypic network could be at play in autoimmune disease. After immunizing animals with

an antibody specific for an autoantigen, they showed that the mice developed the correlating anti-idiotypic antibody. After 4-8 months, the mice generated anti-anti-idiotypic antibodies that displayed the same binding characteristics to the autoantigen as the original immunizing antibody, and the appearance of these antibodies was associated with emergence of typical disease symptoms seen in systemic lupus erythematosus, anti-phospholipid syndrome, and Wegener's granulomatosis.[106-109] Based on their studies, they postulated that the analogy of their system to human disease is the introduction of bacteria carrying a pathogenic idiotype. This would incite the idiotypic network, and the formation of the anti-anti-idiotype could take months to years, blurring the relationship of the infection to the induction of autoimmunity. This is in contrast to molecular mimicry which implies a short, simple induction of cross-reacting autoantibodies.[109] Additionally, the idea of a bacteria carrying a pathogenic idiotype is not the only source of a complementary peptide, as will be discussed later. However, like Erlanger's work, the experimental evidence suggests that the idiotypic network may be at play in autoimmunity.

Until only a few years ago, the data implicating complementary epitopes and the idiotypic network in autoimmunity remained purely theorotical and experimental. Routsias *et al* showed, for the first time, an intact idiotypic network in humans with autoimmune disease. Autoantibodies to La/SSB complexes (subsellular ribonucleoprotein particles) are usually found in patients with both systemic lupus erythematosus and Sjogren's syndrome. This group had previously mapped the B-cell antigenic determinants of La/SSB, and investigated whether patients possessed anti-idiotypic antibodies by synthesizing peptides specific to these determinants, as well their complementary peptide counterparts.[110, 111] Their patients reacted to La/SSB peptides, as expected, and a subset of these patients had

antibodies to the complementary peptides as well; they also show that the two antibodies could interact in an anti-idiotypic fashion. Moving to mouse studies, the group found that immunization of the animals with either peptide (sense or complementary) induced antibody formation first against the immunogen, and then against its complement in a temporal manner.[112] Further, T-cell help was required in the generation of anti-idiotypic antibodies.[112, 113] In their discussion, the authors propose that molecular mimicry of a sequence complementary to La/SSB could be the triggering factor for this autoimmune response: sound familiar?

Work by Pendergraft *et al* in our group began with the aforementioned discovery that PR3-ANCA patients had antibodies to complementary PR3, and this observation led to our Theory of Autoantigen Complementarity.[73] In addition to demonstrating an intact idiotypic network in PR3-ANCA patients, this network was recapitulated in mice; immunization of mice with a human complementary PR3 peptide resulted in the generation of anti-PR3 antibodies that bound human PR3 in both ELISA assays and indirect immunofluorescent staining of neutrophils. Our theory, and the experimental evidence collected by Pendergraft supporting the theory, is an amalgamation of all of the past work discussed above, and is directly in line with what earlier observations in the field have predicted. It is interesting to note that Routsias *et al* was conducting almost identical experiments in parallel with ours in a different disease setting, suggesting this may be a general etiology of autoimmune disease.[110]

The Theory of Autoantigen Complementarity states that the inciting immunogen leading to autoimmune disease is a peptide or protein complementary to the autoantigen. One can consider two possible sources of this complementary sequence, either an

endogenous or an exogenous source. It is now known that the human transcriptome contains several thousand antisense transcripts that may serve to regulate gene expression.[114, 115] There is very little evidence that these antisense transcripts can be translated. However, there is precedence of such translation, as an antisense transcript-encoded self-antigen was found to be expressed on a human kidney tumor.[116] Consider a pathologic setting in which cellular dysregulation occurs and the antisense strand of an autoantigen is transcribed and translated; can this "new" protein now be recognized as a foreign antigen and elicit an immune response? Pendergraft *et al* explored this possibility and found that leukocytes from ANCA patients indeed had such antisense transcripts. These were termed pathological antisense transcripts (PATs) as they did not occur in healthy or disease controls – whether these transcripts were translated remains to be investigated. As others have also predicted, our group explored the notion that an invading pathogen could display a mimicking epitope homologous to the predicted complementary sequence of the autoantigen. To identify microbial homologues of complementary PR3, the predicted amino acid sequence was entered into the basic local alignment search tool (BLAST), which identified several microbes that had previously been associated with the development of PR3-ANCA and/or the onset and exacerbation of PR3-ANCA vasculitis. It is worth noting at this point that the *hydropathic profile* may be as important, or even more important, than the primary amino acid sequence of complementary peptides. For example, one of the best results demonstrating a complementary peptide interaction was obtained using a computer-generated complementary peptide that assigned to each residue of the target sequence an amino acid with an opposite hydropathy value, rather than using sequence to dictate the antisense RNA.[117] Additionally, the secondary structure of proteins is not altered by amino acid

substitutions, provided the frequency of hydrophilic and hydrophobic amino acids in the sequence is maintained.[118] The implications are that the complementary protein may be divergent from the predicted antisense RNA sequence but still maintain an inverted hyropathic profile, thus expanding the potential microbial sequences that can be considered complementary.

There is another similar, but different, theory regarding antigenic complementarity in the origins of autoimmune disease as proposed by Root-Bernstein and colleagues.[119] They state that the immune response leading to autoimmunity must arise from a complex of complementary antigens, one of which must mimic a self-determinant. They proposed this was most common in immunosuppressed people with multiple concurrent, chronic infections, thus increasing the probability of contracting a pair of complementary antigens. This would induce the simultaneous formation of anti-idiotypic antibodies, which would continue to perpetuate each other's existence and cross-react with self-determinants. Predicting the order of immune responses in humans is difficult. Regardless, both theories employ similar ideas as to the origin of autoimmune disease.

Evidence has been compiling over the last 30-plus years that complementary protein interactions can explain a growing number of antibody-antigen and antibody-antibody relationships. There are well over 50 individual reports in which complementary proteins or peptides were utilized in biological systems (see Heal *et al* for a review), and the number is growing as these concepts enter the mainstream.[77] Multiple scientists have predicted that these interactions could drive a humoral or T-cell mediated autoimmune disease, and we and others are beginning to show this to be the case. The major goal of the work presented in Chapter 2 of this dissertation was acquiring experimental animal evidence that a polypeptide

complementary to an autoantigen can cause disease in an animal model. We attempted to expand the application of our Theory of Autoantigen Complementarity to determine whether it could explain the onset of MPO-ANCA vasculitis. Studies were carried out investigating the presence of anti-complementary MPO antibodies in patients, thus implicating the relevance of complementary proteins in this disease. We also addressed the hypothesis that complementary MPO could induce disease in a mouse model.

CHAPTER 1

Myeloperoxidase Interacts with Endothelial Cell-surface Cytokeratin 1 and Modulates Bradykinin Production by the Plasma Kallikrein-Kinin System*

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ABSTRACT

During acute inflammation, functional myeloperoxidase (MPO) is released as a result of neutrophil degranulation, and, in some inflammatory conditions, is detectable in the circulation. MPO can also be released from neutrophils and monocytes at sites of vasculitis. One mechanism of resulting cellular injury involves endothelial internalization of MPO, which causes oxidative damage and impairs endothelial signaling. We report the discovery of a protein that facilitates MPO internalization, cytokeratin 1 (CK1), identified using affinity chromatography and mass spectrometry. CK1 interacts with MPO *in vitro,* even in the presence of 100% human plasma, thus substantiating biological relevance. Immunofluorescent microscopy confirmed that MPO added to endothelial cells can co-localize with endogenously expressed CK1. CK1 acts as a scaffolding protein for the assembly of the vasoregulatory plasma kallikrein-kinin system. Thus we explored whether MPO and high molecular weight kininogen (HK) reside on CK1 together or whether they compete for binding. The data supported cooperative binding of MPO and HK on cells such that MPO masked the plasma kallikrein cleavage site on HK, and MPO-generated oxidants caused inactivation of both HK and kallikrein. Collectively, interactions between MPO and the components of the plasma kallikrein-kinin system resulted in decreased bradykinin production. This study identifies CK1 as a facilitator of MPO-mediated vascular responses, and thus reveals a new mechanism by which MPO affects vasoregulatory systems.

INTRODUCTION

Cellular injury due to inflammation is caused by the release of injurious radicals and proteins from granule components of neutrophils and monocytes into the extracellular space. MPO is one of the principal enzymes stored in azurophilic granules of neutrophils and peroxisome-positive lysosomes of monocytes, and comprises 5% and 1% of the cells' total protein, respectively.[2] MPO imparts its antimicrobial properties by converting hydrogen peroxide, released from neutrophils during a respiratory burst, into hypochlorous acid, a powerful oxidant that readily reacts with adjacent thiol, disulfide and amino acid residues.[17]

Increased levels of MPO are detected in serum of patients with inflammatory disease, suggesting that neutrophils and/or monocytes aberrantly degranulate in the vascular lumen.[30, 31, 34, 54, 120] "Free" MPO spilled into the vasculature interacts with endothelial cells and contributes to endothelial dysfunction.[24, 41, 121] Our previous explorations of this process indicated that MPO is internalized by endothelial cells with a resultant rise in intracellular oxidant radicals.[35] Others have extended these findings by demonstrating that MPO transcytoses through the endothelium and concentrates in the subendothelial matrix.[29] Internalized MPO modulates vascular signaling and vasodilatory functions by decreasing the bioavailability of nitric oxide via multiple mechanisms.[24, 34] For example, hypochlorous acid can chlorinate L-arginine thus reducing functional substrate for endothelial nitric oxide synthetase, and substrate radicals produced by MPO can catalytically consume nitric oxide. [24, 34]

As a continuation of our previous work, our aim was to identify proteins on the endothelial cell surface that specifically interacted with MPO, with the goal of finding proteins that facilitate its internalization. We discovered that cytokeratin 1 is an endothelial

binding partner for MPO. Cytokeratin 1 (CK1) is part of a possible endothelial receptor complex containing urokinase-like plasminogen activator receptor (uPAR) and the receptor for the globular head of complement 1q protein (gC1qR). This multiprotein receptor complex is a platform for assembly and activation of the vasoregulatory plasma kallikrein-kinin system.[122, 123] Circulating high molecular weight kininogen (HK) binds to this complex and serves as the endothelial receptor for the zymogen prekallikrein. An endothelial membrane- and matrix-associated enzyme, prolylcarboxypeptidase, activates prekallikrein, which then cleaves the nonapeptide bradykinin from HK.[124] Because both MPO and HK interact with CK1, MPO might interfere with the functionality of the kallikrein-kininogen system.

The results herein provide a novel layer of interaction between MPO and endothelial vasoregulatory systems that could modulate vascular function and/or potentiate vascular injury during inflammation. Functional studies indicate that CK1 can facilitate internalization of MPO and moreover, this interaction has the potential to disrupt normal endothelial processes.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) and EGM basal media with Singlequot supplements were purchased from Cambrex (Rockland, MD). Cells were typically used between passages 5-10. EA.hy926, an immortalized endothelial hybridoma cell line, was kindly provided by Cora-Jean Edgell, Ph.D. (University of North Carolina at Chapel Hill) and cultured in

Gibco/Invitrogen (Carlsbad, CA) Dulbecco's Modified Eagle Medium with penicillin-streptomycin and 10% fetal bovine serum. Both cell lines were grown in a 5% CO_2 humidified incubator. EA.hy926 cells were used in addition to HUVEC in immunofluorescence colocalization studies and in internalization blocking experiments. Reproducibility of the data using two different endothelial cell lines lends confidence in its validity and implies generality in endothelial systems.

Antibodies

Mouse and rabbit anti-human MPO antibodies were purchased from DAKO (Carpinteria, CA). An additional mouse anti-human myeloperoxidase antibody was purchased from Abcam (Cambridge, MA). Mouse anti-proteinase 3 antibodies were purchased from Lab Vision Neomarkers (Fremont, CA) and Research Diagostics, Inc (Concord, MA). Rabbit anti-CK1 antibody was purchased from Covance Research Products (Berkley, CA), and the mouse pan-cytokeratin antibody (clone PCK-26) was from Sigma (St. Louis, MO). Affinity purified goat anti-CK1 antibody was prepared as previously characterized.[125, 126] Normal total IgG of all species was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Chemicon (Temecula, CA), and Alexafluor 488- and 569-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Alkaline phosphatase-conjugated streptavidin was purchased from Pierce (Rockford, IL). Alkaline phosphatase-conjugated Affinipure $F(ab')_2$ fragment donkey anti-mouse IgG was from Jackson Immunoresearch Laboratories (West Grove, PA). The alkaline phosphatase substrate kit was either from Bio-Rad Laboratories (Hercules, CA) or Pierce.

Purified Proteins

MPO and catalase were purchased from Calbiochem (San Diego, CA). Proteinase 3 was purchased from Weislab AB (Lund, Sweden). Recombinant CK1 ($rCK1_{31}$) was prepared as previously reported.[126] Pooled human cytokeratins and L-methionine were purchased from Sigma. High molecular weight kininogen, plasma kallikrein, Factor XII (SA 27.77 U/mg), and high molecular weight kininogen-deficient human plasma were purchased from Enzyme Research Laboratories (South Bend, IN). PilB, a methionine sulfoxide reductase derived from *Neisseria gonorrhoeae*, was generously provided by Dr. Nathan Brot at Cornell University Joan and Sanford I. Weill Medical College.

Immunoprecipitations of MPO and CK1

HUVEC lysates were prepared as either whole cell or membrane fraction lysates. To prepare the whole cell lysate, HUVEC were washed with ice cold PBS and scraped in lysis buffer (25 mM HEPES, 12.5 mM $MgCl_2$, 150 mM KCl, 0.5% NP40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and Roche (Almeda, CA) complete EDTA proteinase inhibitor cocktail at 4˚C. Glycerol was added to 10%. Lysates were pooled and split evenly into 3 tubes. Insoluble debris was centrifuged and separated from lysate. Membrane fraction lysate was prepared using Pierce's MEM-PER isolation kit as instructed with the addition of sodium fluoride, sodium orthovanadate, and the Roche protease inhibitor cocktail. The pooled membrane fraction was dialyzed in PBS + 1% NP40 overnight for 3 nights, changing the dialysate each night. Glycerol was added to 10% and the pooled lysate split evenly into 3 tubes. Protein A/G beads (Pierce) and normal mouse IgG were added to each tube for 1 hr at 4˚C and then centrifuged to pre-clear the lysate. Ten micrograms of purified proteinase 3 or MPO was added to the lysates for 5 hours at 4˚C, and mouse anti-

MPO or anti-PR3 antibodies were added to the appropriate tubes overnight. Protein A/G beads were added to the tubes for 1 hr at 4˚C. The beads were washed with lysis buffer and PBS and any protein complexes were eluted from the beads directly in Laemmli buffer. The samples were subjected to SDS-PAGE and then stained with Coomassie Brilliant Blue R-250. Unique bands in the MPO immunoprecipitation lane were excised and analyzed by MALDI-TOF at the University of North Carolina's Proteomics Core Facility using methods published by Parker et al.[127]

Mouse anti-MPO antibody (100 μg) was immobilized on Pierce's AminoLink Plus Coupling Gel as instructed. HUVEC were washed with ice cold HBSS and scraped at 4˚C into lysis buffer (formulation above) containing 0.5% Triton X-100 and 10% glycerol. The lysate was sheared with a 22g needle and the insoluble debris centrifuged and separated. The lysate was split into 2 tubes, one of which received 2.5 μg purified MPO. The tubes were rocked at 4˚C for 3 hours after which immobilized mouse anti-MPO was added to the tubes overnight. The gel-containing lysates were spun and washed in Pierce's Handee Cup spin columns with 10 column volumes of lysis buffer and eluted using the AminoLink plus kit. Laemmli buffer was added to each elution fraction and the fractions were subjected to SDS-PAGE and western blot analysis.

Two micrograms of purified MPO was mixed with either 2 μg recombinant CK1, 2 μg recombinant control protein, or nothing in IP buffer (25mM HEPES, 12.5 mM $MgCl_2$, 150 mM KCl, 0.5% NP40, 10% Glycerol) overnight at 4˚C with constant mixing. Four micrograms of either mouse anti-MPO or mouse anti-pan CK antibody was added to the mix, as well as antibody alone in IP buffer, and allowed to mix overnight at 4˚C. Protein A/G

beads (Pierce) were added for 1 hour, washed, and protein complexes were eluted directly in Laemmli buffer. Elutions were subject to SDS-PAGE and western blot analysis.

ELISAs Demonstrating MPO Binding to CK1 and HK

ELISA plates were coated with 5 μg/ml of either rabbit anti-CK1 or normal rabbit IgG overnight at 4˚C. Wells were blocked with a 0.2% fish gelatin buffer (150 mM NaCl, 50 mM Tris base, 0.05% Tween 20, 0.2% fish gelatin, 0.02% sodium azide, pH 7.6) for 4 hours at room temperature, and all subsequent steps were carried out in this blocking solution. CK1 was captured overnight at 4˚C from a mix of solubilized human keratins (10 μg/ml) derived from human epidermis. Purified MPO was presented to the captured CK1 at 5 μg/ml at room temp for 4.5 hours. The wells were exposed to mouse anti-MPO antibody at a 1:100 dilution and each of three sera from patients with MPO-specific anti-neutrophil cytoplasmic autoantibodies (MPO-ANCA) diluted at 1:100 in duplicate overnight at 4˚C. The CK1-MPO complexes were detected by alkaline phosphatase-conjugated secondary antibody incubation for 1 hour at room temperature followed by the addition of substrate. Plates were read at 405 nm after 1 hour using a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA).

ELISA plates were coated with either rabbit anti-CK1 antibody or normal rabbit IgG at 5 μg/ml overnight at 4˚C. The wells were blocked in a 1% fish gelatin buffer for 4 hours at room temperature and then exposed to a 10 μg/ml solution of solubilized human keratins in blocking buffer overnight at 4˚C to capture CK1. Plasma was obtained from healthy subjects in Becton Dickinson Vacutainer tubes K3 EDTA 12mg (purple top) (Franklin Lakes, New Jersey). Solutions of 0, 25, 50, 75, and 100% plasma were prepared with or without the addition of 5 μg/ml MPO. The plasma solutions were prepared using 1% fish gelatin buffer that was drawn into the Vacutainer tubes in order to keep the EDTA concentration constant

throughout this stage of the experiment. The plasma solutions were placed in the wells for 4 hours at room temperature. The captured MPO was labeled with the DAKO mouse anti-MPO antibody at a 1:100 dilution at 4˚C overnight. The next day, secondary antibody was applied for 1 hour at room temperature followed by substrate. The plates were read at 405 nM 3 hours after the addition of substrate.

ELISA plates were coated with either rabbit or mouse anti-MPO antibody, normal rabbit or mouse IgG, at 5 μg/ml overnight at 4˚C. The wells were blocked with 0.2% fish gelatin buffer for 1 hour at room temperature, and all subsequent steps were carried out in the blocking buffer. MPO was captured at 5 μg/ml for 1 hour at room temperature. Wells were washed thoroughly and 2 μg/ml biotinylated HK was added with or without a 75-fold excess of unlabeled HK in duplicate. Unlabeled HK only was also added to wells in duplicate. After 1 hour at room temperature, the wells were washed and alkaline phosphatase-conjugated streptavidin was added for an additional hour. Following addition of substrate for 1 hour, the plate was read at 405 nm. Similar experiments were conducted using ELISA plates directly coated with either MPO or BSA.

ELISA plates were coated with either rabbit anti-MPO antibody or normal rabbit IgG (5 μg/ml) overnight at 4˚C. The wells were blocked with 1% fish gelatin buffer for 2 hours at room temperature. HK-deficient plasma was replenished with a physiologic concentration (80 μg/ml) of biotinylated high molecular weight kininogen (bHK). The HK-deficient plasma was added to the wells with or without bHK overnight at 4˚C. Following thorough washing, alkaline phosphatase-conjugated streptavidin was added to the wells for 1 hour at room temperature. Subsequently, substrate was applied and the plate was placed at 4˚C

overnight. Spectrophotometer readings were taken at 405 nM after resting the plates at room temperature for 6 hours.

Immunofluorescent Co-localization of MPO and CK1

EA.hy926 cells were plated on glass coverslips, grown to approximately 90% confluence, washed, and treated with MPO (10 μg/mL) for 10 minutes in serum-free DMEM. The cells were washed in PBS, fixed in 4% paraformaldehyde, and permeabilized with acetone. Goat serum was used as a block (at 10% in TBS with 0.05% Triton-X100) and antibody dilution buffer (at 1% in TBS with 0.05% Triton-X100), and the cells were labeled with rabbit anti-CK1 and mouse anti-MPO antibodies. Alexafluor-conjugated secondary antibodies were used for detection: the Alexafluor 488 was goat anti-rabbit, and the Alexafluor 568 was goat anti-mouse. The coverslips were mounted on slides with Antifade and analyzed using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope with the accompanying software suite.

HUVEC were plated to approximately 90% confluence (140,000 cells per well) and allowed to attach overnight to 0.1% gelatin-coated glass cover slips in 12 well culture dishes. Cells were washed twice with pre-warmed, phenol red-free EGM basal media with or without 2% fetal bovine serum, and MPO was added at 15 μg/mL for 12 minutes at 37˚C. Cells were then placed on ice and washed in cold PBS, and then fixed in cold 2% paraformaldehyde for 45 minutes on ice. The wells were then washed and the cells were then permeabilized with ice cold 1:1 methanol:acetic acid on ice for 10 minutes. Goat serum was used as the blocking agent (at 10% in TBS with 0.05 % Triton-X100) and the antibody dilution buffer (at 1% in TBS with 0.05% Triton-X100). The coverslips were blocked for 1 hour at room temperature. The cells were then labeled with the Abcam mouse anti-MPO

antibody (1:250 dilution) for 1 hour at room temperature, followed by rabbit anti-CK1 (1:80 dilution) in the same conditions. Alexafluor-conjugated secondary antibodies were used for detection: the Alexafluor 488 was goat anti-rabbit, and the Alexafluor 568 was goat anti-mouse. The secondaries were prepared at a 1:500 dilution and applied simultaneously. The coverslips were mounted on slides with antifade and analyzed using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope with the accompanying software suite.

Antibody Blocking Experiments

EA.hy926 cells grown in 12-well culture dishes, or HUVEC grown in 6-well dishes were washed with serum-free, phenol red-free DMEM. Cells were pretreated in the serum-free media with blocking antibodies. One experiment used a blocking mix consisting of 16.67 μg/ml goat-anti CK1 (which blocks HK binding to endothelial cells) and a 50-fold molar excess of Factor XII (which also binds CK1) for 45 min at 37˚C.[125] Other experiments used the pan-cytokeratin antibody or normal mouse IgG at 16.67 μg/ml for 45 min at 37˚C. MPO (0.5-2 μg/ml) was added subsequently to the cells in the presence of the blocking antibodies for 10 minutes. The cells were washed with the serum-free media and trypsinized. When the cells released from the plate, either trypsin neutralizing solution (Cambrex) or serum-containing medium was added and the cells were collected. The cells were processed using Caltag Laboratories Fix and Perm Kit (Invitrogen) and stained for MPO using the rabbit anti-MPO antibody in conjunction with an Alexafluor 488 chicken anti-rabbit secondary antibody. The stained cells were analyzed using a FACScan flow cytometer linked to a CellQuest software system (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

HK Binding Studies on HUVEC

HUVEC were plated to confluence in EGM basal media (*without* the Singlequot supplement) in 96-well tissue culture wells that were pre-coated with a 1% gelatin solution. Eighteen to 24 hours after plating, the cells were washed with a HEPES-carbonate buffer (154 mM NaCl, 5.6 mM KCl, 3.4 mM $NaHCO_3$, 5.5 mM Dextrose, 5 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% gelatin, pH 7.4) and biotinylated HK was added to the cells at 20 nM in HEPES-carbonate buffer either alone, or in the presence of a 50-fold molar excess of unlabeled HK or MPO for 30 minutes at 37˚C. Unbound proteins were washed away and the cells were treated with alkaline phosphatase-conjugated streptavidin at room temperature for 1 hour. The cells were washed and the substrate was applied and absorbance read at 405 nM after 1 hour at room temperature.

Immunofluorescent Co-localization of MPO and HK on Endothelial Cells

HUVEC were grown to 80% confluence on 0.1% gelatin-coated glass coverslips. Biotinylated HK (50 nM) was pre-mixed with a 5-fold molar excess of MPO in EGM basal medium. The cells were gently washed with the EGM basal medium and the protein mix added for 30 minutes at 37°C. Control groups included each protein added individually or no protein. The cells were placed on ice and washed thoroughly in cold PBS and then fixed in 1.5% paraformaldehyde for 40 minutes at room temperature. Some coverslips were dipped in acetone to permeabilize the cells. The coverslips were blocked in 10% chicken serum for 45 minutes. Subsequent steps were carried out in 1% chicken serum for 1 hour at room temperature in the order listed with thorough PBS washes in between each step: alexafluor 488-conjugated streptavidin diluted 1:500, rabbit anti-MPO at a 1:2500 dilution, and

alexafluor 569-chicken anti-rabbit secondary antibody diluted 1:1500. The coverslips were then mounted in Antifade medium.

ELISAs Measuring the Effects of MPO on Bradykinin Production

To quantitate bradykinin released in the presence of MPO, HK (50 nM) was incubated with 5 nM, 50 nM, or 500 nM MPO in PBS for 20 minutes at room temp in microcentrifuge tubes. Kallikrein (50 nM) was added to the mix for 20 minutes at room temp, and the samples were frozen at -80˚C for future analysis. Samples were thawed and analyzed for bradykinin release using Dainippon Pharmaceuticals MARKIT-M Bradykinin ELISA kit (Osaka, Japan). Our experimental samples were handled for analysis per the manufacturer's instructions specific to urine specimens. Amount of reagents added was adjusted to accommodate smaller volumes.

To measure the effect of the MPO-H_2O_2-Cl^- system activity on bradykinin production, HK (50 nM) was incubated with MPO (10, 50, or 250 nM) and 0.45 μM H_2O_2 in PBS in the presence or absence of catalase (10-fold molar excess to MPO) for 20 minutes at room temperature in microcentrifuge tubes. L-methionine (100 μM) was added to the mix for 20 minutes to quench HOCl activity. Kallikrein (50 nM) was then added for 20 minutes at room temperature and the samples were frozen at -80˚C for future analysis. Samples were thawed and analyzed for bradykinin release using the aforementioned bradykinin ELISA kit.

To determine the direct effects of HOCl on bradykinin production, HK (50 nM) was pre-treated with increasing concentrations of HOCl in PBS at room temp for 20 minutes in microcentrifuge tubes. A 20-fold excess of L-methionine (compared to HOCl concentration) was added to the mix for an additional 20 minutes. Subsequently, kallikrein was added to 50

nM for 20 minutes at room temperature and the samples were frozen at -80˚C for further analysis. Bradykinin release was measured in the samples using the bradykinin ELISA kit.

The unknown values were determined using a 7-point standard curve with an R-squared value of 0.95

Kallikrein Protease Activity

Kallikrein (50 nM) was treated with HOCl or HOCl that was pre-incubated with a 20-fold excess of L-methionine (to quench oxidative properties) at varying concentrations in microcentrifuge tubes for 15 minutes. Subsequently, L-methionine was added to those tubes that received non-quenched HOCl. Samples were transferred to a 96-well plate and Chromogenix S-2303 plasma kallikrein-specific chromogenic substrate (Diapharma, Westchester, OH) was added, mixed, and OD 405 nM was read after 30 minutes of rocking at room temperature. Values were adjusted by subtracting background OD values of HOCl + L-methionine without kallikrein.

Methionine Sulfoxide Reductase Assay

HK (50 nM) was pre-treated with 25 μM HOCl in PBS for 20 minutes at room temperature, and unreacted HOCl was quenched with 500 μM L-methionine (a 20-fold molar excess) for an additional 20 minutes. PilB (50 nM) was added to the reaction mix in the presence of 15 mM dithiothreitol and 30 mM Tris-HCl (ph 7.4) for 1.5 hours at 37˚C. After cooling the tubes to room temperature, plasma kallikrein was added to the reaction at 50 nM for 25 minutes, and kallikrein activity was stopped with 0.5 μM Diisopropylfluorophosphate (DFP). The samples were deproteinized using reagents provided with the Dainippon MARKIT-M bradykinin ELISA kit, and analyzed for bradykinin release using Bachem

Peninsula Laboratory's (San Carlos, CA) Bradykinin EIA kit as per manufacturer's instructions.

RESULTS

Identification of CK1 and Ck9 as Endothelial MPO-binding Proteins

To identify endothelial cell surface proteins that bind MPO, purified MPO was incubated with HUVEC membrane lysates, and subsequent complexes were immunopurified using an anti-MPO antibody. For comparison, proteinase 3, also a cationic neutrophil granule protein, was added to an aliquot of endothelial lysates and analyzed in parallel for binding partners. Two proteins were identified that specifically bound MPO and not proteinase 3 under these conditions (Figure 1.1a). MALDI-TOF analysis determined these to be cytokeratins 1 (67 kD) and 9 (62kD) (Figure 1.1b).

CK1 was verified to physically bind MPO, using an anti-MPO affinity column to capture CK1 in complex with MPO in the context of HUVEC protein lysates. Western blot analysis showed CK1 specifically eluted with MPO (Figure 1.1c). Mixing of purified MPO and CK1 further confirmed the specificity of this interaction (Figure 1.1d). MPO specifically bound CK1 while a negative-control recombinant protein did not.

We next examined whether the interaction of MPO and CK1 was limited to native human MPO, and moreover, whether the complex was detectable using human anti-MPO antibodies from patients with MPO-specific autoantibodies.[60] Sandwich ELISAs demonstrated that CK1 complexed with human native MPO, recombinant human MPO, and native mouse MPO. MPO was detected only when an anti-CK1 antibody was present to

capture CK1 as compared to when a normal rabbit IgG was used as the capture antibody (Figure 1.1e). The mouse anti-human MPO antibody reacted with only the native MPO protein.

MPO interacts with several blood proteins such as ceruloplasmin (its natural inhibitor), albumin, and the lipoprotein apo-A1.[36, 128, 129] The above studies were carried out in total cell lysates or in serum-free buffer to test specificity. We asked if the MPO-CK1 interaction was influenced by the presence of plasma proteins, as found in an in vivo environment. In a competition assay, purified MPO binding to immobilized CK1 was slightly competed away by addition of plasma from volunteer 1 and saturated at 25% plasma (Figure 1.1f). This effect was more pronounced in volunteer 2, but saturated at 50%. In parallel, plasma was added to immobilized CK1 without exogenously added MPO to determine if circulating plasma MPO would bind. Free MPO in plasma bound CK1 and, interestingly, binding was maximal at the concentration coinciding with the saturation point with excess added MPO, indicating all available CK1 was complexed.

To test this interaction at the cellular level, cells expressing surface CK1 were treated with MPO in the absence or presence of plasma and analyzed for the ability of these proteins to colocalize. By immunofluorescence microscopy, MPO colocalized with CK1 expressed on EA.hy926 cells and on HUVEC (Figure 1.2). Widespread colocalization was noted in HUVEC on the cell surface and intracellularly in both the absence and presence of serum (2%). Based on this data, MPO binding to CK1 is highly likely to occur in vessels.

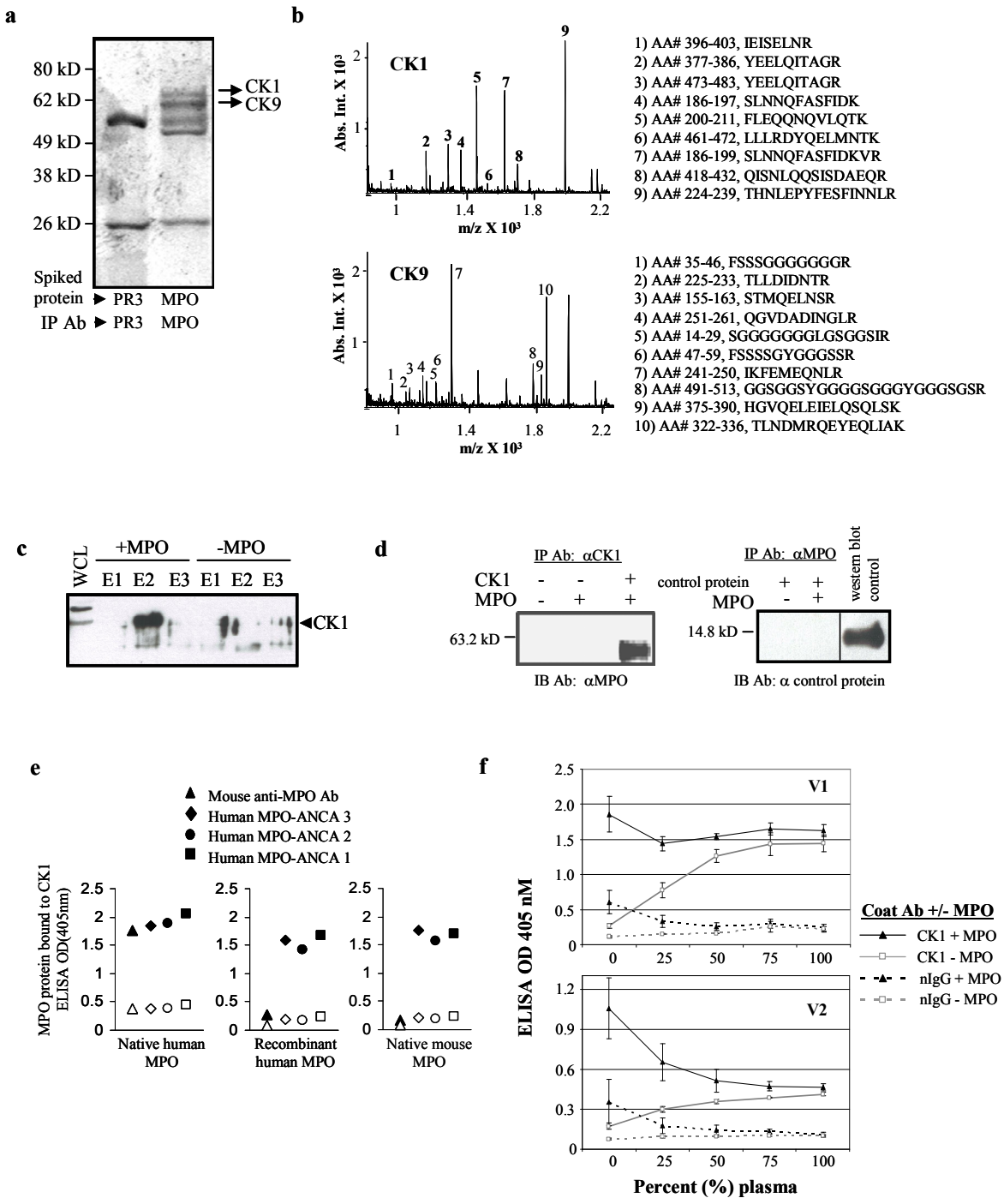

Figure 1.1. Cytokeratins 1 and 9 Specifically Bind MPO. *Note: Figure legend on following page*

Figure 1.1. Cytokeratins 1 and 9 Specifically Bind MPO. **a)** Coomassie stained gel of immunopurified MPO or proteinase 3 (PR3) complexes resolved by SDS-PAGE. Mass spectrometry analysis of the unique bands in the MPO-spiked membrane fraction identified cytokeratins 1 and 9. The spectra are shown in **b**) The tryptic peptides are numbered and displayed with the corresponding sequences. **c)** Western blot analysis verified that CK1 binds to MPO. HUVEC whole cell lysates were incubated with purified MPO, and passed over a monoclonal MPO-specific antibody immobilized on Aminolink Plus coupling gel. The proteins eluted from the beads were subjected to SDS-PAGE and western blot analysis using cytokeratin 1-specific antibody. E1, E2 and E3 are sequential elution fractions from the beads. **d)** *In vitro* mixing of purified proteins resulted in the binding of MPO to CK1, but not but not to a control recombinant protein (BMP7). Western blot analysis (IB) of immunopurified complexes (IP) indicated that an anti-CK1 antibody (Ab) immunoprecipitated MPO, but only when complexed with CK1. MPO does not bind the control recombinant protein demonstrating the specificity of the MPO-CK1 interaction. **e)** Sandwich ELISAs: the different shapes represent the antibody used to detect MPO binding. Solid black indicates the ELISA well was coated with a CK1 antibody, and open shape indicates the well was coated with normal IgG. Antibody-captured CK1 was incubated with purified MPO, recombinant MPO, or purified mouse MPO. Results show that purified MPO was detected by the monoclonal MPO antibody, but this antibody did not react with recombinant MPO or mouse MPO. Anti-MPO antibodies from three patients with ANCA disease (MPO-ANCA) recognized the CK1-MPO complex with all three forms of MPO protein. **f**) CK1 captures MPO directly from human plasma. ELISA wells were coated with either anti-CK1 antibody or normal IgG. CK1 was then captured and incubated with or without MPO in increasing amounts of plasma drawn from two healthy volunteers (V1 and V2).

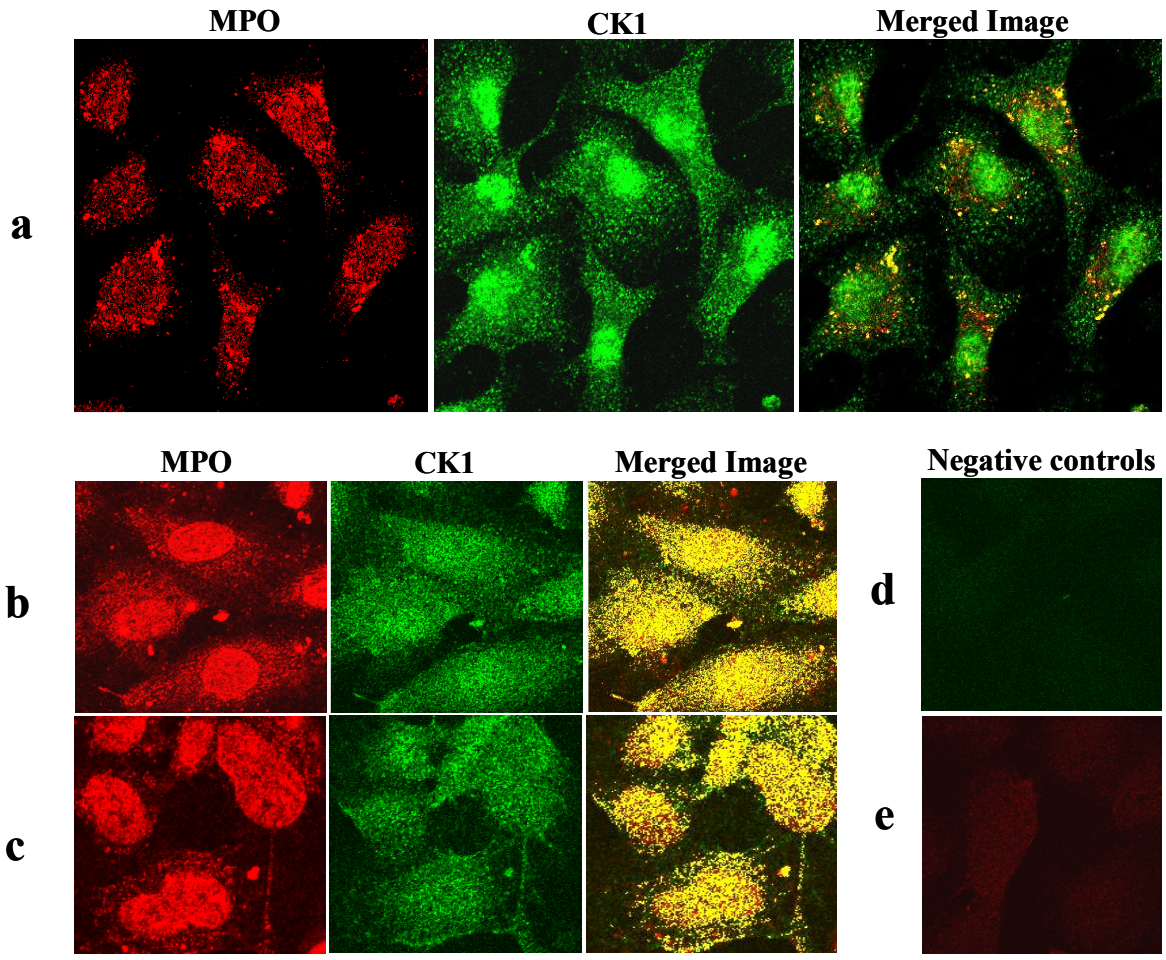

Figure 1.2. MPO and CK1 colocalize in living cells. a) EA.hy926 cells were incubated with MPO in serum-free medium. MPO was detected with the Dako mouse anti-MPO antibody, and CK1 was detected using a polycloal anti-CK1 antibody. The yellow foci in the merged image indicate areas of colocalized CK1 and MPO. **b** and **c**) HUVEC were also exposed to MPO in serum free (**b**) or serum-containing (**c**) medium. MPO was labeled in this experiment with a mouse-MPO antibody from Abcam. CK1 was labeled with the same polyclonal antibody used in **a**. Widespread colocalization was noted in both conditions. **d**) Negative control for the CK1 label on HUVEC (anti-rabbit secondary antibody only). **e)** Negative control for the MPO label on HUVEC (cells not treated with MPO but exposed to the anti-MPO antibody and its corresponding secondary antibody). All images were produced at 60X magnification.

Internalization of MPO by Endothelial Cells is Blocked by Interfering with the MPO-CK1 Interaction

To test whether CK1 participates in the uptake of MPO, we performed a blocking experiment using anti-CK1 antibodies. Following antibody treatment, cells were analyzed for uptake of added MPO by flow cytometry. Experimental design included a trypsinization step to remove residual MPO not internalized by the cells. MPO uptake was reduced in the presence of anti-CK1 antibodies but not IgG controls (Figure 1.3). Blocking of MPO internalization was concentration dependent, i.e. saturation of the system with MPO overwhelmed the antibody blocking effects (Figure 1.3a). A blocking mix consisting of a polyclonal goat anti-CK1 antibody and FXII reduced the percentage of MPO-positive EA.hy926 cells from 42.1% to 6.1% when the cells were treated with 2 μg/ml MPO, but did not block when treated with 5 μg/ml MPO. Similar results were obtained when a monoclonal antibody specific for type II cytokeratins was used as a blocking antibody on HUVEC. Pre-treatment with this antibody resulted in more than a 50% decrease in the number of MPO-positive cells (Figure 1.3b). This monoclonal was used to block CK1 on EA.hy926 cells, however, in this experiment normal mouse IgG was tested to assure that the mouse IgG, in general, was not responsible for blocking MPO. The CK antibody decreased the number of MPO-positive cells from 33% (with the normal mouse IgG) to 12.7% (Figure 1.3c).

MPO Directly interacts with HK

To investigate potential effects of MPO binding to CK1 on vascular processes, we asked if MPO would compete with HK for binding to CK1, since HK is known to also bind

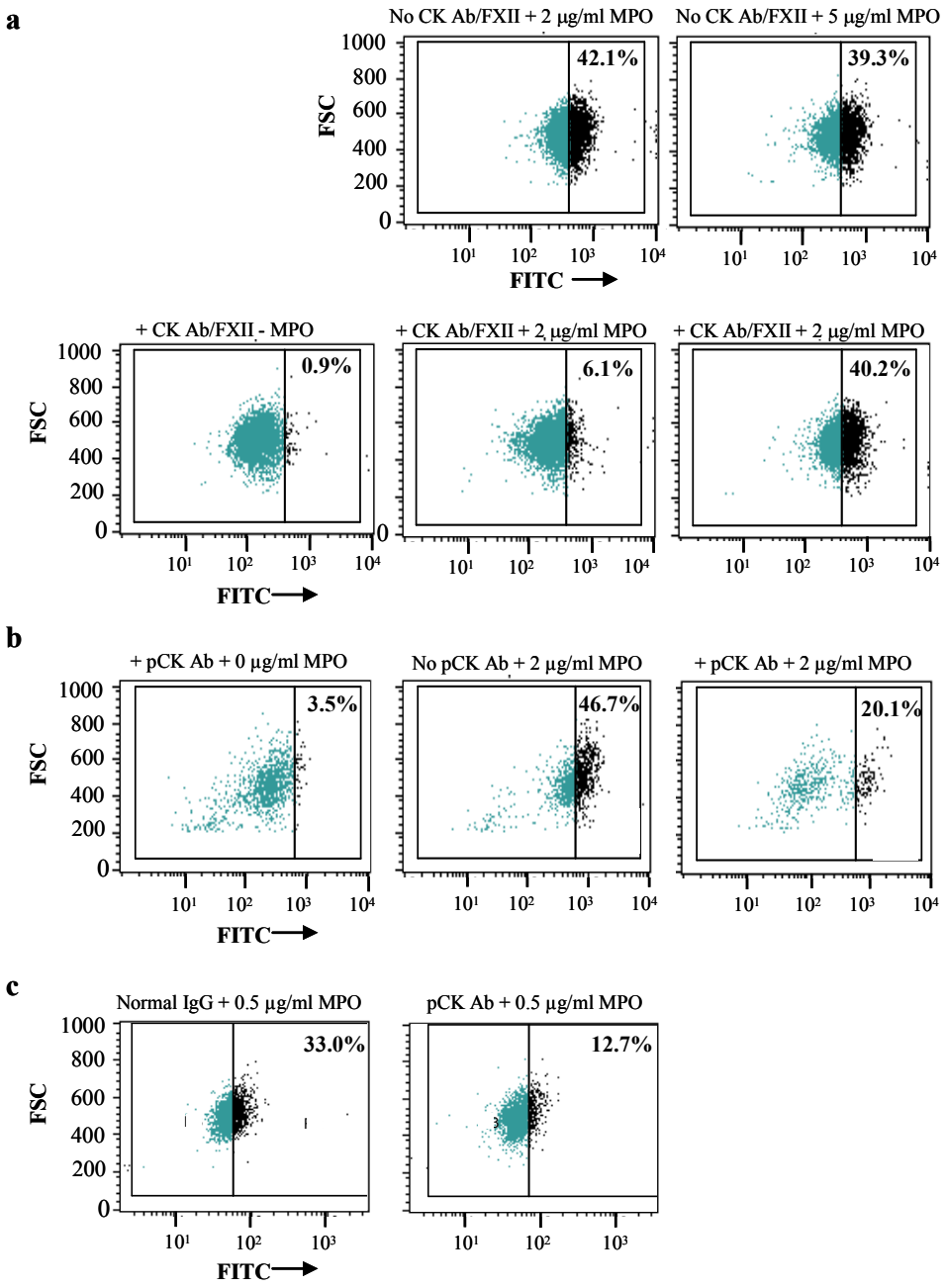

Figure 1.3. Internalization of MPO by Endothelial Cells is Reduced by Blocking the MPO and CK1 Interaction. Experiments are flow cytometry analyses of endothelial cells labeled with a polyclonal anti-MPO antibody. **a)** EA.hy926 cells were pre-treated with a CK1-blocking mix consisting of factor XII (FXII) and a goat anti-CK1 antibody (CK Ab) raised against a kininogen binding site of CK1. Cells were then exposed to 2 or 5 µg/ml MPO for 10 minutes. **b)** HUVEC were pre-treated with a pan-CK (pCK Ab) antibody and then 2 µg/ml MPO was added for 10 minutes. **c)** EA.hy926 cells were pre-treated with either a pan-CK (pCK Ab) antibody or normal mouse IgG before 0.5 µg/ml MPO was added for 10 minutes.

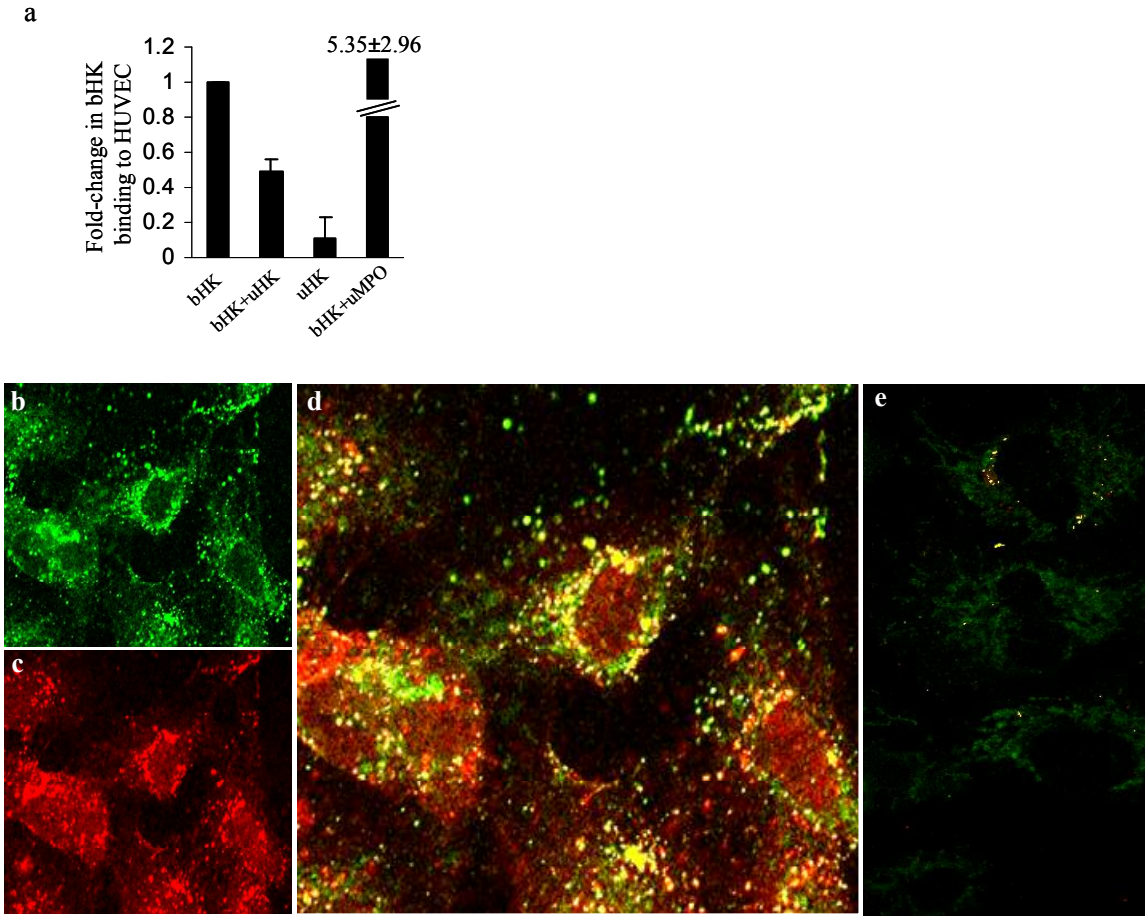

Figure 1.4. **MPO Enhances Kininogen Binding to Endothelial Cells and the Proteins Co-localize on the Endothelial Cell Surface and Intracellularly. a**) MPO enhances kininogen binding. HUVEC were treated with biotinylated high molecular weight kininogen (bHK) either alone, or in the presence of a 50-fold molar excess of unlabeled high molecular weight kininogen (uHK) or MPO for 30 minutes at 37˚C. Bound bHK was detected using alkaline phosphatase-conjugated streptavidin. Unlabeled HK competed with bHK binding resulting in 50% less bHK protein bound. MPO enhanced bHK binding by 5.35-fold. **b-e**) MPO and kininogen colocalize in/on endothelial cells. HUVEC were either treated with bHK and purified MPO (**b-d**) or protein-free medium (**e**). The cells were labeled using both alexafluor 488-conjugated streptavidin (**b**) and an anti-MPO antibody with corresponding alexafluor 568 secondary antibody (**c**). **d)** The merged image of B and C showing a high degree of colocalization between MPO and bHK. The merged image of cells that were treated with protein-free medium is shown in **e.** All images were produced at 60X magnification.

CK1.[130] Rather than compete for CK1 binding, a more than 5-fold increase in binding of biotinylated HK was observed in the presence of MPO (50-fold excess), while the controls using a 50-fold excess of unlabeled HK blocked biotinylated HK binding by 40% (Figure 1.4a). Moreover, by immunofluorescence microscopy, MPO and HK were found to colocalize at the surface membrane and in the cytoplasm of endothelial cells (Figure 1.4b). We next asked if enhanced binding could be due to a direct interaction between HK and MPO. Data from a direct ELISA (Figure 1.5a) and from a capture ELISA (Figure 1.5b and c) verify that MPO and HK have the capability to physically interact. Importantly, we also showed that immobilized MPO bound kininogen from plasma (1.Figure 5d), thus demonstrating that this interaction is favored in a physiologic matrix.

MPO Interferes with the Plasma-Kallikrein Kinin System

To further explore MPO-mediated effects on the plasma kallikrein-kinin pathway, we tested whether MPO affected bradykinin production. We hypothesized that MPO binding to HK would interfere with its cleavage by kallikrein to produce bradykinin. Bradykinin production from HK by plasma kallikrein was monitored in an *in vitro* system with and without MPO. The results indicate that a 10-fold molar excess of MPO (500 nM) to HK reduced bradykinin production by 30% compared to equimolar concentrations (50 nM) (Figure 1.6a). The data indicate that MPO's binding to HK physically hinders the accessibility of the kallikrein cleavage site on HK. We next asked if MPO's peroxidase function would influence bradykinin production. The data indicate that when hydrogen peroxide (H_2O_2), the substrate of MPO, is present, inhibition of bradykinin production is enhanced showing a significant reduction at 250 nM (Figure 1.6b) versus 500 nM MPO

without H_2O_2. Supporting that the conversion of H_2O_2 and Cl^- to hypochlorous acid by MPO was involved, the addition of catalase, an H_2O_2 scavenger, reversed this effect (Figure 1.6c).

Effects of HOCl of Kallikrein and HK

HOCl, an antimicrobial oxidant produced by MPO, has long been implicated in the injury caused by inflammation, as it readily reacts with - and alters - proteins and their functions.[17] Thus, proteins in close proximity of MPO could be affected by MPO-produced HOCl. We first evaluated the effects of HOCl on the protease activity of plasma kallikrein, using a colorimetric activity assay. Kallikrein activity was inhibited 50% at an 80-fold molar excess of HOCl to kallikrein and completely inhibited at a 200-fold excess (Figure 1.7a). A 20-fold molar excess of L-methionine to HOCl quenched its oxidant activity,[17] as thiols and thioether groups of methionine are the most reactive substrates for HOCl oxidation.

Kininogens oxidized by chloramines (stable breakdown products of HOCl) cannot bind prekallikrein or be cleaved by kallikrein, an effect attributed to a modification of the critical Met-361 residue adjacent to the internal kinin sequence in kininogen to methionine sulfoxide.[131, 132] HOCl can mediate this same methionine-specific chemistry, thus we hypothesized HOCl would oxidize HK preventing bradykinin release by kallikrein.[50, 133] Addition of HOCl to HK inhibits bradykinin production in a dose-dependent manner (Figure 1.7b). The addition of PilB, a methionine sulfoxide reductase, reversed the decrease in bradykinin production and verified that a HOCl-oxidized methionine was responsible for this reduction.

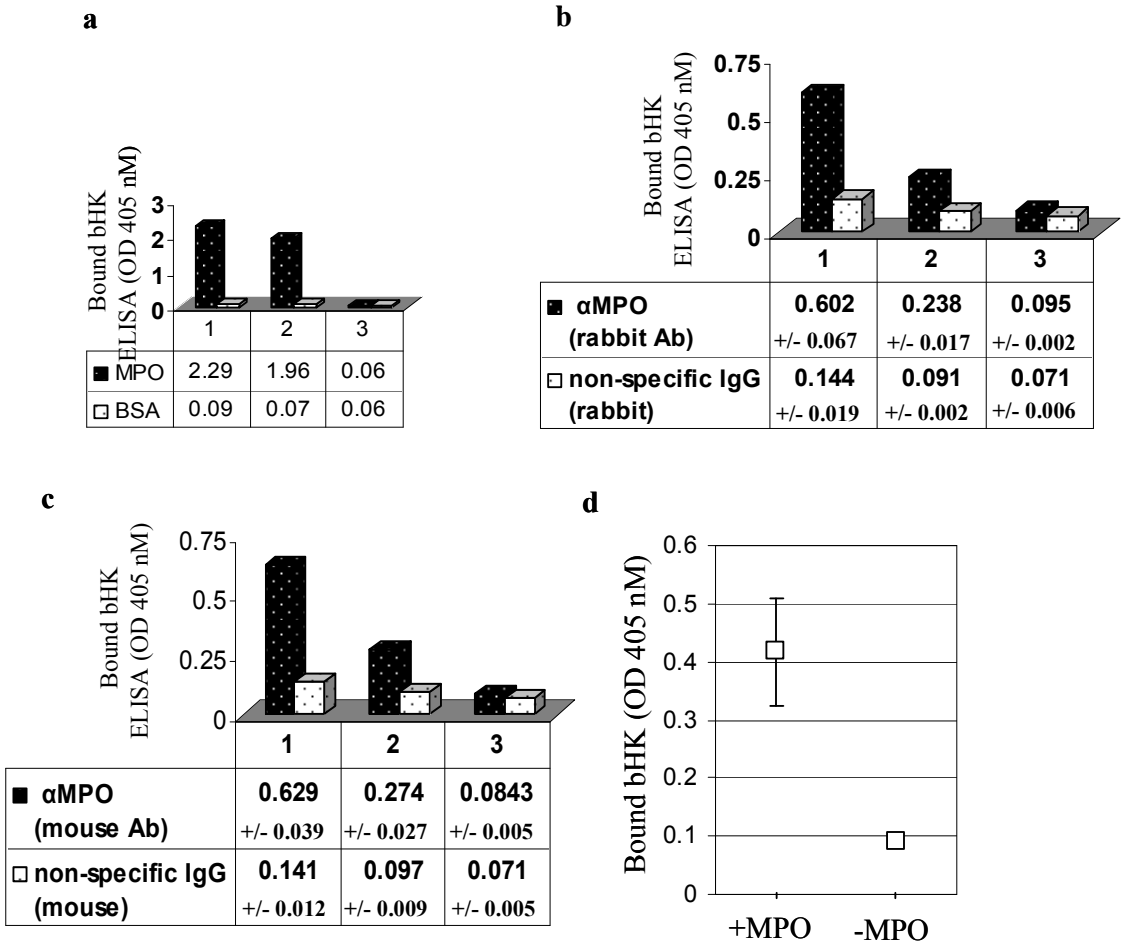

Figure 1.5. **MPO Binds Kininogen**. **a)** Direct ELISA: Column 1: Biotinylated kininogen (bHK) binds immobilized MPO but not BSA as detected by alkaline phosphatase-conjugated streptavidin; Column 2: bHK binding is reduced in the presence of excess unlabeled kininogen (uHK); Column 3: Unlabeled kininogen alone was negative. **b)** Sandwich ELISA: further validation of the direct interaction between MPO and kininogen. Column 1: monoclonal MPO antibody-captured MPO binds bHK as detected by alkaline phosphatase-conjugated streptavidin; Column 2: Competition of bHK binding in the presence of excess uHK; Column 3: Unlabeled kininogen alone was negative. Shown are the averaged results of three independent trials +/- standard deviations. **c)** Sandwich ELISA: validation using a second MPO antibody. Column 1: polyclonal MPO antibody-captured MPO binds bHK as detected by alkaline phosphatase-conjugated streptavidin; Column 2: Competition of bHK binding in the presence of excess uHK; Column 3: Unlabeled kininogen alone was negative. Shown are the results of three independent trials +/- standard deviations. **d)** MPO captures bHK from plasma. HK-depleted plasma was replenished with bHK to physiologic levels. Immobilized MPO was used as bait to capture bHK from plasma. Shown are the results (with standard error bars) of three independent experiments.

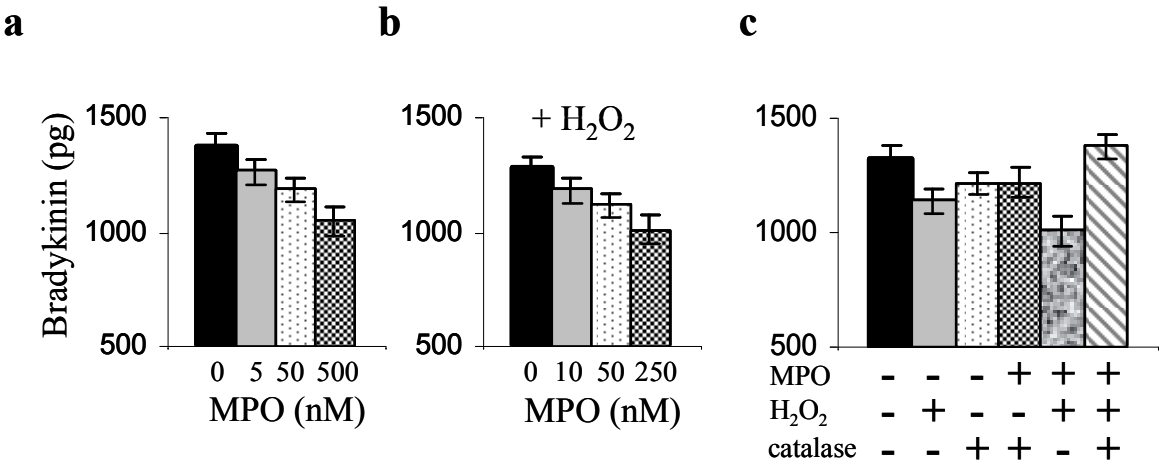

Figure 1.6. **MPO Interferes with the Plasma Kallikrein-Kinin System: Bradykinin Production is Diminished.** **a)** MPO interfered with bradykinin production through steric hindrance at high concentrations. Shown are the results of four independent experiments measuring the bradykinin liberated from 50 nM HK in the presence of MPO. **b)** Provided with its substrate (H2O2), MPO affects bradykinin production at lower concentrations. Shown are the results of three independent experiments. **c)** The enzymatic activity of MPO (conversion of H2O2 to HOCl) hinders bradykinin production, which is rescued in the presence of catalase. Shown are the results of three independent experiments.

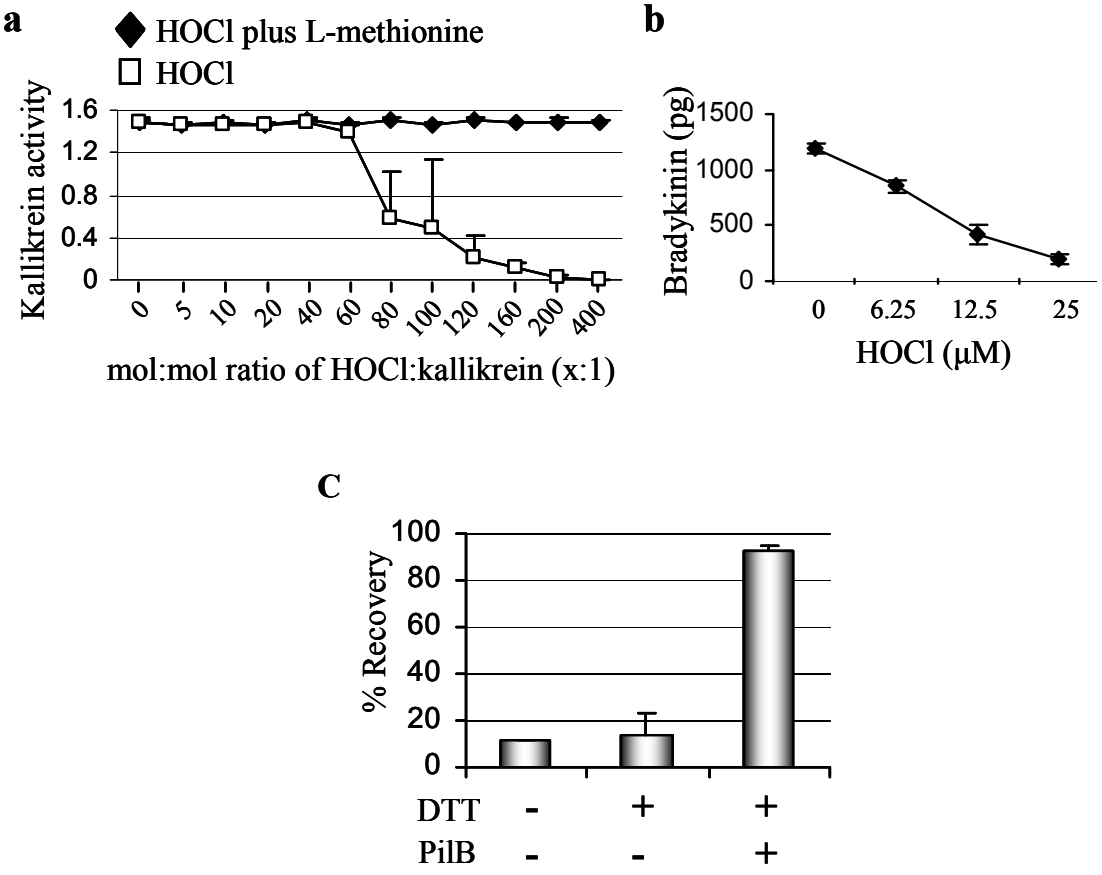

Figure 1.7. Hypochlorous Acid Inhibits Bradykinin Production. **a)** The proteolytic activity of kallikrein is abolished by HOCl dose-dependently, but is protected when HOCl is quenched with L-methionine (20-fold molar excess). Shown are the results (with standard deviations) of three separate experiments. **b)** HK is oxidized by HOCl resulting in a product uncleavable by active kallikrein. HK was pre-treated with increasing concentrations of HOCl. A 20-fold excess of L-methionine was added (20 min) prior to addition of active kallikrein. Bradykinin production was assessed by ELISA. Shown are the results from four separate experiments. **c)** HOCl inactivates kininogen by oxidizing a critical methionine residue. HOCl-oxidized HK was treated with a methionine sulfoxide reductase (PilB), and the inhibition of bradykinin production was reversed.

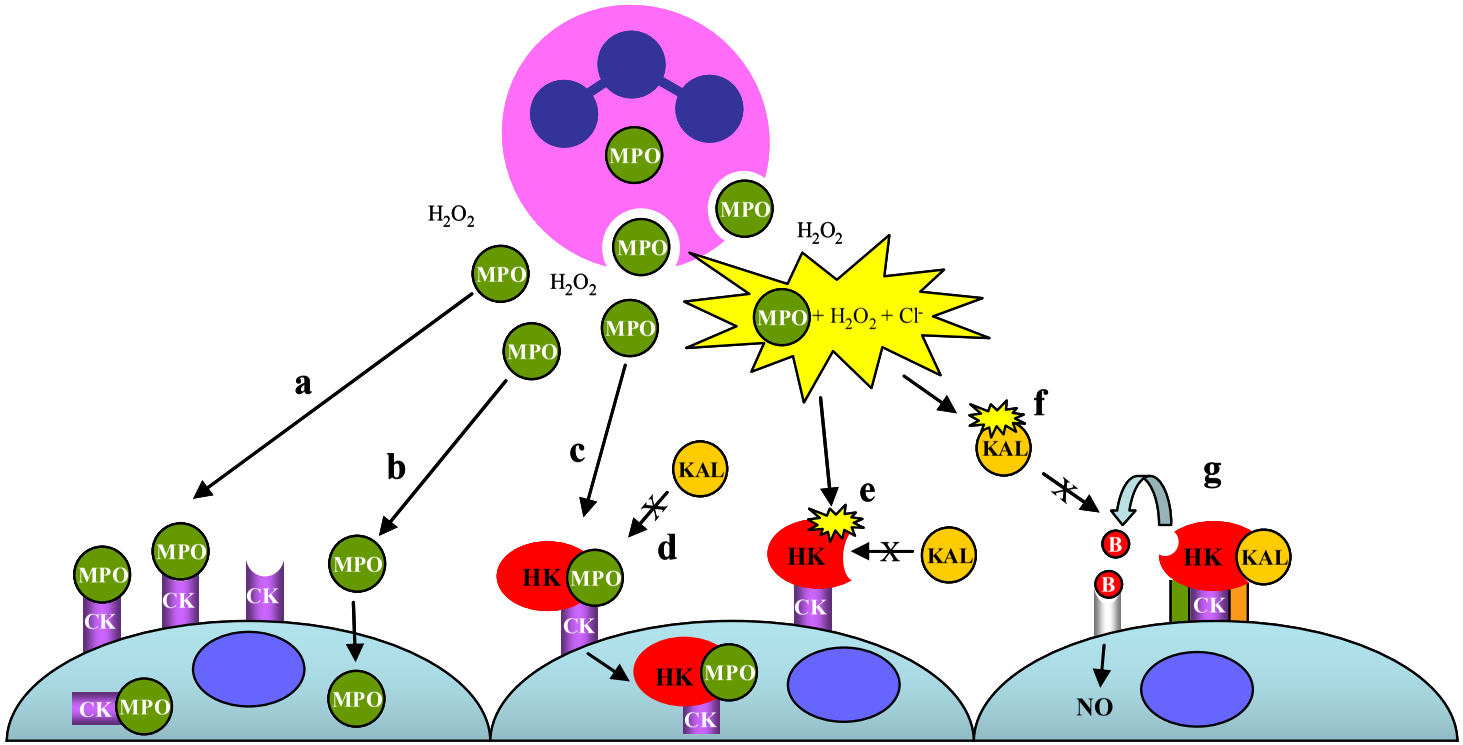

Figure 1.8. Proposed Schematic for the Interactions of MPO with Endothelial Cells and the Plasma Kallikrein-Kinin System. When a neutrophil releases its granule constituents and oxygen radicals at sites of inflammation, MPO can leak into the lumen of the vessel. **a**) Endothelial cells bind and internalize MPO, in part through interactions with the cell-surface protein CK1; MPO and CK1 enter the cells in complex. **b**) MPO can also enter cells through other mechanisms which have yet to be fully characterized. **c-f**) MPO can modulate the action of the plasma kallikrein-kininogen system. **c**) MPO associates directly with high molecular weight kininogen (HK) and increases the amount of HK bound to the cells; this complex appears to internalize. **d**) When MPO and kininogen are coupled, kallikrein is unable to cleave HK. MPO utilizes the hydrogen peroxide generated during a neutrophil's respiratory burst to oxidize chloride and produce hypochlorous acid (represented by the yellow star). **e**) Hypochlorous acid can oxidize and inactivate HK by altering kallikrein's cleavage site

DISCUSSION

MPO has long been viewed to function primarily as a bactericidal enzyme. A new paradigm presented here extends this perspective and suggests that MPO is profoundly involved in the regulation of cellular homeostasis, and may play a central role in the regulation of vasodilatation through direct interactions with components of the kallikrein-kininogen system (diagrammed in Figure 1.8). We propose that in the inflammatory microenvironment, MPO is internalized by endothelial cells through a direct interaction with the endothelial surface protein CK1 (Figure 1.8a), known as a scaffold for the plasma kallikrein-kinin system.[122, 125] CK1 appears to tether MPO into close proximity of HK and the two proteins are internalized in complex (Figure 1.8c). Moreover, MPO and HK form a complex such that blocks the HK site normally cleaved by kallikrein (Figure 1.8d). In addition, MPO's peroxidase activity causes oxidation and inactivation of HK protein (Figure 8e) and inactivates plasma kallikrein's activity (Figure 1.8f). The end result is a reduction in bradykinin liberation from HK (Figure 1.8g). Manipulations of bradykinin levels at sites of inflammation could have profound effects on vascular tone and other mediator functions of bradykinin.

Of central importance is the finding that CK1 facilitates MPO internalization by endothelial cells. This is in concordance with reports that MPO uptake is energy-dependent, a characteristic of receptor-mediated endocytosis.[29] Cytokeratins are typically thought of as structural intermediate filaments, though descriptions of the functions of CK1 in endothelial cells imply that it is more than merely structural. CK1 functions as part of the cells' environmental response pathways sensitized to oxidative stress; endothelial CK1 surface protein expression is upregulated in such conditions.[125, 134] While this is the first

report of a specific endothelial MPO-binding protein, multiple groups have investigated the entry of free MPO into endothelial cells. In our study, internalized MPO was not exclusively localized to CK1, suggesting other possible mechanisms for cell association and trafficking of MPO. Cell surface glycosaminoglycans have been implicated in MPO internalization.[29] Additionally, MPO binds to albumin through both sequence- and charge-specific mechanisms; the albumin-bound MPO is nonspecifically internalized by albumin-binding proteins.[36] However, our group and others have observed substantial MPO internalization in albumin-free conditions, supporting CK1-binding as a mode of entry.

MPO, because it is highly cationic, is often argued to interact with the endothelium solely through charge-based interactions. The mechanism through which CK1 and MPO interact is yet to be determined. In light of the reports that HK is not internalized when bound to CK1 through its cationic domain 5, we would argue that internalization of CK1-bound MPO is unique and specific.[135, 136] This would be fundamentally different from mechanisms mediating MPO's adherence to endothelial cell-surface glycosaminoglycans or its internalization coupled to albumin.

We questioned whether MPO and HK reside on CK1 together or whether they compete for binding. The data support cooperative binding on cells, which would tether MPO and HK into close proximity. For reasons yet to be determined, MPO caused a substantial increase in cell-bound HK. One speculation is that MPO is transporting HK to additional sites for binding; MPO can bind through both CK1-dependent and independent interactions. It is tempting to speculate that investigators will find additional situations where MPO and HK functionally overlap. For example, it was reported that MPO and HK

individually bind CD11b/CD18 integrin and interestingly, the outcomes were functionally opposing.[137-139]

The ultimate biologic mediator produced by the plasma kallikrein-kinin system is the vasoactive peptide bradykinin, which is cleaved from HK by kallikrein. Bradykinin binds at least two endothelial receptors to induce vasodilation and an anti-thrombotic state by stimulating endothelial nitric oxide production, prostacyclin synthesis, and tissue plasminogen activator release.[130, 140, 141] Although MPO increases HK bound to endothelial cells, we do not believe this augmentation correlates with increased bradykinin production based on our studies. It may be that MPO binding and internalization of HK serves to sequester HK from kallikrein and hinder HK's effector functions in the inflammatory milieu. Beyond the physical interference of MPO with the HK-kallikrein interaction, there was strong evidence that protein damage caused by the methionine-specific oxidizing agents produced by MPO could be responsible for decreased bradykinin production.[50, 133] There was a direct effect of biologically relevant concentrations of HOCl on kallikrein protease activity, and the oxidation of HK rendered it resistant to kallikrein proteolysis. HOCl concentrations below 15 μM are sublethal, whereas activation of the endothelial apoptosis machinery begins at a 30-50 μM dose and oncotic cell death is provoked above 100 μM.[51] The data show that even sublethal concentrations of HOCl overtly affect the ability of this system to produce bradykinin, and doses beyond 25 μM totally inhibited bradykinin production (not shown). HOCl is a highly reactive oxidant, so the proximity of MPO to HK resulting from their direct interaction and their shared interaction with CK1 could be very important when considering these experimental results.[17]

During acute and chronic inflammatory states, MPO is released into the vessel wall and is functional, based on published studies demonstrating chlorotyrosine in vessel walls.[44] Only MPO can mediate this chemistry under physiologic conditions. Given the large amount of MPO per neutrophil (5% of the dry weight), the large number of neutrophils infiltrating during acute inflammation, and the altered flow in vessels associated with procoagulant activity, we propose that concentrations of MPO in the inflammatory milieu can approach the 500mM range utilized in these studies.[3] Furthermore, in this microenvironment, the data indicate the potential for MPO-kininogen interactions to interfere with the kininogen-kallikrein process. The effects of MPO's enzymatic activity could be even more pronounced than we are reporting if nitration of tyrosine residues affects kininogen processing.[29, 142] MPO can mediate tyrosine nitration in inflammatory conditions through a reaction with nitrite, a stable end-product of nitric oxide metabolism. A hydrogen peroxide dependent reaction, nitrite is oxidized by MPO to form the nitrogen dioxide radical, a species capable of nitrating both free and protein-bound tyrosines.[25, 26] Such reactions have been associated with the inactivation of endothelial angiotensin-converting enzyme as well as the structural alteration of endothelial matrix-associated fibronectin.[25, 28, 29]

Previous studies showed that MPO remains active following internalization and catalytically consumes NO, thereby limiting the endothelial vasodilatory response.[24] In comparison, our work unveils a mechanism whereby MPO may *prevent* endothelial NO generation in the inflammatory milieu by limiting the production of bradykinin, the most potent known inducer of endothelial NO.

A limitation of these studies is the lack of an *in vivo* or *ex vivo* model to demonstrate that MPO can interfere with bradykinin production and thus impair vascular function.

Although observed in a cellular system, it is difficult to extrapolate the consequences of the MPO and CK1 interaction to the inflamed tissue environment. This question will be a focus of future studies. Another point that needs further exploration is the composition of the internalized CK1, MPO, and/or HK complexes.

In summary, CK1 is an endothelial receptor that aids in the internalization of MPO. Additionally, MPO directly interacts with HK, which also binds CK1 protein. The products of MPO's enzymatic activity can oxidize and inactivate both HK and plasma kallikrein. The collective interactions between MPO and the plasma kallikrein-kinin system result in a decrease in bradykinin production. Whether the molecular interactions discovered here are part of the normal processes of an inflammatory response or whether they are pathophysiological mediators of organ injury, or both, is yet to be determined. It may be that leakage of small amounts of MPO localized at the vessel wall play a role that is ultimately beneficial to the host. The converse may be true when MPO is released in excess quantities during inflammation. Regardless, the importance of MPO in vascular biology is further demonstrated by these studies.

CHAPTER 2

Explorations into Complementary Proteins as the Etiologic Agents of Myeloperoxidase-specific Antineutrophil Cytoplasmic Autoantibody-mediated Disease

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ABSTRACT

The Theory of Autoantigen Complementarity implicates proteins complementary in sequence and/or structure to autoantigens as a cause of autoimmune disease. In this chapter, we demonstrated that MPO-ANCA patients have antibodies specific to a recombinant complementary MPO protein. The presence of these antibodies is consistent with the development of MPO-specific antibodies as a result of an anti-idiotypic response against the anti-complementary antibody. We could not optimally test this hypothesis using human patient material. Thus, we used a mouse model of MPO-ANCA glomerulonephritis, attempting to induce disease through immunization with recombinant complementary proteins and synthetic peptides. Though these models were unsuccessful, they allowed us to rule out potential pathogenic epitopes. A mass spectrometry-based epitope mapping technique was employed to determine the murine MPO epitope recognized by disease-causing anti-murine MPO antibodies. This technique revealed a 24 amino acid peptide. However, this peptide was minimally immunogenic and anti-peptide antibodies did not cross-react with native murine MPO. While it appears that human patients were exposed to a complementary MPO protein some time along their disease course, proving causation in an animal model will be a challenge until pathogenic epitopes and interacting complementary proteins are identified.

INTRODUCTION

The origins of autoimmune disease remain elusive, though increasing evidence suggests that complementary proteins play a role. The Theory of Autoantigen Complementarity, as proposed by our group, begins with the exposure to a peptide or protein that is complementary in sequence and/or shape to a known autoantigen. This complementary antigen is recognized as foreign to the host and elicits a humoral response. The idiotypic network of antibody regulation is initiated, and an anti-antibody is generated whose binding site reflects the "internal image" of the complementary protein; therefore, this anti-antibody is an autoantibody that cross-reacts with the autoantigen leading to disease manifestations. In support, we and others have demonstrated an intact idiotypic network in patients with autoimmune disease, implying that the patients with PR3-ANCA, systemic lupus erythematosus, and Sjogren's syndrome may have been exposed to a complementary autoantigen prior to the onset of disease.[73, 110] Pendergraft *et al* have recapitulated the idiotypic network *in vivo* by showing that mice immunized with a complementary PR3 peptide developed an antibody reactive to PR3.

There is an old saying, "Which came first: the chicken or the egg?" This remains an outstanding question relative to studies implicating the idiotypic network in autoimmunity. With regards to PR3-ANCA, do patients harbor the anti-complementary PR3 antibody primarily as a result of the immune response generated against complementary PR3, or did this anti-complementary PR3 antibody develop secondarily as an anti-idiotype to the autoantibody. The latter suggests the presence of anti-complementary PR3 antibodies is merely an epiphenomenon. Importantly, animal models suggest the former is possible,

however there exist no studies in which disease is induced in an animal model by the immunization of a complementary autoantigen.

The work in Chapter 2 attempts to broaden the support for our Theory of Autoantigen Complementarity. We asked if there was evidence of an immunological encounter with a complementary protein in MPO-ANCA patients, as was seen in PR3-ANCA patients. We found that a subset of MPO-ANCA patients harbor antibodies specific for a recombinant complementary MPO polypeptide, substantiating the general applicability of this proposed mechanism. Are these anti-complementary MPO antibodies involved in the pathogenesis of the disease? The major goal of this study was to induce disease in an MPO-ANCA mouse model by producing pathogenic anti-MPO IgG through immunization of the mice with several complementary MPO antigens. Additionally, a new epitope mapping technique was employed to define a pathogenic MPO epitope using anti-MPO antibodies that cause disease in mice.

MATERIALS AND METHODS

Antibodies, Antigens, and Reagents

The His-probe (Rabbit anti-histidine fusion peptide) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), the rabbit anti-MPO antibody was from DAKO (Carpinteria, CA), the horseradish peroxidase goat anti-rabbit IgG antibody was from Chemicon (Temecula, CA), and the alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was from Pierce Biotechnology (Rockland, IL). Alkaline phosphatase-conjugated goat anti-human IgG and donkey anti-mouse IgG were purchased from Jackson

Immunoresearch Laboratories (West Grove, PA). The alkaline phosphatase substrate kits were purchased from Bio-Rad Laboratories (Hercules, CA) or Pierce Biotechnology, and the Western Pico Chemiluminescent Peroxidase Substrate Kit was from Pierce Biotechnology.

Native murine MPO was prepared from WEHI cells as previously described, and generously provided by Hong Xiao.[69] Bovine Serum Albumin was purchased from New England Biolabs (Beverly, MA). Fish gelatin was from Sigma (St. Louis, MO).

Murine MPO and complementary MPO peptides were ordered from Alpha Diagnostic International (San Antonio, TX).

Cell Culture

HEK 293 cells were grown in DMEM-F12 supplemented with 2 mM L-Glutamine, 5% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), penicillin/streptomycin, and Genetecin for selection when needed. All reagents were from Gibco (Invitrogen, Carlsbad, CA)

Human Recombinant cMPO proteins: Site-directed Mutagenesis, Subcloning, and Recombinant Protein Production

Two complementary MPO (cMPO) proteins were created for these studies. Upon analyzing the antisense mRNA sequence of MPO in a 5'→3' direction, we found that the antisense codon corresponding to serine 485 was a STOP signal. Therefore, we utilized Strategene's QuikChange II XL Site-Directed Mutagenesis Kit (La Jolla, CA) to mutate the last base of the serine-485 codon from "a" to "c" in order to change the complementary amino acid from a termination signal to an glycine. The instructions provided with the kit were followed to produce the MPO cDNA mutant. Human MPO cDNA was used as the

starting material for site-directed mutagenesis. The forward primer sequence used to generate mutated MPO cDNA was 5'TCCTACAATGACTCCGTGGACCCACGCATC-3' and the reverse primer sequence was 5'GATGCGTGGGTCCACGGAGTCATTGTAGGA-3'.

To create plasmids containing the antisense MPO sequence as the sense strand of the plasmid, we created PCR primers containing endonuclease restriction sights that would allow us to amplify specific antisense MPO fragments from the mutated MPO cDNA and insert them into a mammalian expression vector. The forward primers contained a HindIII site, and the reverse primers contained an XhoI site as well as a STOP codon. One set of primers amplified a complementary MPO polypeptide from the mutated cDNA corresponding to sense amino acids 420-697 (cMPO(420-697)): 5'CCCAAGCTTGATCTGGGCCAGGGCCTGTC-3' (forward primer sequence) and 5'--CCGCTCGAGCTACTTCGGGAGCACAACCGGCT-3'(reverse primer sequence). Another set of primers amplified a sequence corresponding to amino acids 279-392 (cMPO(279-392)): 5' Cccaagcttgcggttggtgaggagacaggg 3' (forward primer sequence) and 5' CCGCTCGAGCTAGTCAACTGCGAGACCAGCTGC 3' (reverse primer sequence). The translated cMPO(279-392) sequence did not contain any STOP signals and did not require prior site-directed mutagenesis. The PCR products were gel purified using Qiagen's Gel Purification Kit (Valencia, CA), and the products and parent plasmid were digested with HindIII and XhoI (New England Biolabs, Beverly, MA) to create compatible ends. The digested PCR products were gel purified and ligated into the digested parent plasmid, a pcDNA3 eukaryotic expression vector (Invitrogen) previously engineered to express a BM40 secretion signal peptide and a hexahistidine tag, both C-terminal to the complementary protein.

The cMPO proteins were produced by electroporating HEK 293 cells with the appropriate vectors using a Bio-Rad Gene Pulser. The cells were placed in Genetecin-containing media to select for cells containing the cMPO plasmids. The surviving cells were passaged and expanded into Corning CellSTACK "factories" (Acton, MA) for mass production of recombinant protein. When the cells reached confluence, the medium was replaced with serum-free medium for one week. Expression of the BM40 signal peptide induced the cells to secrete the protein into the serum-free medium. The proteins were precipitated from 4 liters of collected cell-free medium with ammonium sulfate (313 g/L) at 4°C overnight, resuspended, and dialyzed into PBS containing 20mM immidizole. The cMPO proteins were partially purified using HisTrap HP columns (GE Healthcare) and analyzed for purity by coomassie-stained SDS-PAGE, and for specificity by immunoblot specific for the hexahistidine tag.

Murine Recombinant cMPO Proteins: Site-directed Mutagenesis, Subcloning, and Recombinant Protein Production

The murine recombinant polypeptides m-cMPO(460-718) and m-cMPO(253-449) were engineered in parallel with the human cMPO recombinant proteins and prepared in the exact same manner save for a few changes. Two mutated m-MPO full length cDNAs were prepared, each expressing one mutation; Strategene's QuikChange II XL Site-Directed Mutagenesis Kit (La Jolla, CA) was utilized to introduce an "a" to "c" mutation of the third base of the leucine 646 codon in one preparation, and the same mutation was introduced to leucine 323 in another; these mutations changed the complimentary STOP signals to glutamic acids. The primers used for the site-directed mutagenesis were: 5'CTCAGTTTAGGAAGCTCCGTGATGGTGATCGGTT-3' (L646 forward),

5'AACCGATCACCATCACGGAGCTTCCTAAACTGAG-3' (L646 reverse), 5'CTCAGTTTAGGAAGCTCCGTGATGGTGATCGGTT-3' (L323 forward), and 5'AACCGATCACCATCACGGAGCTTCCTAAACTGAG-3' (L323 reverse). The instructions provided with the kit were followed to generate mutated MPO cDNAs, and the UNC Genome Analysis Facility verified the sequence of each. To PCR amplify the cDNA fragments encoding our m-cMPO proteins from the mutated MPO cDNA, one set of primers amplified a complementary murine MPO polypeptide corresponding to sense amino acids 460-718 (m-cMPO (460-718)), and the other set corresponded to sense amino acids 253-449 (m-cMPO(253-449)). The primer sequences were: 5'CCCAAGCTTGGTCTCCTTCCAGGAAGTCA-3' (m-cMPO(460-718) forward), 5'CCGCTCGAGCTAGTAGACCCTCGAATCGCCAA-3' (m-cMPO (460-718) reverse), 5'-CCCAAGCTTGGTCTCCTTCCAGGAAGTCA-3' (m-cMPO(253-449)forward), and 5'CCGCTCGAGCTAGTAGACCCTCGAATCGCCAA-3' (m-cMPO(253-449) reverse). All of the other methods involving production of the m-cMPO proteins were identical to those for human cMPO.

ELISAs to Determine Whether Patients Harbored Antibodies Reactive with cMPO(420-697)

An initial screen was conducted to compare MPO-ANCA patient reactivity (n=25) against cMPO(420-697) to a small set of healthy control sera (n=5). Afterwards, another set of patients (n=19) was screened and compared to a different, and larger, set of healthy controls (n=26) to increase the statistical power of the experiment. One patient sample was repeated in both experiments.

Prior to performing the ELISAs, it was determined that the cMPO(420-697) could be diluted by half prior to coating the ELISA wells with only modest loss of reactivity using the His-probe (not shown). ELISA wells were coated overnight with rocking at 4°C with cMPO(420-697), MPO at 5 μg/ml, or 0.5% Fish gelatin solution as a sham; all dilutions were in ELISA coat buffer. Between all subsequent steps, the wells were washed at least 3 times with a PBS-Tween solution. The wells were blocked with 1% fish gelatin buffer for 2 hours at room temperature. Healthy control and ANCA patient sera were spun at 10,000 RPM for 10 minutes at 4°C to remove aggregates and precipitate prior to use. The sera were diluted 1:100 in the fish gelatin buffer and incubated in the wells for 2 hours at room temperature. The secondary antibody was added to the wells for 1 hour at room temp followed by the addition of substrate for 1 hour. The wells were read in a microplate reader at OD 405 nm.

Patients were considered positive for reactivity to cMPO(420-697) if their OD 405 was higher than 2 standard deviations above the mean OD 405 of the normals.

Murine Recombinant Protein Immunizations and Serum Collection

Approximately 100 μl of blood was collected from MPO -/- mice on a C57BL/6J background by tail nicks prior to immunizations. The blood was allowed to clot for at least one half hour at room temperature and then spun in a microcentrifuge at 10,000 RPM for 10 minutes to separate the serum from the clot. The serum was collected and stored at -20°C for later use.

Partially purified recombinant m-cMPO was dialyzed in PBS, and mixed into an emulsion in a 1:1 ratio with TiterMax (Norcross, GA). Each mouse was immunized intraperitoneally with 200 μl of the emulsion; each mouse received 100 μl of the partially

purified m-cMPO that was at an approximate concentration of 19 μg/ml. The negative control mice received 10 μg of BSA and the positive control mice received 10 μg of native murine MPO, both in the same volume as the m-cMPO injections. The same immunogens were prepared in TiterMax for boost immunizations at weeks 2 and 6. The boost immunizations were injected intramuscularly at the tail base, 100 μl each on both sides of the tail. The appropriate mice received 100 μl of the partially purified m-cMPO, and 10 μg of MPO and BSA were delivered to control mice. The mice were bled weekly, and the blood was processed to obtain serum as described above. Each set of mice had a mix of males and females.

ELISA to Analyze for the Induction of the Idiotypic Network

Prior to performing ELISAs to analyze serum reactivity to m-cMPO, a direct ELISA determined that m-cMPO could be diluted 25-fold and still be recognized by the His-probe antibody, with only 15% less reactivity compared to a neat m-cMPO solution (data not shown).

ELISA wells were coated with either 50 μl each of m-cMPO diluted 1:25, 5 μg/ml native murine MPO, 5 μg/ml BSA, or coat buffer only at 4°C with rocking overnight; all dilutions were in ELISA coat buffer. The wells were blocked with 150 μl of a 1% fish gelatin solution for 1 hour at room temperature. The mouse serum was centrifuged at 5,000 RPM for 10 minutes at 4°C prior to usage to remove any immunoglobulin aggregates or precipitate. The serum was diluted 1:100 in the fish gelatin solution and 50 μl was added to the wells for 2 hours with rocking at room temperature. Fifty μl of 1:2500 diluted alkaline phosphatase-conjugated secondary antibody was added to the wells for 1 hour at room

temperature, followed by substrate. The wells were washed at least 3 times between each step. Following substrate exposure for 1 hour, the ELISA plates were read at 405 nM.

Sense and Complementary Peptide Immunizations and Serum Collection

Approximately 100 μl of blood was collected from MPO -/- mice on a C57BL/6J background by tail nicks prior to immunizations. The blood was allowed to clot for at least one half hour at room temperature and then spun in a microcentrifuge at 10,000 RPM for 10 minutes to separate the serum from the clot. The serum was collected and stored at -20°C for later use.

The initial immunizations were delivered in Freund's complete adjuvant (Difco Laboratories, Detroit, MI), and each set of mice received 100 μg of total protein. One set of mice (n=5) received 50 μg of each antisense peptide, m-cMPO(484-508) and m-cMPO(694-718). One set of mice (n=5) received 50 μg of each corresponding sense peptide, m-MPO(484-508) and m-MPO(694-718). Another set of mice received both pairs of sense and antisense peptides. M-MPO(484-508) and m-cMPO(484-508) were mixed in PBS at room temp for 1 hour to form a sense-antisense peptide pair, as were peptides m-MPO(694-718) and m-cMPO(694-718). The two pairs of peptides were then mixed together and emulsified in Freund's adjuvant for delivery. The negative control mice included a set (n=4) that received 100 μg BSA set, and 2 positive control mice were immunized with 10 μg native m-MPO. All mice were immunized intraperitoneally, and all sets had a mix of males and females.

The boost immunizations were carried out in Freund's incomplete adjvant, also delivered intraperitoneally, at weeks 4 and 8. These boosts used exactly half the amount of

proteins as the initial immunizations with the exception of the 10 μg of native m-MPO used in the positive controls. Serum was collected and stored weekly as described above.

Splenocyte Transfer, Urine, Serum, and Tissue Collections

Splenocytes were isolated from all immunized sets of MPO -/- mice by disrupting the pooled spleens into cold RPMI 1640 medium and then washing twice with the medium. Red blood cells were removed with lysis buffer (Sigma, St. Louis, MO) followed by washing with cold RPMI 1640, and the splenocytes were resuspended in sterile PBS for injection. A suspension of 1 X 10^8 splenocytes in 500 μl of PBS was delivered intravenously to each RAG-2 -/- mouse. Males and female mice were mixed in each set (n=4), and each set reflected the initial immunization scheme (i.e. one set of RAG-2 -/- mice received splenocytes from the m-cMPO peptide immunized MPO -/- mice, etc.). There were only 2 positive control RAG-2 -/- mice that received splenocytes from the native m-MPO-immunized MPO -/- mice.

The RAG-2 -/- mice were bled as described above (including a bleed prior to the splenocyte transfer) on Days 7, 10, and 17. Mice were immobilized on Day 16 and directly urinated on a dipstick to test for hematuria. On Day 12, the positive control mice appeared physically ill and were anesthetized with Avertin for euthanasia. All other RAG-2 -/- mice were euthanized on Day 18. The mice were terminally bled and samples of their kidney, lung, spleen, and liver were fixed in 10% formalin and processed for light microscopy. All specimens were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) stains. Pathologic evaluation was carried out without knowledge of the experimental conditions.

ELISA to Analyze the Induction of the Idiotypic Network in Peptide-immunized MPO Knockout Mice

ELISA plates used for the MPO -/- mice were coated overnight at 4°C with either 10 μg/ml sense MPO peptides (5 μg/ml of each sense MPO peptide mixed), 10 μg/ml complementary MPO peptides (5 μg/ml of each complementary MPO peptide mixed), 5 μg/ml native murine MPO, or 1% goat serum as a sham. ELISA wells used to analyze the RAG-2-/- mice used a total peptide concentration of 5 μg/ml and 5 μg/ml fish gelatin as a sham. The wells were rinsed after coating (and after every subsequent step) with at least 3 washes of a PBC-Tween solution. For the MPO -/- mice the wells were blocked with 1% goat serum, and the wells for the RAG-2-/- were blocked with 1% fish gelatin (the blocking solutions for each respective experiment also served as the antibody diluent). The wells were blocked at least 2 hours at room temperature. The serum from each mouse was centrifuged at 10,000 RPM for 10 minutes at 4°C. Serum was diluted 1:100 in the appropriate diluent and applied to the wells at room temperature for 2 hours. Secondary antibody was applied for 1 hour at room temperature followed by the addition of substrate for 1 hour.

Epitope Mapping Pathogenic Murine Anti-MPO Antibodies

A collaboration to epitope map the pathogenic murine MPO-ANCA by a combination of epitope excision and Matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS) was established with the UNC-Duke Michael Hooker Proteomics Center, as described in Parker *et al.*[143] Briefly, total IgG containing pathogenic ANCA was prepared by pooling sera from terminal bleeds of 3 different MPO -/- mice that had been immunized with native murine MPO. The pooled sera were filtered through a 0.22μm filter and passed over a HiTrap Protein G Column (GE Helthcare) to purify total IgG. The total

IgG was immobilized on CNBr-activated Sepharose beads. Affinity-purified murine MPO was linked to the immobilized antibodies and submitted for analysis. The murine MPO was affinity purified using the DAKO rabbit anti-human MPO, which recognized mouse MPO.

A series of proteolytic steps were used to cleave fragments from the immobilized antibody-bound MPO; the epitopes attached to the antibody are protected from proteolysis and stay attached to the antibody. The beads are then washed and the MALDI matrix solution desorbs the bound fragment from the immobilized antibody. This fragment(s) is subjected to MALDI analysis to determine which peptides are left bound to the antibody following each digestion step. This can be accomplished because the immobilized antibodies themselves are fairly resistant to proteolysis, perhaps due to its high glycosylation status.[143] A list of predicted enzyme cleavage sites can be generated for each peptidase used, and one can compare the resulting MALDI mass spectrum obtained from the analysis to the predicted cleavage fragments to identify the antibody-bound fragment. The initial enzyme used was endoproteinase Lys-C, an infrequent cutter that generated "reasonably" sized fragments to identify. Subsequently, trypsin-TPCK is used to further hone the bound eptitope. Finally, the beads are subjected to carboxypeptidase Y digestion for fine-scale mapping of the C terminus, and aminopeptidase M for the N terminus. The results are confirmed by MS/MS, comparing spectra from the antibodies alone to the antibodies with digested MPO bound.

m-MPO(302-325) Peptide Immunizations and Serum Collection

MPO null mice were pre-bled as described above. One set of mice (n=3) was immunized with 50 μg of the epitope mapped peptide, m-MPO(302-325), prepared in Complete Freund's Adjuvant. Two mice were immunized with adjuvant alone as a sham

control, and one mouse was immunized 10 μg native murine MPO as a positive control. Mice were bled weekly by tail nicks as described above.

The m-MPO(302-325) mice were boosted at week 4 with 75 μg peptide prepared in Incomplete Freund's Adjuvant. The shams were immunized with adjuvant alone and the positive control was boosted with 5 μg murine MPO.

ELISAs to Analyze Anti-m-MPO(302-325) Antibody Cross-reactivity with Native MPO

ELISA plates were coated with 5 μg/ml murine MPO, 10 μg/ml m-MPO(302-325) peptide, or 5 μg/ml fish gelatin overnight at 4°C overnight. Each well was blocked for 2 hours at room temperature with 1% fish gelatin buffer. Serum samples were prepared by centrifuging at 12,000 RPM for 10 minutes and then diluted 1:100 in the fish gelatin buffer. The wells were exposed to the sera for 2 hours at room temperature, followed by the alkaline phosphatase-conjugated donkey anti-mouse secondary at a 1:3500 dilution for 1 hour. Substrate was applied and the plate incubated at 4°C overnight and read at 405 nM the following morning.

Prior to conducting a competition ELISA, the dilution at which the MPO-immunized mouse serum and pooled total IgG (of 3 MPO null mice immunized with MPO) had 50% reactivity against native murine MPO was determined. The pooled total IgG was the same used to epitope map the m-MPO(302-325) peptide. ELISA plates were coated for 2 hours at 37°C, and then blocked with 1% fish gelatin for 1 hour at room temperature. A 5-fold dilution series starting at a 1:25 dilution, and a 1:2 dilution series starting at 1:50, was prepared using the serum and total IgG in the fish gelatin buffer. The serum sample had 50% reactivity at 1:3000 dilution and the total IgG at 1:8500. For the competition

ELISA, the wells were coated 5 μg/ml murine MPO or fish gelatin. The wells were blocked with fish gelatin for 2 hours at room temperature. The antibody solutions were prepared at the dilutions mentioned above in the presence of 6.25 to 100 μg/ml m-MPO(302-325) peptide for 1 hour 45 minutes at room temperature before being applied to the wells for 1 hour. A nonspecific, competition control peptide was used that corresponded to complementary proteinase 3. The secondary antibody was applied for 1 hour at room temperature, followed by substrate and readings at 405 nM.

RESULTS

Rationale for the Design of Human Recombinant MPO

There was no defined pathogenic epitope for human MPO-ANCA for which to design a correlating complementary protein. However, epitope mapping studies by our lab and others revealed an immunodominant region consisting of 230 amino acids in the C-terminus of the heavy chain, so we focused our efforts on this region of human MPO.[144, 145] We decided to engineer a large, 278 amino acid cMPO polypeptide corresponding to sense amino acids 420-697, roughly covering the entire C-terminal half of the heavy chain (Figure 2.1). Unfortunately, translation of the antisense strand of MPO cDNA (read 5'→ 3') revealed multiple STOP codons, including one at sense amino acid serine-698. We decided to utilize site-directed mutagenesis to mutate the wobble base of serine-698 to change the antisense codon from a STOP to a glycine, allowing us to produce the cMPO(420-697) polypeptide in full. While cMPO(420-697) may not be translated as such in vivo, it allowed us to incorporate the entire C-terminal half of the heavy chain, thus providing as many

conformational complementary epitopes as possible in one protein. The epitope mapping study by Fujii et al both confirmed that the C-terminal of MPO was immunodominant, and revealed an ANCA-reactive region in the N-terminus of the heavy chain; an additional complementary protein was constructed corresponding to this region, amino acids 279-392. Together, the two cMPO polypeptides we produced span the majority of the MPO heavy chain sequence as diagramed in Figure 2.1.

A Subset of MPO-ANCA Patients have Antibodies Specific for cMPO(420-697)

An initial ELISA screen was conducted following the first round of cMPO(420-697) purifications. Because a limited amount of protein was purified, we screened more patients than healthy controls to gain insight as to whether this protein would be recognized in the patient population. We found 7 of 25 patients tested in this initial screen were reactive to the cMPO(420-697) protein (Figure 2.2). Though tantalizing, the results were statistically insignificant due to the low numbers of normals (5) tested (p value = 0.55 using Fisher's Exact Test).

A second screen was conducted analyzing more healthy controls (n=25) than patients (n=19). In this screen, 6 of 19 patients were positive and these results were statistically significant (p value = 0.03 using Fisher's Exact Test) (Figure 2.3). Two positive samples in this ELISA were from patients that tested positive in the initial screen; one sample was an exact repeat, and the other was from the same patient drawn on a different date. Interestingly, the cMPO(420-697) antibody seems to have persisted in this patient over several months.

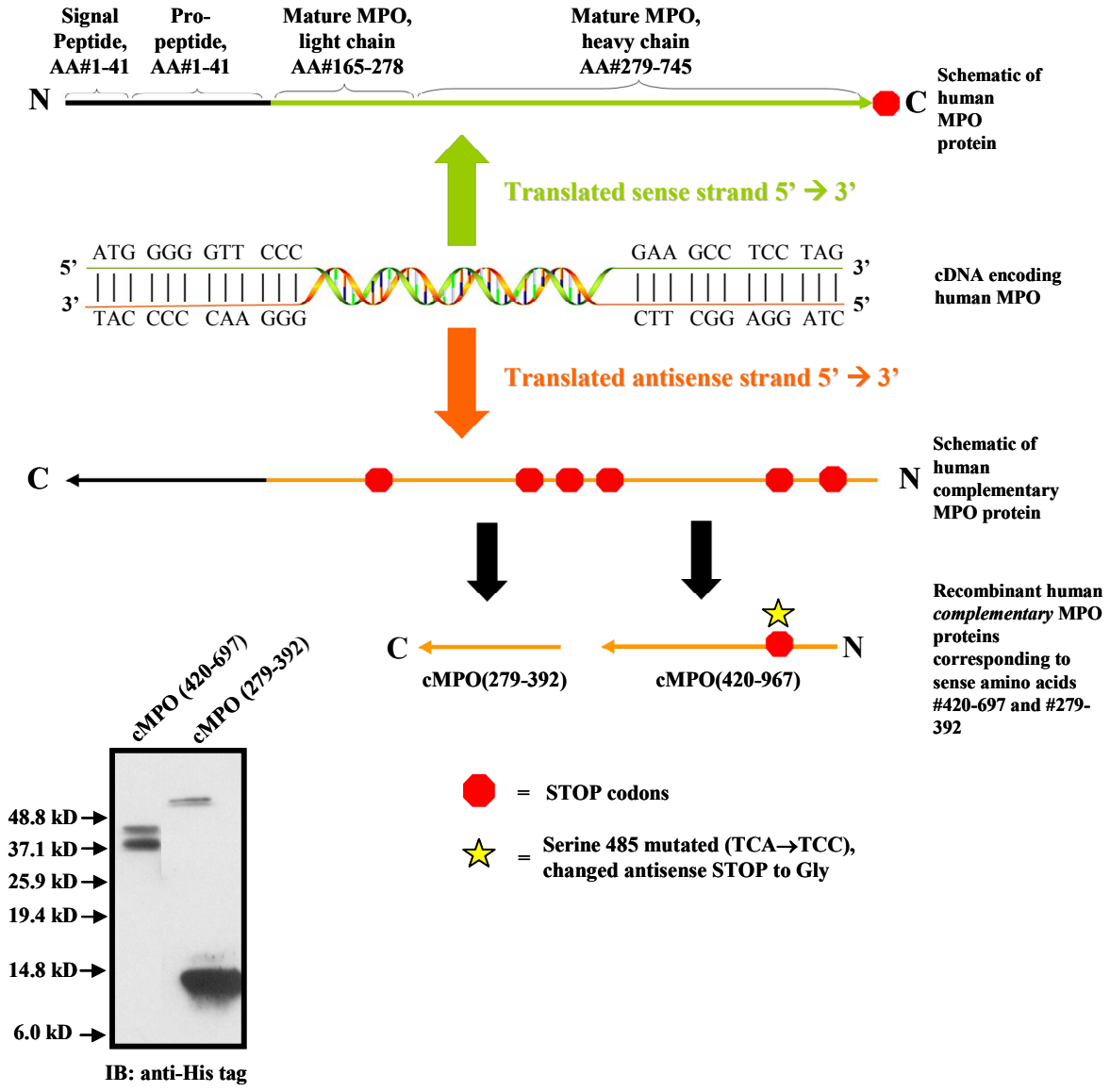


Figure 2.1. Design and Production of Recombinant Complementary Myeloperoxidase Polypeptides. We produced complementary protein fragments encoded by the *antisense* sequence (read 5' → 3', in frame) of the MPO gene, corresponding to sense amino acids 420-697 [cMPO(420-697)] and 279-392 [cMPO(279-392)]. Several STOP codons were introduced when translating the antisense sequence; site-directed mutagenesis was employed to mutate the 3rd base in the serine 485 codon (TCA→TCC) to change the antisense STOP to a glycine residue. This cDNA was inserted into a vector and transfected into a HEK 293 cell secretion system. The expressed recombinant protein fragments contain an amino-terminal histidine tag for purification and immunodetection. Inset is an immunoblot of the purified recombinant proteins, using an anti-histidine tag antibody.

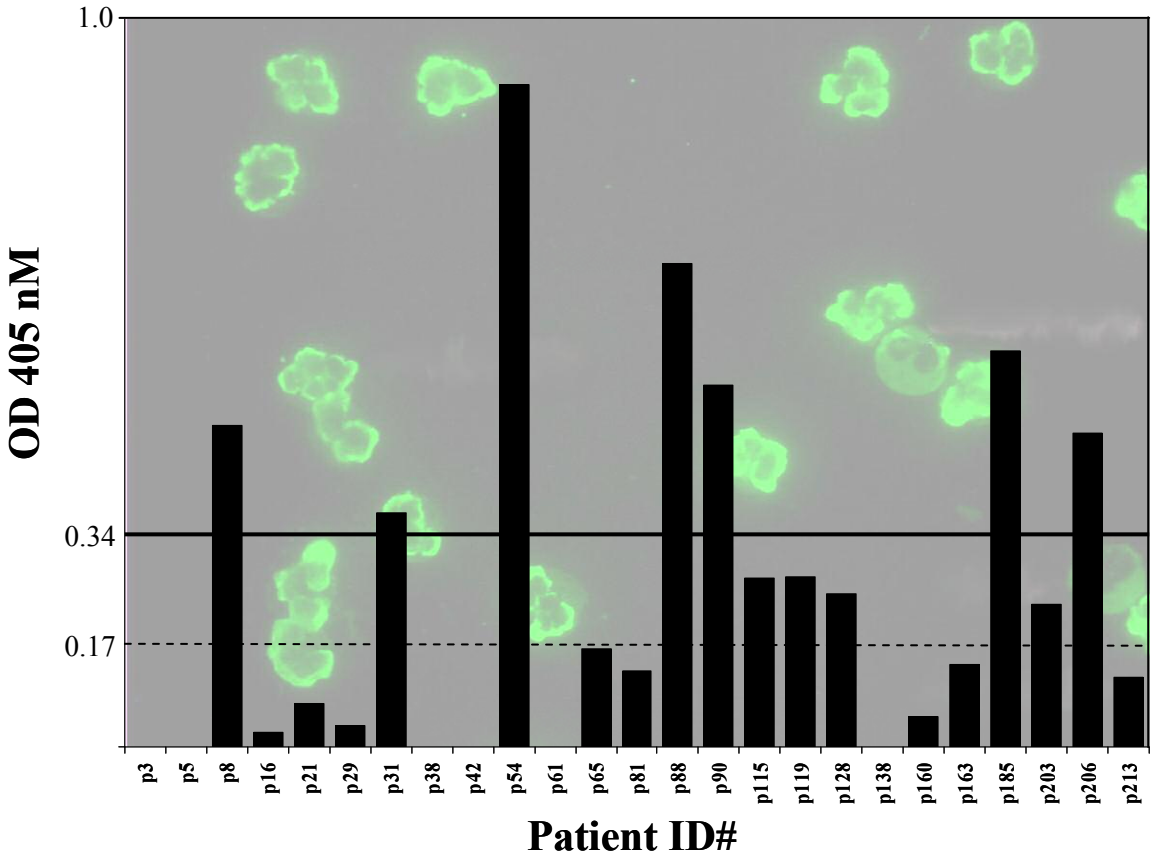


Figure 2.2. An Initial Screen Reveals that MPO-ANCA Patients may have Antibodies Reactive with cMPO(420-697). MPO-ANCA patient (n=25) and healthy control (n=5) sera were screened for reactivity against cMPO(420-697). The mean OD value of the healthy controls was 0.17 (dashed line). Patients are considered positive if their sera results in an OD405 greater than 0.34, two standard deviations of the mean of the healthy controls (solid line). A p-value of 0.55 was acquired using Fisher's Exact Test, meaning that the results of this ELISA were insignificant due to a low number of control samples.

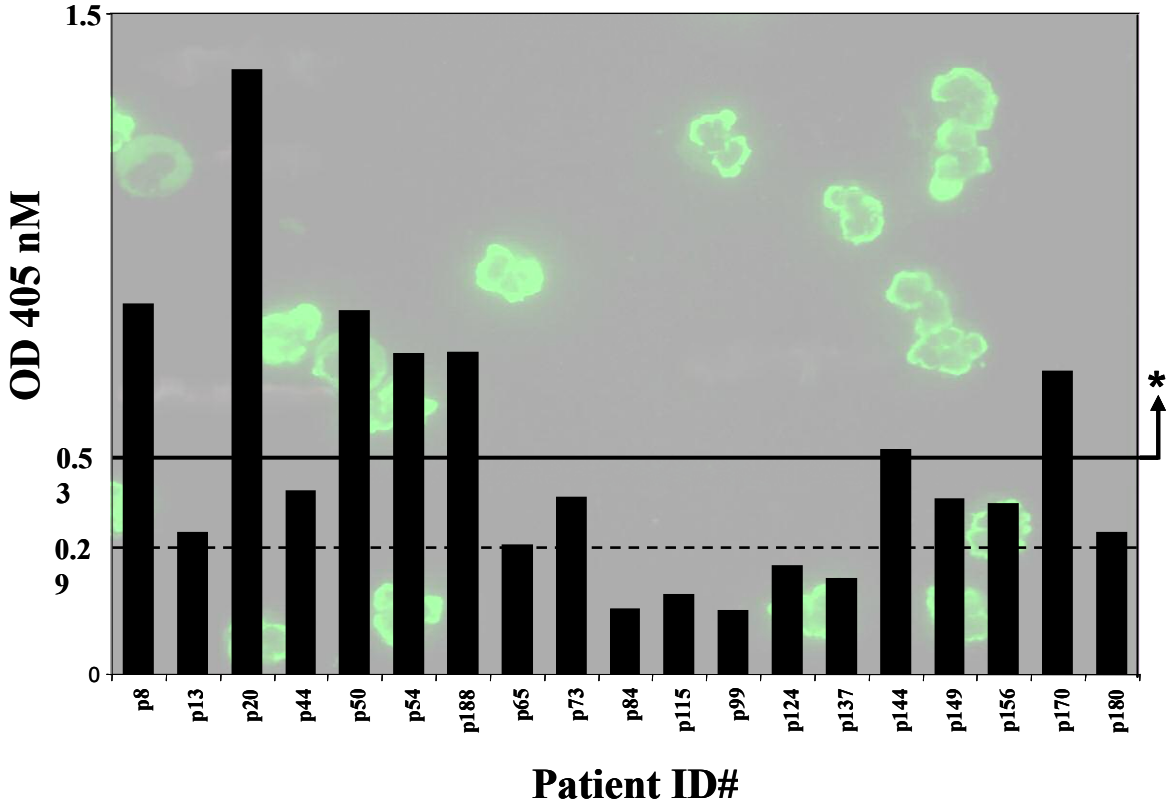


Figure 2.3. MPO-ANCA Patients have Antibodies Reactive with cMPO(420-697). MPO-ANCA patient (n=19) and healthy control (n=26) sera were screened for reactivity against cMPO(420-697). The mean OD value of the healthy controls was 0.29 (dashed line). Patients are considered positive if their sera results in an OD405 greater than 0.53, two standard deviations of the mean of the healthy controls (solid line). A p-value of 0.03 was acquired using Fisher's Exact Test, meaning that the patient values over 0.52 are statistically significant, and indicated by the star.

Rationale for the Design of Recombinant Murine MPO

The murine anti-MPO antibodies that caused disease in the MPO-ANCA glomerulonephritis mouse model were not epitope mapped.[69] As there was not a defined pathogenic epitope for murine MPO, we correlated information from human studies to design our potentially pathogenic m-cMPO. Our two recombinant m-cMPO proteins were designed similar to the human sequences, together spanning the majority of the m-MPO heavy chain; this is based on the assumption that, as in humans, the murine MPO heavy chain harbors the major antigenic region(s).

Like the human sequence, analysis of the translated protein sequence from the antisense m-MPO RNA revealed several stop codons (Figure 2.4). Where leucine 323 and 646 are coded in the sense strand by a "cta" codon, translation of the corresponding 5'→3' antisense sequence ("tag") codes for a STOP codon. We decided to mutate the "wobble" bases of the leucine 323 and leucine 646 codons in order to change the corresponding antisense stop codons to glutamic acids. While m-cMPO(460-718) and m-cMPO(253-449) may not be translated as such *in vivo,* it allowed us to incorporate the entire c- and n-termini of the heavy chain; these regions were previously shown to have ANCA reactivity in human patient samples.

Production and Purification of m-cMPO(460-718)

The recombinant protein was partially purified using nickel chromatography and analyzed for purity by coomassie-stained SDS-PAGE. Three column elution fractions contained the majority of the m-cMPO(460-697) protein, as visualized at approximately 30kD(not shown). However, these fractions also had a considerable amount of high molecular weight contaminating proteins that did not react with the His-probe antibody by

western. We determined the concentration of m-cMPO in the preperation to be approximately 19 μg/ml by comparison to known amounts of a control protein. The 3 fractions containing the cMPO were combined and utilized for immunizations.

Mice Immunized with m-cMPO(460-718) did not Produce Anti-MPO Antibodies

The initial goal of this study was to induce the idiotypic network in MPO-/- mice, so that mice immunized with m-cMPO(460-718) would develop anti-cMPO antibodies as well as an anti-anti-cMPO antibodies. The anti-anti-cMPO antibodies would be anti-idiotypic antibodies that could recognize native mouse MPO. The ultimate goal of the study was to induce pauci-immune glomerulonephritis in MPO +/+ mice by transferring splenocytes from the m-cMPO-immunized mice that developed anti-MPO antibodies, thus demonstrating for the first time that complementary proteins can induce disease. Immunized MPO-/- mice sera were analyzed for the presence of anti-MPO antibodies over a time course spanning 10 weeks (Figure 2.5). Mice were tested for their humoral responsivity to their respective immunogens as well; all mice except one BSA control mouse was highly reactive to their immunogen. At no time over the 10-weeks did the m-cMPO-immunized mice generate an anti-MPO response above background or when compared to the BSA controls (Figure 2.5c). Because of the lack of anti-MPO antibodies, we did not harvest and transfer splenocytes using this model. Though these results were similar when using synthetic peptides, we did splenocyte transfers using the peptide-immunized mouse splenocytes, as described below.

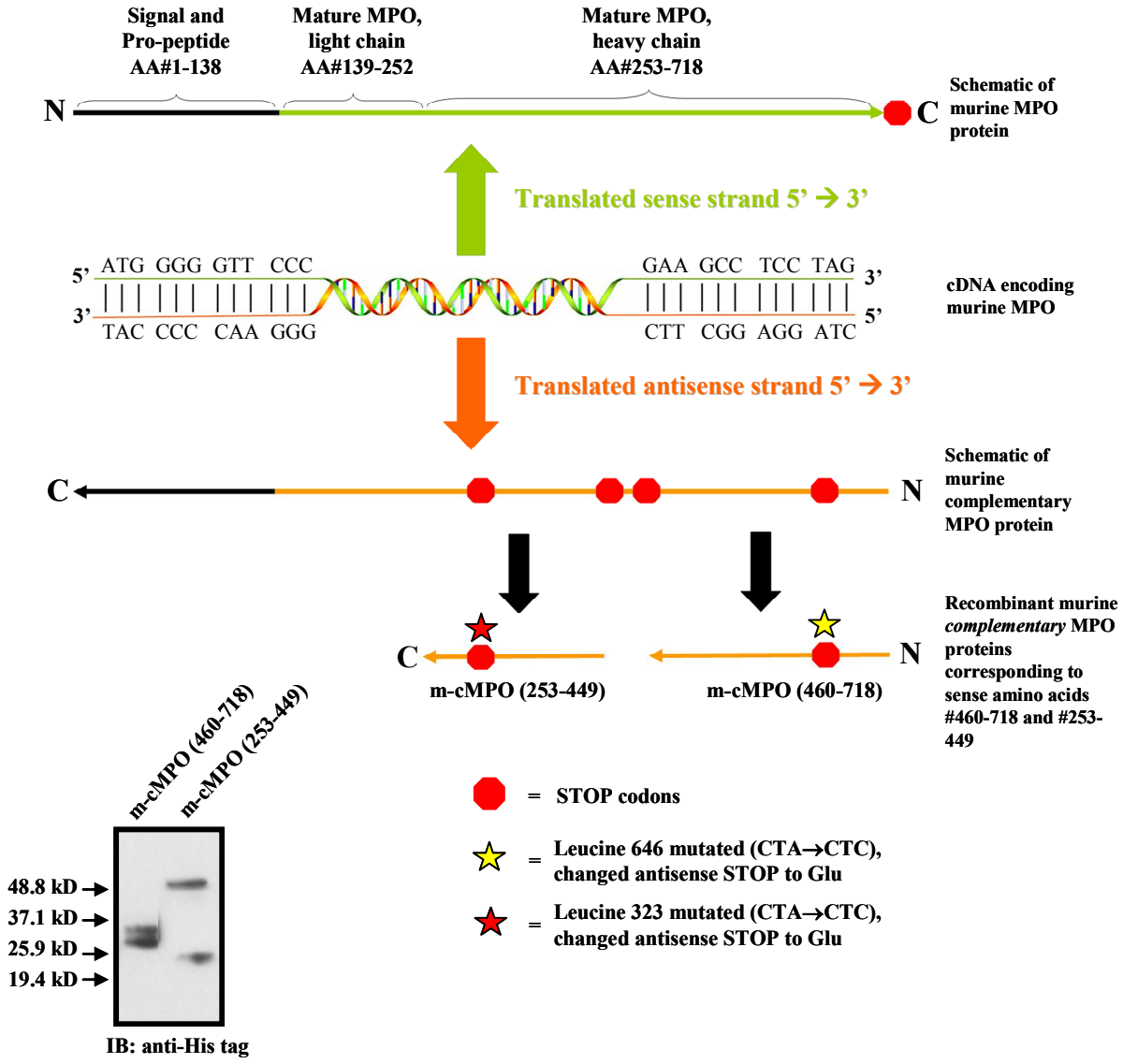


Figure 2.4. Design and Production of Recombinant, Complementary Murine Myeloperoxidase Polypeptides. We produced complementary protein fragments encoded by the *antisense* sequence (read 5' → 3', in frame) of the MPO gene, corresponding to sense amino acids 460-718 [m-cMPO(460-718)] and 253-449 [cMPO(253-449)]. Several STOP codons were introduced when translating the antisense sequence; site-directed mutagenesis was employed to mutate the 3rd base in leucines 323 and 646 codon (CTA→CTC) to change the antisense STOP to a glutamic acid residue. This cDNA was inserted into a vector and transfected into a HEK 293 cell secretion system. The expressed recombinant protein fragments contain an amino-terminal histidine tag for purification and immunodetection. Inset is an immunoblot of the purified recombinant proteins, using an anti-histidine tag antibody.

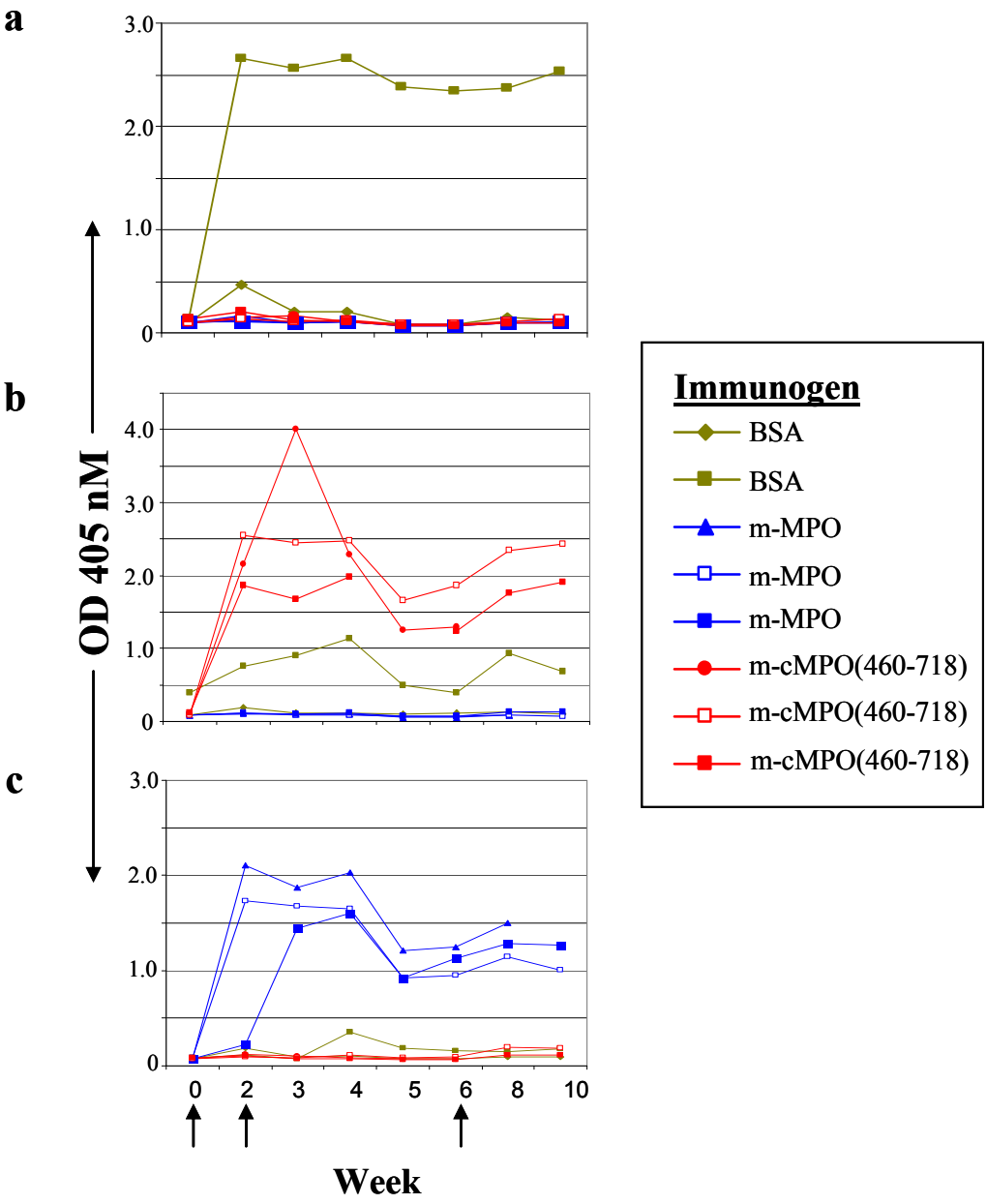


Figure 2.5. Mice Immunized with m-cMPO(460-718) did not Produce Anti-MPO Antibodies. MPO knockout mice were immunized with either m-cMPO(460-718), murine MPO (m-MPO), or bovine serum albumin (BSA) on weeks indicated by arrows. Sera was acquired weekly and analyzed for reactivity to a) BSA, b) m-cMPO(460-718), and c) m-MPO by ELISA.

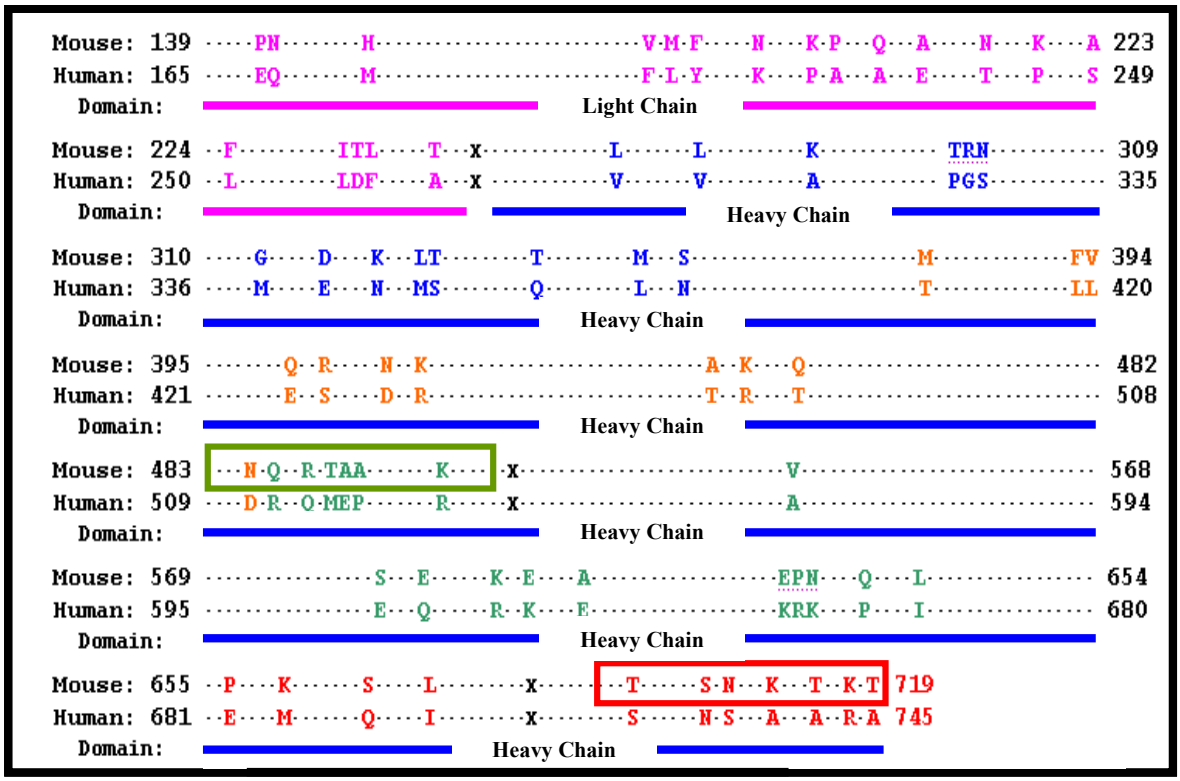
Rationale for the Selection of m-cMPO Peptides

The first experiment, conducted in an attempt to generate anti-MPO antibodies by immunizing mice with a complementary MPO protein, was not successful. This experiment used a partially purified recombinant protein that we were unable to produce large amounts of in a reasonably concentrated, pure form. The m-cMPO(460-718) was less than 25% of the total protein in the partially purified mixture, and the mice were probably immunized with only 2 μg of m-cMPO. The HEK 293 production system proved highly inefficient and expensive for this particular recombinant protein, so we purchased synthesized m-cMPO peptides in an effort to acquire highly purified and concentrated antigen.

Again, there was no defined pathogenic epitope for the disease-causing murine anti-MPO antibodies, so we gleaned what we knew from human studies to design these peptides. Studies from our laboratory showed that despite the 80% amino acid identity between human and mouse MPO, the majority of human ANCA do not bind murine MPO. This implied that the pathogenic human epitopes could be in the regions of amino acid sequence dissimilarity, and perhaps the same could be said for murine MPO epitopes. The study by Erdbrugger et al identified a broad region at the C-terminus of the MPO heavy chain to be most reactive to human patient ANCA.[144] Additionally, a previous graduate student in our laboratory attempted to epitope map MPO by producing random protein fragments. That study revealed a region from amino acids 522-683 that reacted with every ANCA tested; however, this region also reacted with several normal sera. When testing another polypeptide corresponding to amino acids 550-617, which was internal to the highly reactive 522-683 fragment, the same ANCA lost much of the reactivity. Therefore it was determined that the highly reactive epitope on the MPO molecule could be between amino acids 522-549, or

between amino acids 618-683. This information was considered and four 25-mer peptides were conceptually designed for potential use in these studies. Using computer based molecular modeling software, the peptides were analyzed to predict their surface exposure on the MPO molecule. Murine MPO had not yet been crystallized, however such data did exist for human MPO.[146, 147] Analysis of the human regions of MPO homologous to the previously designed murine MPO peptides revealed two surface-exposed peptides corresponding to amino acids 484-508 and 694-718 (Figure 2.6). A limitation in this human modeling information lied in the fact that the selected mouse peptide sequences diverged from the human by 7 out of 25 amino acids in each. These regions may fold differently in the native murine MPO molecule and may or may not be exposed on the surface, as we predicted. Regardless, pairs of sense and complementary peptides corresponding to the selected regions were ordered.

The individual mice in this study were immunized with a set of peptides for multiple reasons. Primarily, because we did not know the pathogenic epitope, this method permitted the exposure of multiple epitopes to each animal as we intended to do with the recombinant m-cMPO(420-697). Additionally, work associated with our laboratory was focused on creating a monoclonal anti-murine MPO antibody that could induce disease in our MPO-ANCA animal model. This work has not yielded disease induction as accomplished *via* passive transfer of total IgG from m-MPO-immunized MPO null mice. It is thought that, to be pathogenic, the antibodies may be polyclonal. We hypothesized that immunizing with multiple peptides would give us the best chance of eliciting a pathogenic polyclonal anti-MPO response.



Peptide Sequences for amino acids 484-508

Sense: FRLNNQYRPTAANPRVPLSKVFFAS
Comp: AGKKDLAEWDFSGVGRCGPVLIVQAE

Peptide Sequences for amino acids 694-718

Sense: SNTYPRDFVSCNTLPKLNLTSWKET
Comp: GLLPGSQVQFRKGVTADKVSGVCVG

Figure 2.6. Murine MPO Peptide Sequences Targeted for Sense and Complementary Peptide Study. Shown is an alignment of human and murine MPO amino acid sequences, with only the disparate amino acids shown. All identical amino acids appear as dots. Sense peptides and their complementary counterparts were synthesized corresponding to amino acids #484-508 (green box) and #694-718 (red box), and the sequences of the sense and complementary (Comp.) pairs are shown. Figure adapted from Erdbreugger *et al*.

Root-Bernstein and colleagues suggest that complementary proteins play a role in autoimmune disease, but their ideas differ slightly from our initial theory.[119] They predict that the induction of an autoimmune response comes about when two exogenous antigens that share complementary binding regions interact. At least one of these complementary regions is the mimic of a self-antigen. The complex becomes more immunogenic than either antigen individually and induces the simultaneous generation of antibodies specific for each complementary site; these antibodies can interact in an anti-idiotpic fashion and create a self-sustaining network of antibodies which can interact with "self" and incite autoimmunity. We attempted to address Root-Bernstein's theory by immunizing mice with pre-incubated sense and complementary peptide pairs, thus exposing the mice to a complementary peptide complex. We predicted that this complex would be highly immunogenic and incite a more potent humoral response, and the mice receiving these splenocytes would have worse disease.

Mice Immunized with m-cMPO peptides did not Produce Anti-MPO Antibodies

The sera from peptide-immunized mice were analyzed at week 0, 2, and 10 for the development of anti-MPO antibodies (Figure 2.7a-d). It was important that not only the complementary peptide-immunized mice develop anti-MPO antibodies to support our theory, but also for the sense peptide-immunized mice sera to recognize native MPO; this would demonstrate that we chose an appropriate exposed epitope on the surface of the MPO molecule. By ELISA, we showed that the mice responded very well to the peptide immunogens both after the first and last immunizations. However, there was almost no

reactivity to native murine MPO, both in the sense and complementary peptide immunized mice.

RAG-2 Knockout Mice that Received Splenocytes from Peptide-immunized Mice did not Develop Crescentic, Necrotizing Glomerulonephritis

The positive control RAG-2 -/- mice that received splenocytes from MPO-immunized MPO -/- mice were sacrificed at day 12, as they appeared dramatically, physically ill. Experimental mice were sacrificed at day 17 when mice from multiple groups began to show visible signs of illness.

Sera from the terminal bleeds of the RAG-2-/- mice were analyzed for the presence of anti-MPO antibodies (Figure 2.8). While it did not appear that the mice had circulating antibodies that reacted with the peptides, all mice, including the BSA negative controls, had some reactivity to native MPO (Figure 2.8a). Other than the native MPO-immunized splenocyte sera, the m-cMPO peptide- and BSA-immunized splenocyte sera had the highest reactivity to native MPO.

Urine analysis revealed that all mice had some degree of hematuria, the BSA controls had the lowest hematuria scores overall (Table 2.1). However, the scores were inconsistent within groups and urine was not collectable from every animal.

The pathologic evaluation of disease consisted of determining the percent of glomeruli with necrosis and crescents within a given microscopic field. The positive control (native MPO-imunized) mice had crescents in 99% of glomeruli and more than 50% of these glomeruli were necrotic; this data indicated our model was working well and the anti-MPO antibodies induced ANCA disease (Table 2.1). All mice, including the BSA negative controls, had a background mesangial cell proliferation that we commonly see when utilizing

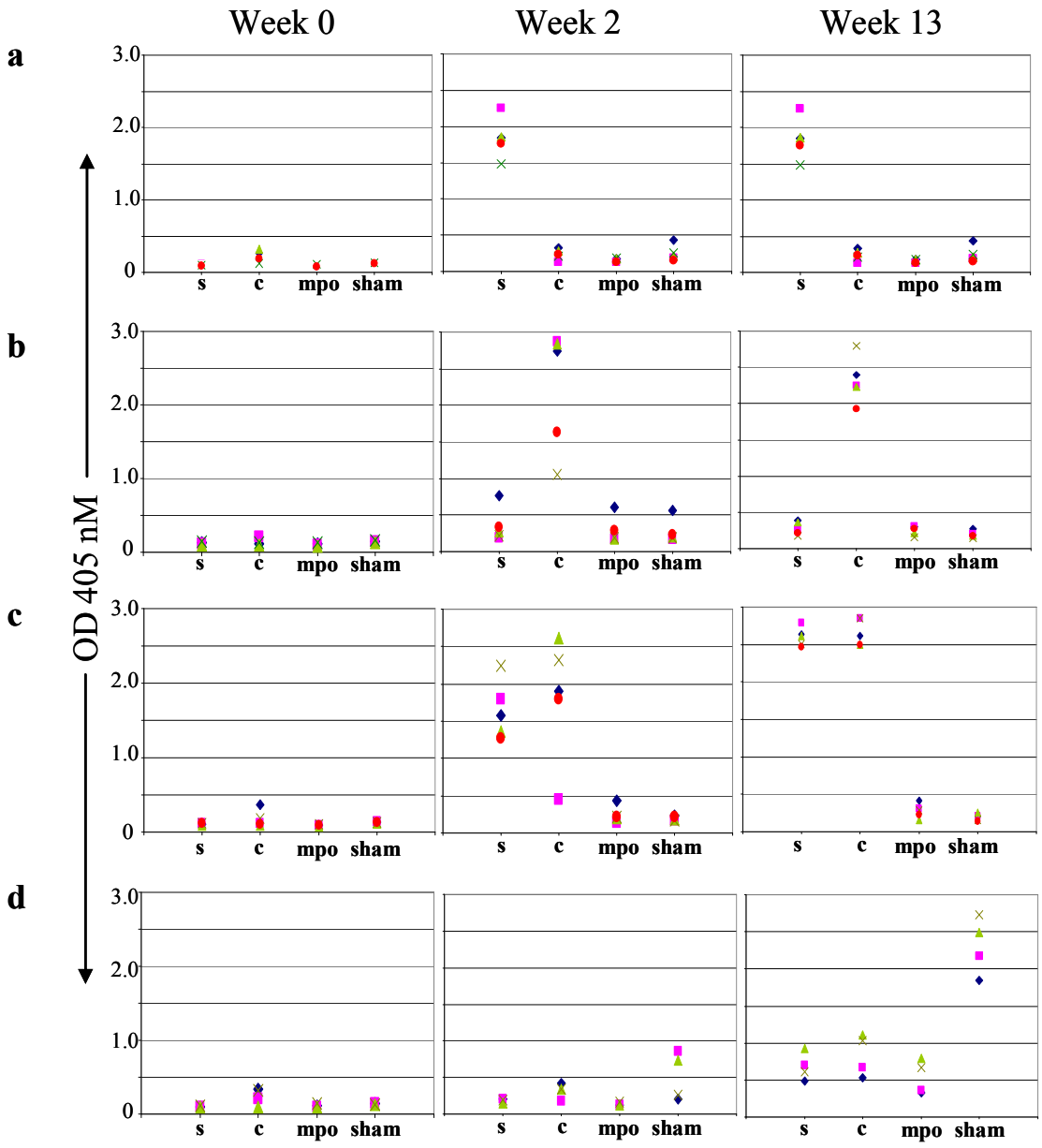
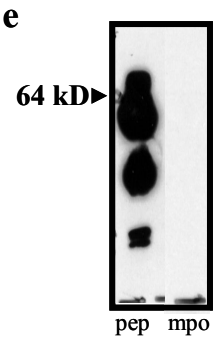


Figure 2.7. Peptide-immunized Mice Sera Reactivity to Immunogens and Native Murine MPO. MPO knockout mice were immunized with a) sense peptides (n=5), b) complementary peptides (n=5), c) sense-complementary peptide complexes (n=5), or d) bovine serum albumin (n=4). Sera was acquired and tested for reactivity against the sense peptides (s), complementary peptides (c), native murine MPO (mpo) or goat serum blocking buffer (sham) at weeks 0, 2, and 13. e) Serum from a sense peptide (pep)- and a native murine MPO (mpo)-immunized mouse was analyzed for reactivity to denatured, reduced native MPO by western blot.



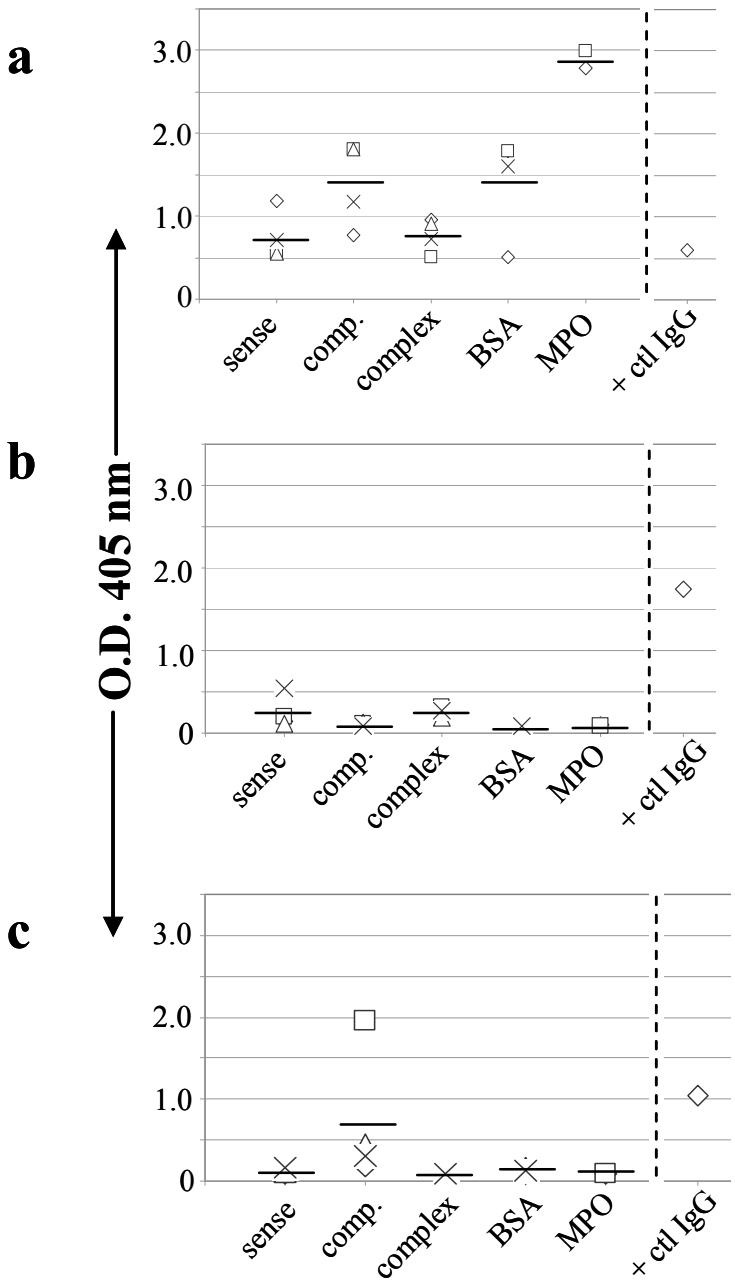


Figure 2.8. ***RAG-2*** **Knockout Mice Sera Analyzed for Reactivity to Immunogens.** Sera from mice acquired on the day of sacrifice was analyzed for reactivity by ELISA against **a**) native murine MPO, **b**) sense peptides, and **c**) complementary peptides. The mice are grouped according to the splenocytes received from the MPO knockouts: either those from mice immunized with sense MPO peptides (sense), complementary MPO peptides (comp), sense-complementary peptide complexes (complex), bovine serum albumin (BSA), or native murine MPO (MPO). The OD 405 nM value is displayed for each mouse with the mean value for each group indicated by the line.

Table 2.1. Histopathology and Urinalysis from Splenocyte-transferred RAG-2 -/- Mice

Mouse #	Splenocytes	Necrosis (%)	Crescents (%)	Proliferation (0-4+)	Urinalysis* (0-3+)
1	sense	2	3	2	no urine
2	sense	1	0	2.5	2.5
3	sense	3	2	2.5	3
4	sense	1	1	2	1.5
1	complementary	1	0	2	1.5
2	complementary	0	0	2	3+
3	complementary	0	0	1.5	0
4	complementary	0	0	1.5	1.5
1	complex	2	1	2.5	2
2	complex	0	0	2	0
3	complex	0	0	2	3+
4	complex	0	1	2	no urine
1	bsa	0	0	2.5	1.5
2	bsa	1	2	2	0.5
3	bsa	0	1	2	1.5
4	bsa	1	0	2	no urine
1	native mpo	55	99	2	no urine
2	native mpo	65	99	2	no urine

**Urinalysis for hematuria using Roche Chemstrip 10UA*

the splenocyte transfer model.[69] The RAG-2 -/- mice that received splenocytes from MPO -/- mice immunized with complementary peptides (alone or complexed weth sense peptides) did not develop disease. The RAG-2 -/- mice that received splenocytes from MPO -/- mice immunized with sense peptides developed the most apparent pathologic abnormalities, but none of those mice developed more than 3% necrosis or crescents.

Anti-m-MPO(302-325) Antibodies did not Cross-react with Native Murine MPO

As the recombinant protein and peptides were not pathogenic, we attempted to map a pathogenic region of murine MPO using immunoglobulin from MPO -/- mice immunized with purified murine MPO. The mass spectrometry measurements after the first two digestion steps (LysC and trypsin) yielded the same fragment: LysC digestion yielded amino acids 219-326, and trypsin revealed a more defined fragment, amino acids 302-325 (Figure 2.9). The results after the tryptic digest were confirmed by MS/MS.

M-MPO(302-325) peptide was ordered from Alpha Diagnostics, Inc., and engineered with a N-term cysteine residue for use in linking to carrier proteins or purification columns.

Prior to receiving the boost at week 4, the m-MPO(302-325) peptide-immunized mice did not make anti-peptide antibodies, nor anti-native m-MPO antibodies (not shown). The native m-MPO-immunized mouse generated a potent humoral response to MPO, indicating that the adjuvant and procedure were sound. Analysis of sera at week 1 and 2 following the boost revealed that the m-MPO(302-325) peptide-immunized mice made low titer antibodies (Figure 2.10). However, the reactivity of this serum was only 2-fold over the pre-immune bleeds and the antibodies did not cross-react with native MPO.

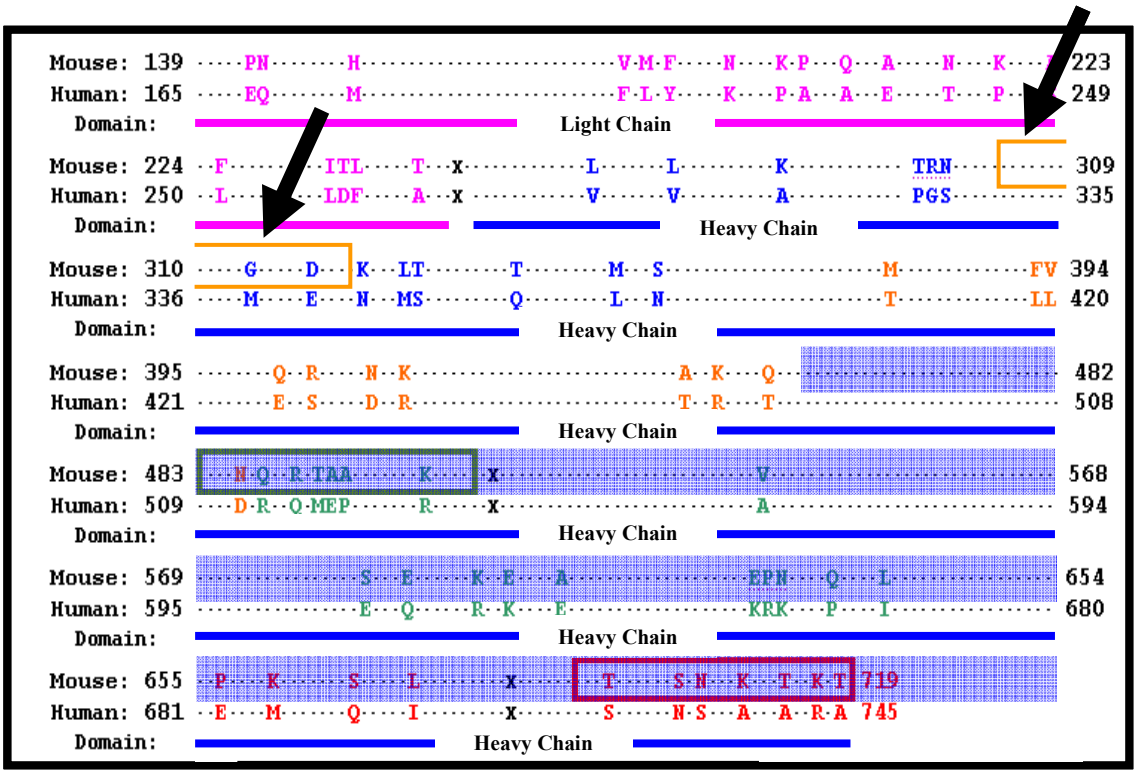
Because the m-MPO(302-325) peptide was weakly immunogenic and did not incite anti-MPO antibody production in the mice, we attempted to use the peptide to compete away

binding of MPO-reactive immunoglobulin to MPO as an additional measure of its utility in future studies. Compared to control peptide, MPO(302-325) was unable to compete away immunoglobulin binding even in extreme molar excess (Figure 2.11). The total pooled IgG used in this ELISA was the same used to epitope map this peptide, which we expected to readily bind the peptide; it does not. These results provide some uncertainty as to the validity of the epitope mapping technique and whether it will be useful in future work. It must be taken into consideration that this mapping was done once and not repeated in this study.

DISCUSSION

Previously, our group published work supporting autoantigen complementarity as an inciting factor in the development of PR3-ANCA-mediated disease.[73] Expanding these findings, the work in this chapter demonstrates that MPO-ANCA patients have antibodies specific to complementary MPO. This broadens the application of our Theory of Autoantigen Complementarity, which states that the exposure to proteins complementary to an autoantigen may elicit an immunologic cascade leading to autoimmunity. However, it remains unproven whether complementary proteins are etiologic agents of disease. A major goal of this research was to induce disease in a mouse model of MPO-ANCA glomerulonephritis by immunization with complementary MPO proteins.

A subset of MPO-ANCA patients had antibodies reactive to recombinant cMPO(420-697), while healthy controls did not. These data imply that these patients were exposed to a complementary protein, which may have contributed to the onset of disease. Alternatively, the anti-cMPO(420-697) antibodies could be anti-idiotopes generated as an immune regulatory response against ANCA. Because we only have serum from patients *after* their



Peptide Sequence for m-MPO(302-325)

CNQINALTSFVDASGVYGSEDPLAR

Figure 2.9. Epitope Mapping Study Reveals a 24-Amino Acid Epitope. Shown is an alignment of human and murine sequences as described in Figure . The epitope mapping study revealed a peptide epitope corresponding to amino acids 302-325 (orange box, black arrows), whose sequence is shown above. For comparison, the previously used recombinant protein, m-cMPO(420-697), which corresponds to the sense sequence shown by the transparent blue box is shown, along with peptides m-MPO(484-508) (green box) and m-MPO(694-718) (red box).

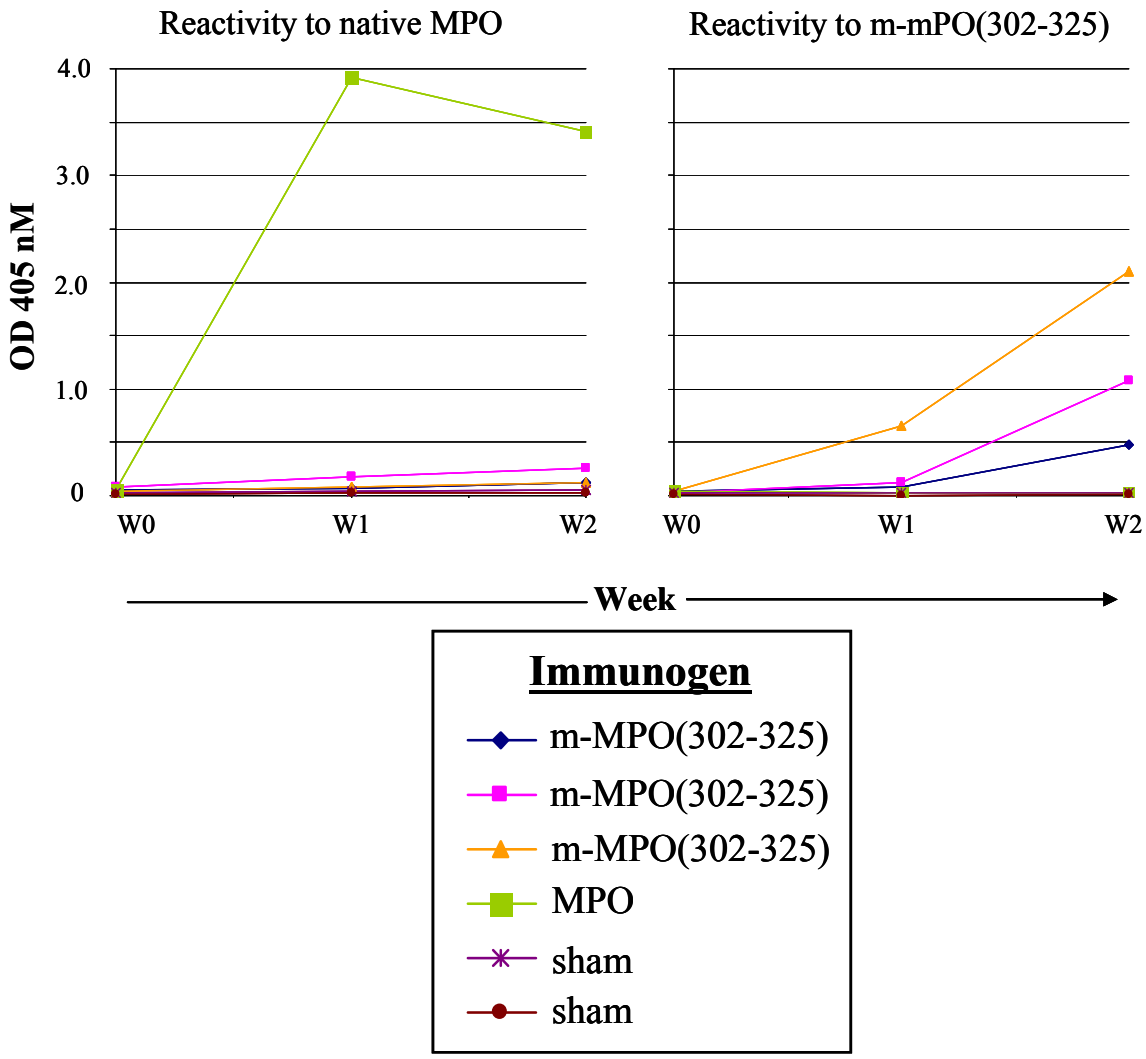


Figure 2.10. m-MPO(302-325) Peptide-immunized Mice do not Have Antibodies that Cross-react with Native Murine MPO. Sera was collected from MPO knockout mice that were immunized and boosted with the epitope-mapped, m-MPO(302-325) peptide (m-MPO(302-325), n=3), native murine MPO (MPO, n=1) and adjuvant only (sham, n=2). Shown is the reactivity from pre-immune sera (W0) and sera from weeks 1 (W1) and 2 (W2) following the booster immunization.

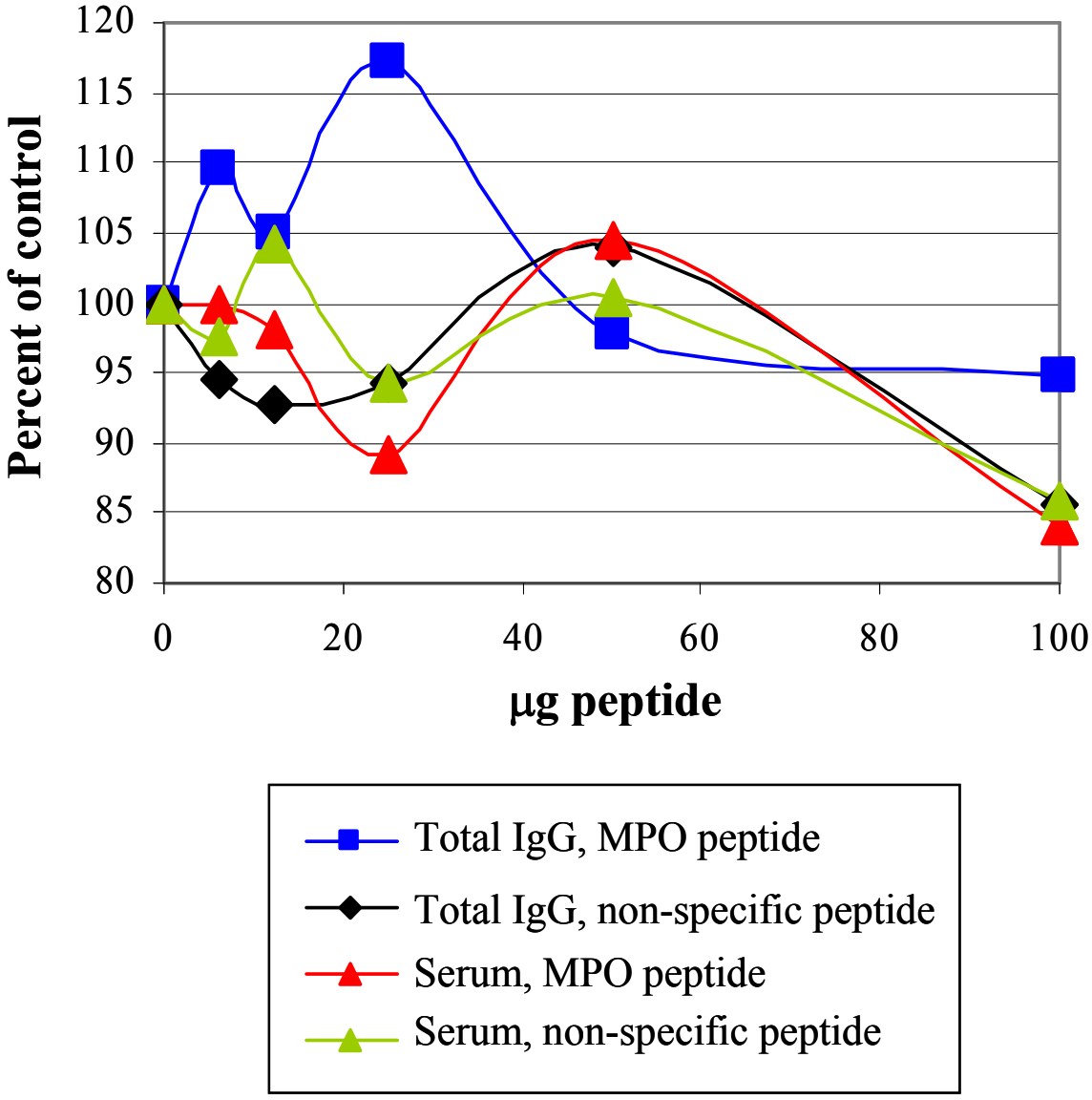


Figure 2.11. m-cMPO(302-325) Peptide Does not Compete with Anti-MPO Immunoglobulin Binding to Native Murine MPO. Increasing amounts of the epitope-mapped, m-MPO(302-325) peptide were used to compete away the binding of two sources of anti-MPO specific immunoglobulin to native MPO. The "total IgG" was the pooled, purified total IgG from three mice that had been immunized with native MPO. This total IgG was that used to epitope map the m-MPO(302-325) peptide. The "serum" was from an MPO knockout mouse that had been immunized with native MPO. The concentrations of the immunoglobulins used were at dilutions that represented 50% reactivity to native MPO. The results are expressed as the percent reactivity (as indicated by OD 405 nM by ELISA) of the immunoglobulin in the presence of peptide versus that in the absence of peptide.

diagnosis as ANCA-positive, we cannot determine the temporal pattern of the appearance of the anti-cMPO antibodies versus ANCA and whether the exposure to the complementary protein came before, simultaneous to, or after the development of ANCA. Such studies are nearly impossible in humans, which is why an animal model is key to establishing our theory as a bona fide mechanism of disease. One way in which this question may be answered is by gaining access to the United States of America Department of Defense serum repository that contains 30 million serum samples prospectively collected from more than 5 million service men and women. If some of these people were diagnosed with an ANCA-mediated disease, a study could be conducted similar to that published by Arbuckle and colleagues in which they analyzed sera for the presence of several autoantibodies associated with systemic lupus erythematosis prior to disease diagnosis.[148] For ANCA patients (or other autoimmune diseases with defined pathogenic targets), we could determine if the anti-complementary protein antibodies were present prior to the development of the autoantibody and onset of disease.

This is not the first report of anti-idiotypic antibodies specific for MPO-ANCA in patients. In a series of studies regarding the mechanism of efficacy of intravenous immunoglobulin (IVIg) treatment of ANCA patients, Lockwood and colleagues determined that not only the IVIg, but patient sera had anti-MPO anti-idiotopes.[149-152] Their studies revealed that Jerne's Network Theory was potentially at play in regulating the autoimmune response as they noted a reciprocal pattern of anti-MPO and anti-idiotypic antibodies over time, including a higher titer of anti-idiotopes during disease remission. They proposed that remission of patients was governed by an increase in the anti-idiotopes in circulation. This work was approached from a therapeutic standpoint, and the researchers did not consider that

the exposure to a complementary protein could have induced this network and thus disease. While not the focus of Lockwood's research, the identification of these antibodies provides further evidence that autoantigen complementarity may be at play is MPO-ANCA vasculitis.

Establishing an animal model of complementary protein-induced disease without a defined pathogenic human MPO epitope proved to be a risky task. Not only does it appear that ANCA are polyclonal among patient populations, none of the multiple reactive regions have been finely mapped. Even if a pathogenic eptitope was known, human ANCA do not cause disease in mice, implying that the pathogenic epitope(s) of human MPO contains areas of sequence and/or structural divergence from the murine MPO molecule.[144] The lack of published data led us to theoretically predict potential pathogenic regions of murine to make corresponding complementary recombinant protein and synthetic peptides. Neither the recombinant cMPO(420-697) or the cMPO peptides induced the idiotoypic network in these mice, and thus the experiments did not generate ANCA by immunization with a complementary peptide. While the intended use of MPO null mice was to supply a permissive environment for the development of anti-idiotopes (anti-MPO), perhaps the use of these mice was a disadvantage because these mice had no antigen except for the anti-cMPO antibody to drive the production of an MPO cross-reactive antibody. Based on Root-Bernstein's ideas of antigenic complementarity in autoimmune disease, a *complex* of complementary antigens, one of which mimics a self-determinant, is required to induce a simultaneous, self-sustaining, and vicious network of autoantibodies and their anti-idiotopes.[119] In this manner, Jerne's idiotypic network is not induced in a stepwise fashion. Rather, the complementary regions of the antigens generate coincident antibodies that interact in an idiotypic fashion. In our recombinant cMPO experiment, we introduced the

complementary protein in the absence of the autoantigen or its mimic, which may have shifted the balance of antibody generation in favor of anti-cMPO. We attempted to address this issue by immunizing mice with a complex of complementary synthetic peptides; however, the anti-peptide antibodies did not cross-react with native MPO, as discussed below. Additionally, we could not find evidence that recombinant cMPO(420-697) could bind MPO (data not shown), which may prove to be a critical element for successfully developing a model of our theory.

A major limitation in using the synthetic peptides was that the antibodies generated against the sense peptides did not cross-react with native MPO, confounding the establishment of our autoimmune disease model. Western blots revealed that the anti-sense peptide antibodies reacted to reduced, denatured MPO, informing us that the peptides were at least proper murine MPO sequences (Figure 2.7d). Murine MPO has not been crystallized, so there was no way to be certain that the selected peptide sequences were exposed on the surface of the native molecule. The shape of these immunogens is of utmost importance. If the sense peptide is not a MPO surface moeity and does not generate antibodies that bind native MPO, then it can be inferred that the complementary peptide would not provide the predicted internal image of the autoantibody (anti-idiotope). Thus, the anti-idiotope generated following immunization with the complementary peptide would not be expected to bind MPO.

It is interesting that the vast majority of murine studies focused on the idiotypic network have utilized the BALB/c strain.[73, 105, 112, 153-155] Our MPO null mice are on C57BL/6J background, so there potentially exist genetic differences that more readily permit immune "regulation" via the idiotypic network in BALB/c mice compared to C57BL/6J.

A promising step forward was the epitope mapping technique that identified a region of MPO bound by pathogenic anti-murine MPO immunoglobulin. A goal of this study was to directly identify a peptide that could be used to generate pathogenic antibodies; once accomplished, we would create the complementary peptide for use in disease models. The 24-amino acid m-MPO(302-325) peptide identified by mass spectrometry was minimally immunogenic, generating antibodies at a low titer only after a booster immunization. This region of MPO is highly conserved, not only among muliple species' myeloperoxidase, but also among other murine peroxidases (i.e. thyroid peroxidase, eosinophil peroxidase, and lactoperoxidase). Therefore, naïve B-cell clones that interacted with this peptide were likely eliminated in the bone marrow due to the interaction with other self-proteins. Additionally, this peptide shared 92% identity with the homologous human sequence, and structural analysis using the National Center for Biotechnology Information's Cn3d software revealed that the majority of this peptide, except for the 4 C-term amino acids, was composed of hidden residues. Regardless, the low titer antibodies failed to cross-react with native MPO. The mapping study was only carried out once. While this attempt did not identify a useful epitope, this approach has great potential and repeat studies should be performed. A considerable pitfall with this technique lies in its questionable ability to identify non-linear epitopes because, as may be the case with pathogenic murine antibodies, human ANCA seem to bind conformational, rather than linear, epitopes.[156]

In the future, we must learn more about both pathogenic epitope specificities and complementary protein interactions to employ a more targeted approach. As an alternative to epitope mapping, we can analyze sequences of known MPO-binding proteins (cytokeratin 1, apoA1, ceruloplasmin, and albumin) for regions of predicted hydropathic complementarity

and determine if these regions mediate protein-protein interactions. These may be potential regions to target for our studies. Finding a set of complementary peptides that interact specifically with MPO may be key to these studies. A large scale study involving the synthesis of overlapping complementary peptides spanning the entire length of the MPO molecule could be employed to screen for peptides that both specificially bind MPO and immunoprecipitate anti-idiotypic antibodies from patient/mouse sera. The strong possibility that the pathogenic epitope is conformational complicates matters, as complementary peptides based strictly on the linear 5'→3' antisense sequence of MPO may not produce an interacting peptide. While we define a peptide pair based on complementary RNA sequences, Bernstein and colleagues define a complementary pair as one in which the "antigens must be capable of stereospecific binding to each other that steroespecific binding must be manifested by the induction of pairs of complementary antibodies (or T-cells) that act like idiotype-antiidiotype pairs."[119] He provides examples of pairs that fit these criteria and cannot be explained by hydropathic complementarity. This proposal broadens what is a complementary peptide. Because MPO is being studied as a potential drug target to reduce oxidative stress in vascular disease, it is possible that companies are conducting high-throughput studies by screening large peptide libraries for MPO-binding specificity. A collaboration could be established with such companies to acquire sequences of MPO-binding peptides. These peptides may represent complementary peptides not able to be predicted based on antisense RNA sequence alone, and could be studied by our group to discover a complementary peptide that incites disease.

We have learned in this research that the idiotypic network is intact in ANCA patients, which is consistent with the exposure to a complementary protein. The initial

animal experiments revealed regions were not pathogenic, and caused us to use a different experimental design. Before progressing to models of disease, we must determine one or more pathogenic epitopes in the mouse model, as well as define a set of complementary proteins that can bind MPO.

EPILOGUE

The inflammatory response is a balance of pro- and anti-inflammatory signals, the latter leading to the resolution of inflammation and the repair of host tissue damaged in the process. In some diseases, such as ANCA vasculitis, this delicate balance is shifted toward a constant pro-inflammatory state with dramatically disrupted resolution and chronic tissue damage. In these inflammatory conditions, the inhibition of bradykinin production by MPO could yield a perpetual impairment of vasodilation by decreasing the bioavailability of nitric oxide. This situation would lead to endothelial dysfunction, a condition underlying diseases as diverse as ANCA vasculitis, atherosclerosis, cirrhosis, and diabetes.[157-160] However, one must consider whether the detrimental interactions reported in Chapter 1 may also play a beneficial role in a *controlled* inflammatory setting. The same constraints on bradykinin production may contribute to homeostasis, serving to counterbalance the vasodilation and edema associated with the initial humoral phase of acute inflammation. The secondary cell-mediated phase is characterized by the recruitment of neutrophils and monocytes to the inflamed site, upon which MPO is released into the vessel to exert its vasomodulatory effects. In this way, MPO may contribute to host defense while keeping inflammatory processes in check.

Dissection of the molecular mechanisms mediating the inflammatory burden is key to revealing targeted, more systemically tolerable, therapeutic targets. Current therapies used to treat ANCA patients are cytotoxic and immunosuppressive: regimen with serious side

effects. Improving vascular function may be one way in which to alleviate disease symptoms and improve outcome in patients. MPO was previously reported to interfere with nitric oxide signaling following its transcytosis through endothelial cells; we provide a new mechanism by which MPO *prevents* nitric oxide production by inhibiting bradykinin release in the vessel. Targeting and inactivating MPO could improve vascular function by protecting the activity of the plasma kallikrein-kinin system and the downstream production of nitric oxide. However, we must validate the functional relevance of plasma kallikrein-kinin system modulation by MPO. Intricate cell based models need to be established to allow plasma kallikrien-kinin system assembly on endothelial cells (in the presence of MPO), as it would in the vessel, and monitoring bradykinin production and downstream signaling. Further, *ex vivo* and/or *in vivo* physiologic models should monitor the true functional effects of MPO's modulation of bradykinin production.

Chapter 2 explored the Theory of Autoantigen Complementarity relative to MPO-ANCA to explain the onset of disease. Though autoantibodies such as ANCA are recognized as pathogenic mediators of disease, the origin of the autoantibodies remains unclear. We found a subset of MPO-ANCA patients that have antibodies reactive to a complementary protein, and our theory predicts that these proteins could incite an immunologic cascade leading to autoimmunity. Because proving causation using human material is extremely challenging, a major effort was put forth to generate a mouse model of complementary MPO-induced glomerulonephritis and vasculitis. However, our attempts were largely unsuccessful. Our results indicate that defining a pathogenic region of MPO is paramount before moving forward with additional animal studies. Anti-glomerular basement membrane disease, also studied by our group, is mediated by autoantibodies and/or T-cells reactive to a very

specified epitope of collagen. While preliminary, studies in our group have suggested that rats immunized with a complementary collagen peptide develop anti-GBM-like disease. These studies used a well-defined peptide representative of the GBM epitope that had been described years earlier. We should continue epitope mapping using the mass spectrometry-based technique described in chapter 2. While the results of the initial attempt were inconclusive, it is a potentially powerful technique that deserves repeating. Additionally, we can epitope map by testing the anti-murine MPO IgG for reactivity against recombinant human-mouse MPO chimeras, which are already produced in our laboratory. This could identify a general region of murine MPO for finer mapping studies. Once we discover a pathogenic target sequence, we can design complementary peptides for our studies.

Scientists have predicted for over 25 years that complementary antigens and the idiotypic network could play a role in autoimmune disease. Proving causation in an animal model is currently the "holy grail" that could validate our Theory of Autoantigen Complementarity and contribute to a better understanding of one of the mysteries of modern medicine: the origin of autoimmune disease.

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