



# Autoantigen complementarity and its contributions to hallmarks of autoimmune disease



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## HIGHLIGHTS

- Autoimmune disease is driven by acquisition of multiple immune-compromising traits.
- Autoantigen complementarity mechanistically promotes disease-associated traits.
- Six key hallmarks are linked with autoantibody-mediated autoimmune disease.

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## ABSTRACT

The question considered is, “What causes the autoimmune response to begin and what causes it to worsen into autoimmune disease?” The theory of autoantigen complementarity posits that the initiating immunogen causing disease is a protein complementary (antisense) to the self-antigen, rather than a response to the native protein. The resulting primary antibody elicits an anti-antibody response or anti-idiotypic, consequently producing a disease-inciting autoantibody. Yet, not everyone who develops self-reactive autoantibodies will manifest autoimmune disease. What is apparent is that manifestation of disease is governed by the acquisition of multiple immune-compromising traits that increase susceptibility and drive disease. Taking into account current cellular, molecular, and genetic information, six traits, or ‘hallmarks’, of autoimmune disease were proposed: (1) Autoreactive cells evade deletion, (2) Presence of asymptomatic autoantibodies, (3) Hyperactivity of Fc–FcR pathway, (4) Susceptibility to environmental impact, (5) Antigenic modifications of self-proteins, (6) Microbial Infections. Presented here is a discussion on how components delineated in the theory of autoantigen complementarity potentially promote the acquisition of multiple ‘hallmarks’ of disease.

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## 1. Introduction

Autoimmune diseases as a whole are a diverse class of over 80 diseases that affect different organs and tissue types. Because of this diversity, we tend to study them as individual diseases rather than as a whole. We proposed that all autoantibody-mediated autoimmune diseases could be characterized by a small number of acquired characteristics. Susceptibility to disease is a multistep process involving a succession of genetic and epigenetic changes. Collectively, these changes dictate physiologic changes whereby novel capabilities, or traits, are acquired. Regardless of the mechanism by which they arise, these traits, or ‘hallmarks’ appear to be shared by most, if not all, autoantibody-mediated diseases (Fig. 1) (Preston and Falk, 2014).

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A mechanism potentially attributable to establishment of ‘hallmarks’ is one discovered serendipitously in the late 1990s termed the ‘theory of autoantigen complementarity’ (Fig. 2). This discovery was made during studies of sera from patients with inflammatory vascular autoimmune disease affecting numerous organs and caused by anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against the neutrophil granule serine protease, proteinase 3 (PR3) (Pendergraft et al., 2004). We discovered that patients harbored not only PR3-ANCA, but antibodies to peptides encoded by the non-coding strand of the gene encoding the autoantigen, PR3. Furthermore, mice immunized with a complementary human PR3 peptide produced antibodies to the peptide as well as antibodies to the peptide’s sense counterpart, PR3. In addition, human and murine antibodies and their idiotype antibody counterparts also bound each other, indicating that they could form anti-idiotypic pairs (Fig. 3). Interestingly, it was later shown that antibodies to complementary PR3 appeared to be specific for a restricted epitope within proteins translated from antisense RNA

sequences corresponding to both middle- and carboxy-terminal domains of PR3 (Fig. 4) (Hewins et al., 2011).

The theory of autoantigen complementarity posits that the immunogen that incites the sequence of events leading to an autoimmune disease is not the autoantigen or its mimic but rather its complementary (antisense) peptide, protein, or its mimic (Fig. 2) (Pendergraft et al., 2004, 2005; Preston et al., 2005; Root-Bernstein, 2007). This complementary protein initiates production of antibodies that in turn elicit an anti-antibody response, referred to as an anti-idiotypic response. The resultant anti-idiotypic antibodies react with the autoantigen, which has an amino acid sequence that is complementary to the sequence of the initiating antigen. The source of this complementary peptide could be from translation of endogenous antisense RNA or of exogenous microbial or fungal DNA that has sequence homology with the non-coding DNA strand of the autoantigen (Pendergraft et al.,

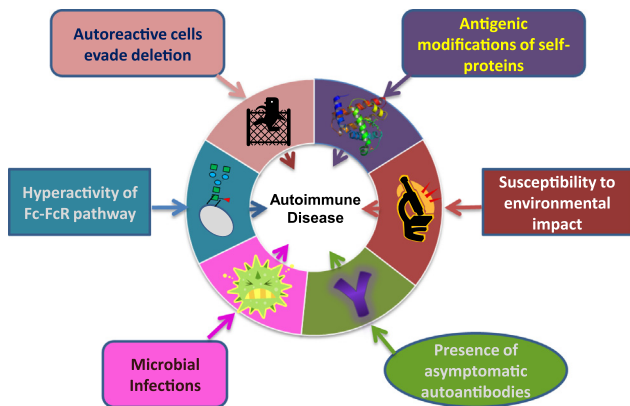


**Fig. 1. Hallmarks of autoimmune disease.** Taking into account current cellular, molecular, and genetic information, six traits, or 'hallmarks', of autoimmune disease are proposed: (1) Autoreactive cells evade deletion, (2) Presence of asymptomatic autoantibodies, (3) Hyperactivity of Fc-FcR pathway, (4) Susceptibility to environmental impact, (5) Antigenic modifications of self-proteins, (6) Microbial Infections.

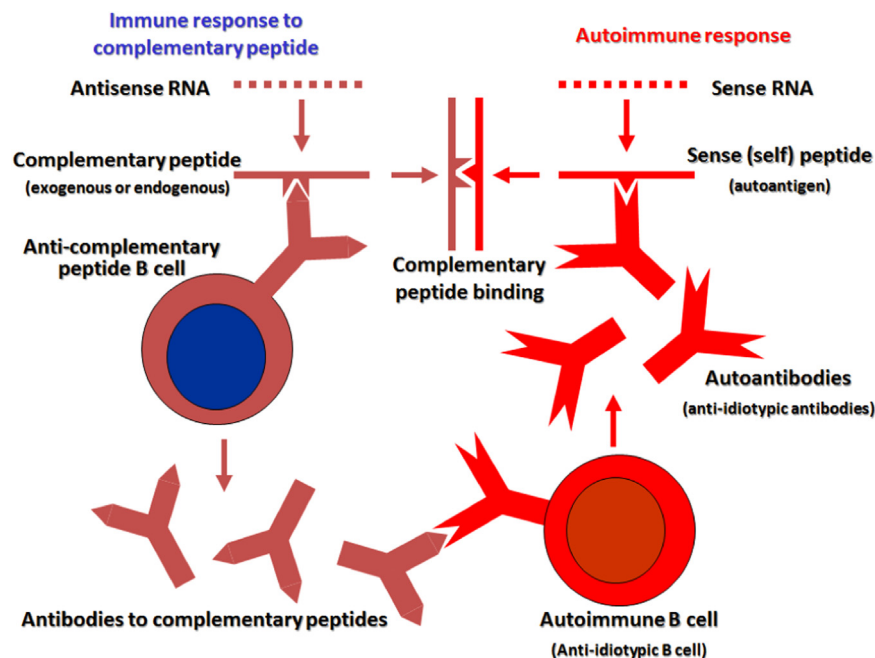


**Fig. 2. Theory of autoantigen complementarity.** The theory of autoantigen complementarity posits that the immunogen that incites the sequence of events leading to an autoimmune disease is not the autoantigen or its mimic but rather its complementary (antisense) peptide, protein, or its mimic. This complementary protein initiates production of antibodies that in turn elicit an anti-antibody response, referred to as an anti-idiotypic response. The resultant anti-idiotypic antibodies react with the autoantigen, which has an amino acid sequence that is complementary to the sequence of the initiating antigen. The source of this complementary peptide could be from translation of endogenous antisense RNA or of exogenous microbial or fungal DNA that has sequence homology with the non-coding DNA strand of the autoantigen.

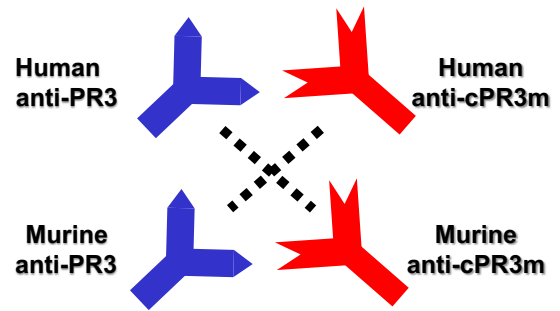


**Fig. 3. Production of both anti-cPR3m antibodies (Ab) and anti-PR3 Ab in cPR3m-immunized mice implies that the anti-idiotypes of the anti-cPR3m Ab are anti-PR3 Ab.** Because human anti-cPR3m Ab bind PR3-ANCA, we asked if mouse anti-cPR3m would also bind PR3-ANCA. Arrestingly, pooled sera from two cPR3m-immunized mice proved to contain antibodies that bound PR3-ANCA from patient 11. Illustrating the specificity of this reaction, the mouse anti-cPR3m Ab did not recognize PR3-ANCA from patient 13; thus, the epitope for recognition must be unique between patients, which would explain the lack of an idiotype interaction. Of note, pooled sera from two sham-immunized mice did not react with PR3-ANCA.

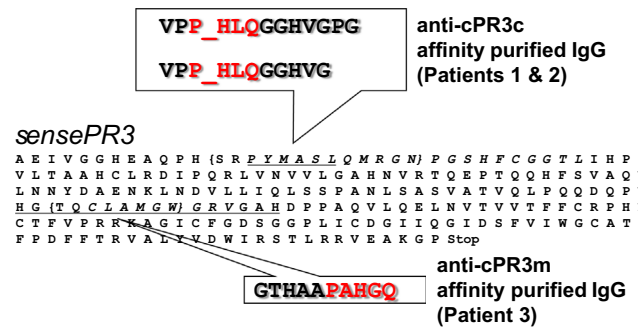


**Fig. 4. Complementary PR3 antibody epitope determination by proteolytic digest and mass spectrometry indicating epitope overlap between cPR3c and cPR3m epitopes.** Regions on sense PR3 protein corresponding to complementary sequences are indicated.

2004, 2005; Preston et al., 2005). It should be noted that the actual molecular complementarity is defined as one would define an antibody binding to an autoantigen akin to a lock and key.

Here, we offer a hypothetical discussion of how autoantigen complementarity, and findings spawned from the application of this theory, contributes to the acquisition of multiple 'hallmarks' of autoimmune disease.

## 2. Contributions of autoantigen complementarity to hallmarks of autoimmune disease

### 2.1. Autoreactive cells evade deletion

It is possible that the idiotypic network could facilitate a mechanism whereby autoreactive cells escape normal surveillance. Recently it was proposed that autoreactive cells are normally controlled by anti-idiotypic interactions in healthy individuals (Schulz et al., 2014). Although it is known that idiotypic antibodies exist and have functional consequences of import, the root immunologic mechanisms underlying their production and control are not fully understood. In 1974, Nobel laureate Niels Jerne proposed the network theory, which stated that active sites of antibodies bind to antigens (idiotypes) and to other antibodies at the same site (anti-idiotypes) (Jerne, 1974). Since this initial proposal of the network theory, it is clear that idiotypes and anti-idiotypes, collectively termed idiotypic pairs, exist and have been validated in animal models (Yu and Talbot, 1995; Routsias et al., 2003) and identified in numerous human diseases (Mendlovic et al., 1988; Dwyer et al., 1983; Routsias et al., 2002, 2011; Stea et al., 2006; Tzioufas and Routsias, 2010). In fact, it has even been proposed that the therapeutic value of intravenous immunoglobulin (IVIg) for humoral autoimmune disease is due to the inherent presence of anti-idiotypic antibodies that neutralize their pathogenic idiotypic counterparts (Blank et al., 2007, 2014). Furthermore, our group identified idiotypic pairs in mice and humans with ANCA vasculitis, and subsequently discovered that these anti-idiotypic antibodies in humans were also capable of binding to and interfering with plasminogen to increase risk of thrombosis in patients (Fig. 5) (Pendergraft et al., 2004; Bautz et al., 2008).

A majority of patients with PR3-ANCA vasculitis, and not MPO-ANCA vasculitis, harbored CD4+ TH1 memory cells responsive to a complementary PR3 peptide, defined by their ability to proliferate and secrete interferon gamma in the presence of peptide (Fig. 6) (Yang et al., 2008). Worth consideration, does exposure to a complementary PR3-like protein stimulate an immune response that, through the

anti-idiotypic pathway, leads to production of self-reactive T cells and autoantibody-producing B cells? Cells derived by this 'back door' mechanism could facilitate evasion of normal surveillance mechanisms.

### 2.2. Presence of asymptomatic autoantibodies

Pathogenic autoantibodies may be triggered by exposure to the autoantigen's complementary protein, which, in an individual with pre-existing asymptomatic autoantibodies, compounds the anti-idiotypic response. Asymptomatic autoantibodies are known to occur prior to the onset of autoantibody-induced ANCA vasculitis (Cui et al., 2010; Xu et al., 2011; Olson et al., 2013). The most interesting example of this phenomenon stems from work using sera samples from the United States Department of Defense (DoD) Serum Repository. Sera samples of members of the military diagnosed with ANCA vasculitis that were obtained before and after disease onset

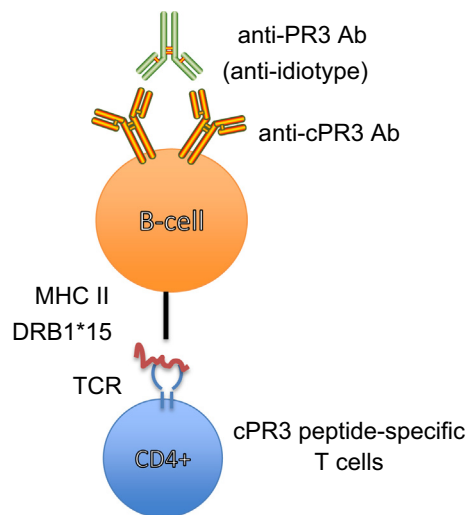


**Fig. 6. Summative illustration of different arms of the immune response to complementary PR3 (cPR3) detected in patients with PR3-ANCA vasculitis.** Memory T cells from patients were activated upon exposure to cPR3 peptide Yang et al. (2008). Ranked linear regression analysis indicated a significant likelihood ( $p=0.009$ ) that anti-cPR3 antibodies and cPR3-specific T cells coexist within an individual, consistent with an immunological history of an encounter with a complementary protein. DRB1\*1501 protein bound only specific complementary PR3 peptides in an *in vitro* assay, in agreement with *in silico* predictions Cao et al. (2011). Anti-cPR3 antibodies were detected and epitope mapped. Anti-cPR3 antibodies were in complex with PR3-ANCA forming an idiotypic pair.

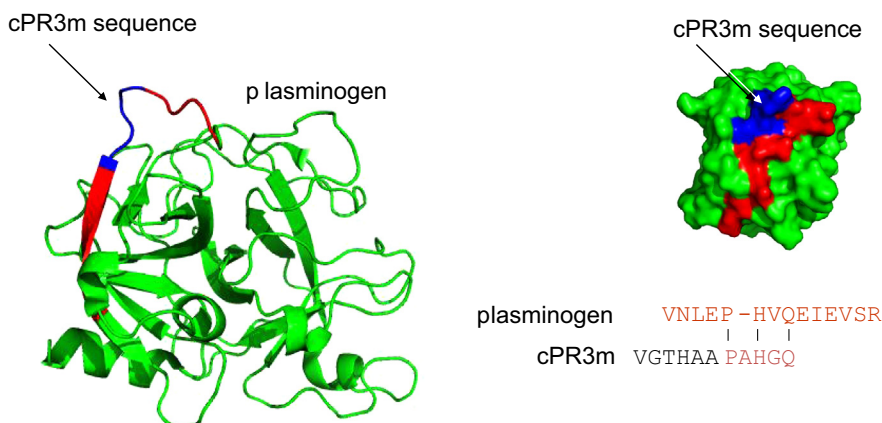


**Fig. 5. Exploration of the etiology and functional consequences of anti-cPR3 antibodies existing in patients with ANCA vasculitis.** The data shown summarize our findings from screening patients' plasmapheresis material for proteins recognized by these antibodies. A protein reactive with patients' affinity-purified anti-cPR3 antibodies was isolated and identified by mass spectrometry as plasminogen. Anti-plasminogen reactivity was found to be highly restricted to plasminogen and not to plasmin. These anti-plasminogen antibodies delayed fibrin clot dissolution *in vitro* and occurred most commonly in patients with PR3-ANCA vasculitis and coincident thrombotic events. This 13mer peptide contains a "PHQ" motif that we proposed to be the basis for cross-reactivity of anti-cPR3m antibodies with plasminogen.

revealed the presence of ANCA prior to the development of clinically overt disease (Olson et al., 2013). One could also propose that testing of the same sera samples from the DoD mentioned previously for the presence of anti-complementary PR3 antibodies would be greatly informative to determine the temporal relationship between ANCA and anti-ANCA before and after disease onset.

Studies to epitope map ANCA revealed an interesting adjacency of epitopes of asymptomatic autoantibodies to epitopes exclusive to active disease (Roth et al., 2013). Potentially, 'non-disease causing' autoantibodies evolve into 'pathogenic' autoantibodies through epitope spreading (Fig. 7). One can envision how anti-idiotypic responses would be permissive for epitope spreading, through the process of producing anti-anti-idiotypes and anti-anti-anti-idiotypes.

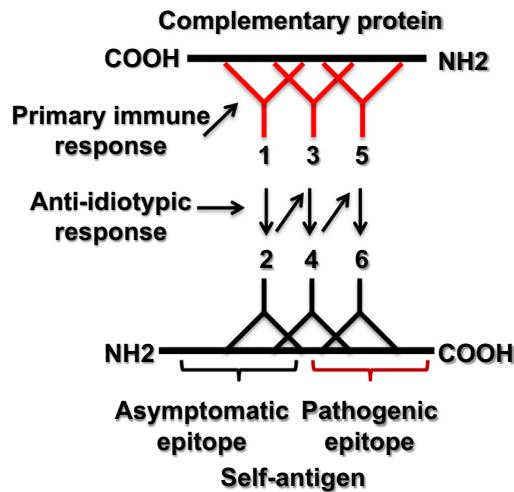


**Fig. 7. Proposal for the anti-idiotypic network as a mechanism for epitope spreading.** Mapping of epitopes on the crystal structure of MPO revealed that epitopes of natural autoantibodies are adjacent to epitopes exclusive to active disease Roth et al. (2013). This proximity of epitope alignment suggests the possibility of epitope spreading from non-pathogenic to pathogenic epitopes as disease develops.

### 2.3. Hyperactivity of Fc–FcR pathway

Normally all antibodies are glycosylated at conserved positions in their constant regions. In fact, at least 36 structurally unique oligosaccharide chains may be attached at each Asn-297 residue in both of the C<sub>H</sub>2 domains of the Fc region. It is clear that the structure of these attached carbohydrates can affect antibody activity. PR3-ANCA disease is associated with hyposialylation and hypoglycosylation of total IgG-Fc, as indicated by elevated levels of G0 truncated N-glycans lacking the terminal galactose (Holland et al., 2002, 2006). This deficit is reported to result in a positive amplification of the Fc/FcR signaling and has been linked with active PR3-ANCA disease affecting numerous organs (Espy et al., 2011). Interestingly, only a percentage of IgG had this deficit, lending to the question of whether these are the pathogenic autoantibodies and/or anti-idiotypic antibodies. Although application of the theory of autoantigen complementarity has not been applied directly to this hallmark, human anti-idiotypes are good immunogens since they can target Fc receptors on antigen-presenting cells, allowing efficient stimulation of both helper and cytotoxic T-cell responses. The immunogenicity of other anti-idiotypes may therefore be enhanced by human Fc targeting of antigen-presenting cells (Durrant et al., 2001).

### 2.4. Susceptibility to environmental impact

We are in the midst of an explosion of interest and energy focused on understanding the epigenetic underpinnings of human disease (Ziller et al., 2013). It is now becoming clear that epigenetic mechanisms are in place to maintain tolerance to self (Zouali, 2013). In fact, predisposition to autoimmune disease may develop due to breakdown of the DNA methylome as individuals age (Johansson et al., 2013). Epigenetic mechanisms link external stimuli and gene expression. Aberrantly expressed antisense transcripts have been reported and may be related to epigenetic dysfunction (Van Den Eynde et al., 1999). Perturbation of epigenetic silencing at the PR3-encoding *PR3* locus was responsible for loss of transcriptional regulation (Ciavatta et al., 2010; McInnis et al., 2014). An 'open'

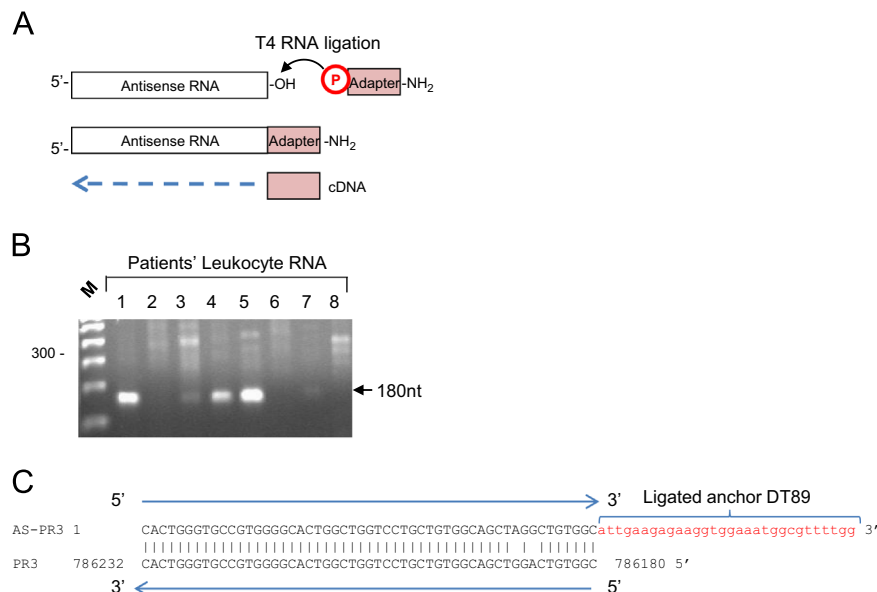


**Fig. 8. Detection of antisense PR3 transcript (AS-PR3) by strand-specific amplification of RNA using ligation-anchored RT-PCR (LA RT-PCR).** (A) Strategy: Before first-strand synthesis of cDNA, total leukocyte RNA was ligated at the 3' end to a short ribonucleotide anchor (DT89) tagging all RNA molecules at the 3' end, which orientates sense vs antisense RNA and generates a universal reverse transcription primer binding site. The anchor ribooligo was synthesized with a terminal –NH<sub>2</sub> group, eliminating the 3' OH, to ensure that ligation occurs only on the 3' ends of the mRNA. (Primers: Reverse transcription (RT) primer DT89-anchor primer sequence; 1st round PCR: FP-DT89 sequence and RP gene specific sequence of PR3 nt600; 2nd round PCR: FP-DT89 sequence and RP gene specific sequence of PR3 nt560.) (B) Patients with PR3-ANCA were positive for antisense PR3 RNA as detected by LA RT-PCR. A band of approximately 180 bp corresponding to the predicted AS-PR3 size was detected in three of eight patients (lanes 1, 4 and 5).

chromatin conformation that facilitates aberrant sense PR3 transcription would theoretically allow transcription from the non-coding strand. Recent work from our group reveals that antisense PR3 (AS-PR3) was detected in 3 of 8 patients with PR3-ANCA disease (Fig. 8). Conceivably, complementary PR3 protein would be produced in these peripheral neutrophils and shuttled to MHC class II complexes. Analysis of the MHC class II ligandome shows that 20% to 30% of self-class II epitopes are derived from cytosolic and nuclear proteins (Dengjel et al., 2005; Nedjic et al., 2009; Fissolo et al., 2009). Neutrophils also have the ability to function as antigen presenting cells (Cao et al., 2011). Peripheral neutrophils from patients homozygous for MHC class II DRB1\*1501 expressed surface DRB1\*15 protein, in response to TNF- $\alpha$  exposure, that bound amino acid sequences of sense-PR3 and of cPR3.

### 2.5. Antigenic modifications of self-proteins

Interactions of sense peptides with their complementary counterparts have implications in modifying self-protein conformation. It is becoming clear that antisense transcription is a naturally-occurring phenomenon that primarily serves to regulate gene expression (Yelin et al., 2003; Pelechano and Steinmetz, 2013); however, it is logical to presume that many of these transcripts can be translated into peptides and/or proteins. Numerous reports describe the phenomenon of peptides derived from complementary DNA strands that appear to form amphiphilic structures that bind one another (Mekler, 1973; Heal et al., 2002). Inhibition of the enzymatic activity of PR3 resulted when bound by complementary PR3 (Pendergraft et al., 2004). Similarly, others have shown specific binding between cystatin C and C4, which contain in their sequences peptides encoded by complementary DNA strands. The sense and antisense peptides were able to mimic or inhibit the interaction between both proteins, indicating that they play a preeminent role in the binding activity (Ghiso et al., 1990). These sense-antisense modifications of proteins could participate in the generation of neoantigens that in turn become autoantigens.

### 2.6. Microbial infections: Microbes carry proteins homologous to complementary proteins

Scientific evidence has linked autoimmune disorders with infection for well over a century. What is the connection? The most consistent microbial trigger in ANCA vasculitis appears to be *Staphylococcus aureus*, which produces a superantigen response that may

play a role in some patients (Stegeman et al., 1994; Mayet et al., 1999; Hellmich et al., 2001; Tadema et al., 2011). The theory of autoantigen complementarity (Pendergraft et al., 2004) is in line with a hypothesis published in 1983 of how a viral infection leads to autoimmune disease (Fig. 9) (Plotz, 1983). The host-cell receptor is envisioned as an internal image of the virus. The variable region of the primary antibody against the viral protein is similar in shape to the host receptor. The anti-idiotypic antibody against the variable region of the primary antibody consequently has a shape similar to the viral protein thus producing an internal image cascade. The anti-idiotypic antibody which now carries the internal image of the viral protein can bind to the host receptor and act as an autoantibody. Proteins complementary to the autoantigen PR3 are carried by many different microbes and viruses (Pendergraft et al., 2004, 2005). Amongst these microbes are Ross River Virus, *S. aureus*, and *Entamoeba histolytica*, all of which have been previously linked with ANCA disease (Stegeman et al., 1994; Davies et al., 1982; Pudifin et al., 1994). Pertinent to this finding, the onset of ANCA vasculitis is commonly associated with a flu-like illness (Falk et al., 1990).

## 3. Outstanding questions of importance and ways to address them

Although considerable progress has been made to understand the theory of autoantigen complementarity as well as the relationship to inherent hallmarks of disease, the majority of this work has been explored in the setting of ANCA vasculitis. Application of this theory to other autoimmune conditions, including those that do not affect the kidneys, is paramount and preliminary work would suggest promise. For example, autoantigens are known for numerous autoimmune conditions. We have shown that many autoantigens have complementary peptides that mirror microbial and fungal proteins (Pendergraft et al., 2005); thus, validation of the theory is possible in other humoral autoimmune diseases. As an example, another inflammatory autoimmune disease affecting the kidneys, anti-glomerular basement membrane (GBM) disease and its systemic counterpart Goodpasture syndrome, has a defined autoantigen with a restricted epitope (Cui and Zhao, 2011). Intriguingly, WKY rats were immunized with a complementary alpha-3 (a3) peptide (c-a3-Gly) comprised of amino acids that 'complement' the well characterized epitope on the a3(IV)NC1 domain of type IV collagen (pCol(24-38)). Within 8 weeks post-immunization, these animals developed crescentic glomerulonephritis, similar to pCol (24-38)-immunized rats, while animals immunized with scrambled peptide were normal. Anti-idiotypic antibodies to epitopes from c-a3-Gly-immunized animals were shown to be specific for a3 protein, binding in a region containing sense pCol(24-38) sequence. Interestingly, anti-complementary a3 antibodies were identified in sera from patients with anti-GBM disease, suggesting a role for 'autoantigen complementarity' in the immunopathogenesis of the human disease. This work supports the idea that autoimmune glomerulonephritis can be initiated through an immune response against a peptide that is anti-sense or complementary to the autoantigen. The implications of this discovery may be far-reaching, and other autoimmune diseases could be due to responses to these once unsuspected 'complementary' antigens (Reynolds et al., 2012). It is also important to note that this theory can apply to autoimmune diseases that affect organs other than the kidney. These autoantibodies attack neutrophils and monocytes causing them to prematurely release their noxious granular constituents; therefore, disease may manifest in any organ bed that is highly vascularized, including but not limited to the kidney, lung, gastrointestinal tract, eye and heart. In addition, theoretically complementary autoantigens can be predicted in silico for many autoantigens. As yet to be researched, it is likely that this theory can and should be applied to

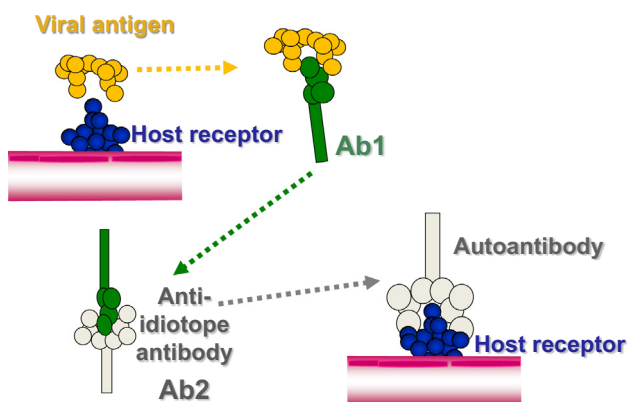


**Fig. 9. Hypothesis of how a viral infection leads to autoimmune disease.** The host-cell receptor is envisioned as an internal image of the virus. The inciting antigen is not the autoantigen, but a viral protein complementary in shape to the host's cell surface protein. This complementary protein elicits an antibody response, which in turn elicits an anti-antibody, referred to as an anti-idiotypic and subsequent anti-idiotypic response. This anti-antibody then reacts with the host's cell surface protein, now becoming an autoantigen. This process relies upon activation of the immune system's idiotypic network.

other defined B and T cell autoantigens in other autoimmune diseases, including systemic lupus erythematosus, bullous pemphigoid, autoimmune thyroid disease and type 1 diabetes.

Another critical area in need of further investigation with regards to the theory of autoantigen complementarity involves the development of specific tests that could specifically differentiate this theory from other theories of autoimmunity. Molecular mimicry is a similar but a different theory that proposes that an antibody to a microbe can cross-react and bind to an autoantigen thereby making it an autoantibody. A simple test here would be to determine in multiple autoantibody-mediated autoimmune diseases if either theory is playing a role by looking for the presence of one cross-reactive autoantibody or a pair of antibodies (idiotype and anti-idiotype). It would also be worthwhile to consider production of transgenic mouse models overexpressing a complementary autoantigen to assess for loss of tolerance and development of autoimmune disease and the presence of two separate antibodies (idiotype and anti-idiotype). We should also entertain the idea that autoantibody binding to autoantigen, akin to a lock and key, could result in conformational change of one or both that could then result in additional immunogenicity and tests to evaluate this are also needed.

Given ongoing massive sequencing efforts, another area of interest lies in the deciphering of the transcriptome. Antisense transcripts exist and primarily serve to regulate gene expression (Pelechano and Steinmetz, 2013); however, analysis of large transcriptomic datasets for antisense transcripts (either human or co-existing microbial) corresponding to autoantigen genes in mice and humans who develop autoimmune disease has yet to be reported. Conversely, re-analysis of microbial DNA sequence datasets to identify homologies between microbial genes and human antisense DNA of autoantigen genes could now also prove to be fruitful given the considerable increase in DNA sequence information.

#### 4. Conclusions

In conclusion, despite an explosion of effort over the last half century, we are yet to understand when and how autoimmune disease develops. The theory of autoantigen complementarity serves as one potential answer in need of additional validation. Furthermore, findings from the application of this theory dovetail nicely with each hallmark of autoimmune disease. These hallmarks of autoimmune disease provide a comprehensive and cohesive foundation for the field that can influence biomedical researchers in their quest for new treatments. We suggest that a holistic approach may provide clarity of mechanism and help to understand how and what treatments would be efficacious for treatment of disease.

#### Disclosures

None.

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