

In the spectrum of immunological diseases affecting various organs by inflammation and/or fibrosis, autoimmune reactions play an important role. Based on different studies both in humans as well as in animal models it becomes obvious that there is a broad range of pathologies that involve not only „primary“ autoimmune reactions but also other pathogenic mechanisms such as postinfectious and autoinflammatory processes. The heterogeneity within the immunological diseases may reflect the variable expression of autoinflammatory, autoimmune, and up to now unknown factors in disease development and manifestation. Based on histological and immunohistochemical examinations, IgG4-related sclerosing disease has been proposed as a novel clinicopathological entity with autoimmune phenomena but unknown etiology (chapter 1). The clarification of the etiopathological mechanisms is required to optimize prophylaxis, diagnostics and therapy. Especially, the application of novel and designer biological therapies (chapter 8) requires a better understanding of the processes that are involved in the genesis of immunological diseases. In chapter 2, some aspects of the role of epigenetic mechanisms and innate immunity in the pathogenesis of autoimmune diseases are described. Regardless of the underlying pathology, disease-associated autoantibodies are important biomarkers for the vast majority of non-organ and organ specific autoimmune diseases. However, to improve our understanding of these diseases and serological diagnostics it is necessary to search for novel autoantibodies, to further evaluate the real clinical relevance of known autoantibodies and to further develop and standardize the detection methods (chapters 3-5). Pathogenetic aspects as well as aspects of the serological diagnostics, including novel autoantibody specificities, novel methodologies and evaluation studies are presented for rheumatoid arthritis, systemic lupus erythematosus, antiphospholipid syndrome, systemic vasculitides, systemic sclerosis (chapter 6) and various organ specific diseases (chapter 7). In summary, the present volume highlights novel insights into the immune dysregulation, pathogenesis, serological diagnostics and biological therapies of autoimmune diseases.

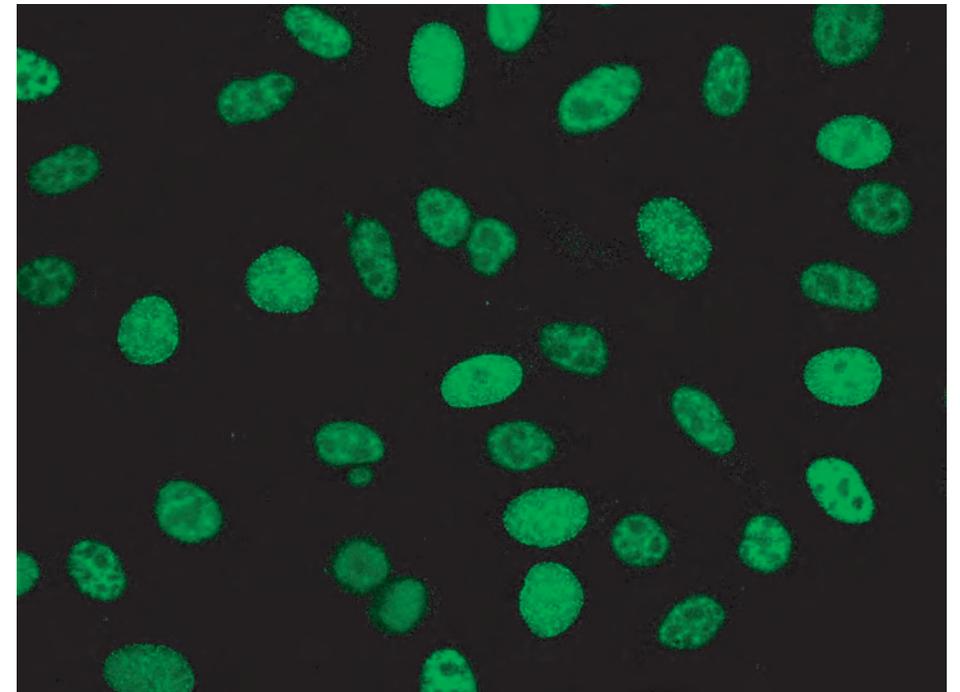
From Pathogenesis to Therapy of Autoimmune Diseases

K. Conrad, E.K.L. Chan, M.J. Fritzler, R.L. Humbel, P. von Landenberg, Y. Shoenfeld (Eds.)

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Report on the 9th Dresden Symposium on Autoantibodies  
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# Preface

In the spectrum of immunological diseases affecting various organs by inflammation and/or fibrosis, autoimmune reactions play an important role. Based on different studies both in humans as well as in animal models it becomes obvious that there is a broad range of pathologies that involve not only “primary” autoimmune reactions but also other pathogenic mechanisms such as postinfectious and autoinflammatory processes. The heterogeneity within the immunological diseases may reflect the variable expression of autoinflammatory, autoimmune, and up to now unknown factors in disease development and manifestation. For example, based on histological and immunohistochemical examinations, IgG4-related sclerosing disease has been proposed as a novel clinicopathological entity with autoimmune phenomena but unknown etiology. The clarification of the etiopathological mechanisms is required to optimize prophylaxis, diagnostics and therapy. Especially, the application of novel and designer biological therapies requires a better understanding of the processes that are involved in the genesis of immunological diseases. An important role in the pathogenesis of autoimmune diseases is discussed for epigenetic mechanisms and components of the innate immunity. The further exploration of those processes including the involved exogenous factors may offer novel prophylactic and therapeutic perspectives.

Regardless of the underlying pathology, disease-associated autoantibodies are important biomarkers for the vast majority of non-organ and organ specific autoimmune diseases. However, to improve our understanding of these diseases and serological diagnostics it is necessary to search for novel autoantibodies, to further evaluate the real clinical relevance of known autoantibodies and to further develop and standardize the detection methods. Pathogenetic aspects as well as aspects of the serological diagnostics, including novel autoantibody specificities, novel methodologies and evaluation studies for rheumatoid arthritis, systemic lupus erythematosus, antiphospholipid syndrome, systemic vasculitides, systemic sclerosis and various organ specific diseases are presented in this volume. We are sure that the novel insights into the immune dysregulation and pathogenesis described and discussed in this volume will stimulate novel concepts to improve diagnostics, prognostics and biological therapies of immune mediated diseases.

The editors



# Proteinase 3 and its receptors: linking innate immunity to autoimmunity in ANCA-associated vasculitides

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## Introduction

Proteinase 3 (PR3) is a multifunctional neutrophil-derived serine protease influencing cell cycle, differentiation, and cell death, and is the main target antigen of autoantibodies in ANCA-associated vasculitides (AAV), especially Wegener's granulomatosis (WG), known as antineutrophil cytoplasmic antibodies (PR3-ANCA). PR3-ANCA is thought to play a critical role in the pathogenesis of vascular damage in AAV. In contrast, it is not clear how the granulomatous inflammation, the hallmark of WG, is driven, and what is the relationship between granuloma and autoimmunity.

Current understanding of the molecular mechanisms by which PR3 regulates inflammatory processes and induces autoimmunity is still lacking. Recently, evidence shows that interactions of PR3 with two new molecules (protease-activated receptor-2: PAR-2 and Interleukin-32: IL-32) actively contribute to regulation of inflammation and immune functions in WG. This review mainly focuses on PR3-mediated dendritic cell (DC) activation and differentiation involving PAR-2 in WG.

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## PR3 and dendritic cells

One of the key questions with respect to the pathophysiology of human autoimmune diseases is how autoreactivity to the particular autoantigen(s) is initiated. The selection of a self-molecule as a target for an autoantibody response might be the consequence of a direct pro-inflammatory interaction of the molecule with a receptor on a gateway immune cell, such as an immature DC (the gateway-receptor model) [1]. PR3 is an ideal candidate for this role as it is not expressed (or quickly inactivated by serine protease inhibitors) in the extracellular space of healthy tissue, however, its level increases during infection, trauma and tissue necrosis. A number of studies demonstrated that at sites of inflammation an increased amount of PR3 is detected in the extracellular space in WG [2, 3, 4]. Most importantly, this protein was most prominently present within the affected tissues of the upper respiratory tract (i.e., nasal granulomatous lesions), which is the place, where the first clinical symptoms of disease occur- and possibly, where autoimmunity is generated [5]. Indeed, in early granulomatous lesions of WG-patients we have found evidence of maturation of autoreactive B-cells, as suggested by ANCA-encoding VH genes [5]. Therefore, granulomatous lesions themselves could represent a (tertiary) lymphoid-like tissue in which the autoantigen is displayed under inflammatory conditions [18]. Furthermore, PR3 was detected on the cell surface of neutrophils and a high membrane PR3 expression is a risk factor for WG [6, 7]. As PR3 can be mobilised upon apoptosis independent from degranulation, expression of PR3 on the surface of apoptotic blebs and ectosomes may render PR3 as an antigenic target.

It was reported that PR3 activates oral epithelial cells through G-protein-coupled protease activating receptor 2 (PAR-2) and actively participates in the process of inflammation such as periodontitis [8]. Furthermore, PARs provide a system that detects tissue injury and triggers a set of cellular responses that contribute to various responses including inflammation [9, 10].

Therefore, we tested the hypothesis whether PR3 possess the capacity to interact and activate PAR-2-expressing antigen presenting cells (APC) and thereby potentially links this inflammatory activity to the initiation of an adaptive immune response.

We demonstrated that PR3 induces phenotypic and functional maturation of blood monocyte-derived iDCs. PR3-treated DCs express high levels of CD83, a DC-restricted marker of maturation, costimulatory molecules CD80 and CD86, and HLA-DR. Furthermore, they become fully competent antigen presenting cells and can induce stimulation of PR3-specific CD4<sup>+</sup> T cells, which produce INF- $\gamma$  and drive the polarization towards a Th1 phenotype [11].

## PR3 and PAR-2

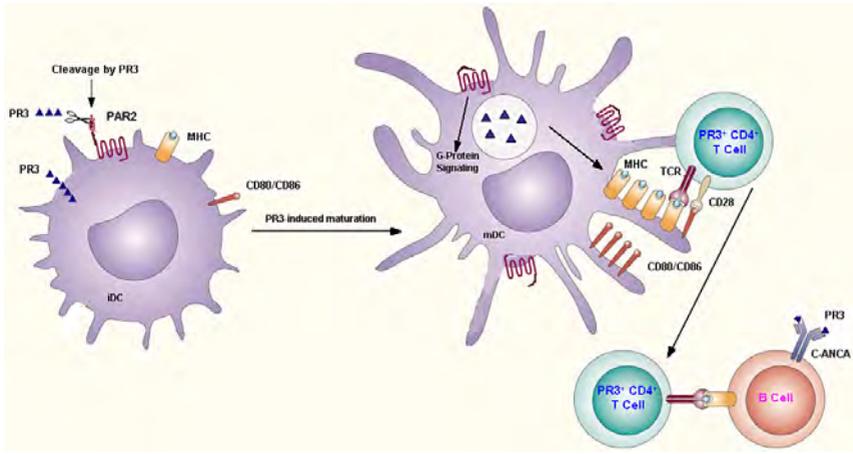
We next examined the pathway of PR3-induced maturation of DCs, with special interest to the PR3-receptor(s). We demonstrated that interaction of PR3 with PAR-2 leads to DC activation and differentiation.

To study the cleavage profile of serine proteases PR3, HLE and CG we used a classical approach: a synthetic peptide corresponding to a region spanning the cleavage site of the PAR-2, residues 32–45 ( $^{32}$ SSKGRSLIGKVDGT $^{45}$ ), was HPLC-separated after the cleavage and analyzed by amino acid sequencing and MALDI mass spectrometry. The results show that PR3 can cleave the synthetic peptide after the valine residue at position 42 (V $^{42}$ -D $^{43}$ ) which results in a C-terminal release of the activating peptide. Thus, PR3 has the potential to cleave the peptide on the opposite site of the tethered ligand (SLIGKV). In contrast, Uehara et al. reported that PR3 cleaves the PAR-2 peptide at the site R $^{36}$ -S $^{37}$  [8]. Differences in purity of the proteases may account for the divergent findings regarding the cleavage site of PR3.

Evidence suggests that the cleavage at the site V $^{42}$ -D $^{43}$  by PR3 may be functionally relevant: (1) a blocking antibody against PAR-2 inhibits the PR3-induced maturation of dendritic cells. (2) the principal mechanism of PAR-mediated activation is through G $\alpha_q$ -proteins, resulting in activation of phospholipase C (PLC). Therefore, the involvement of PAR-2 in DC maturation was further analysed by addition of a specific inhibitor of PLC in combination with PR3 or PAR-2 peptide agonist (PAR-2AP). It was demonstrated that the differentiation of DC by PR3 via PAR-2 activation uses the G $\alpha_q$ -proteins signaling pathway only partially; (3) PR3, but not HLE and CG, induced the expression of PAR-2 on DC, suggesting that this effect is PR3-specific; (4) the PAR-2 agonist peptide SLIGKV-NH $_2$ , corresponding to the PAR-2 tethered ligand, induced maturation of DC. PAR-2AP up-regulated the expression of CD83, HLA-DR, and costimulatory molecules on DC in similar intensity as compared to PR3, suggesting a similar mode of action; (5) HLE and CG digestion of the PAR-2 peptide resulted in different cleavages, but not at the activating site of PAR-2, suggesting that only the cleavage induced by PAR-2 is functionally relevant.

Our results suggested that DC maturation via PAR-2 activation by PR3 with Th1 polarisation may influence the immune response in the tissue microenvironment. In the setting of various non specific nasal tissue injuries (e.g., bacterial infection: *Staphylococcus*, drugs: cocaine), increased numbers of neutrophils that express "Wegener's autoantigen" at high levels are induced, providing the target to focus antigen-specific responses in tissue. PAR-2 may serve a physiological purpose similar to that of TLRs and senses endogenous "danger/alarm" signals in the environment, such as serine proteinase PR3, and its activation influences the development of both innate immune response, namely inflammation, and adaptive immune responses, and namely the decision of the immune system to respond to the self molecules. Thus, the primary role of PR3 as "danger signal" may alert

the immune system and may facilitate and promote tissue repair and restoration. Recently, a number of studies speculated that autoantigens may serve as “danger/alarm signals” and suggested a “beneficial role” of autoimmunity in tissue repair processes (Fig. 1).



**Figure 1.** *The gate-way receptor model:* In WG, expression of PR3 in the extracellular space is increased. PR3 stimulates the expression of PAR-2 on DC and activates PAR-2 resulting in maturation of DC, as indicated by expression of CD80, CD83, CD86 and HLA-DR and these PR3-maturated DCs stimulate CD4<sup>+</sup> T cells to generate increased expression of IFN- $\gamma$ . Hypothetically, T-cell activation by PR3-maturated DCs may finally promote the development of B-cells towards ANCA-producing plasma cells. Modified from [1].

## PR3 and IL-32-alpha

Interestingly, PR3 exhibits a unique property regarding the interaction with interleukin-32, a recently discovered proinflammatory cytokine that induces TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and 2 C-X-C chemokine family members involved in several autoimmune diseases [12]. PR3 is a specific IL-32 $\alpha$ -binding protein, independent of its enzymatic activity. However, cleavage of IL-32 by enzymatically active PR3 enhances activities of this cytokine. Therefore, specific inhibition of PR3 activity to process IL-32 or neutralisation of IL-32 by inactive PR3 or its fragments may reduce the impact of IL-32 on inflammation and autoimmune disease [12]. However, at the moment it is unclear whether PR3 functions primarily as binding protein for endogenous IL-32 $\alpha$  or cleaves IL-32 $\alpha$ , resulting in biologically active fragments.

We are currently investigating IL-32 expression on nasal biopsy from WG patients and circulating blood leukocytes and we detected a high IL-32-alpha intra-

cellular. Interestingly, IL32-alpha is partial colocalized with PR3 in the WG tissue (unpublished data).

## Summary and conclusions

The described observations raise the attractive hypothesis that PR3 expression results in cleavage and activation of PAR-2 on membrane of immune cells with its proinflammatory effects, such as induction of IFN- $\gamma$  production by CD4<sup>+</sup>T cells. Since the IL-32 production is caspase1/IL-18/IFN- $\gamma$  dependent [13], it is possible that the cleavage and activation of IL-32 by PR3 takes also place in DC which results in downstream inflammation. However, PR3 is also an IL-32 binding protein and the neutralising effect of soluble PR3, released from activated and/or dying neutrophils, on the IL-32 activity may represent a negative feedback mechanism at the inflammatory site. Thus, PR3 might have a dual effect in the pathogenesis of WG: first, it can act as an initiator of innate immunity at the frontline and second, PR3 might be involved in the negative feedback mechanisms that suppress ongoing inflammation. Presumably, in patients with genetic and immunoregulatory defects, tissue damage may initiate immune responses via PR3 that persist, despite repair of the damage, and culminate in inappropriate autoimmune, self destructive reactions, as seen in WG patients. Nasal carriage of *S. aureus*, that is associated with an increased rate of relapse [14], could trigger new activity in previously induced lesions.

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# The clinical paradox of esoteric and novel autoantibodies

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## Abstract

Autoantibodies (Aab) are biomarkers found in most autoimmune diseases, but in contrast to genetic biomarkers that reputedly indicate disease predisposition, the role of Aab is much less clear: some are pathogenic, some are disease specific; others antedate the full clinical expression of disease and serve as predictors of disease outcome; some may provide protection against disease; and some serve as signatures of inciting agents of autoimmunity. Over the five decades that followed the first description of Aab in systemic autoimmune rheumatic diseases (SARD), a few Aab have become best known as diagnostic biomarkers but even fewer are used as classification criteria for SARD. Because of growing evidence that some Aab antedate the clinical diagnosis, significant effort is being expended on gathering evidence about their value as predictors of disease onset and outcome. The ever growing lists of Aab associated with SARD have presented significant challenges to physicians and laboratory clinicians alike. The rapid expansion of knowledge about Aab has led to assumptions that many, if not most, of the newer Aab have little clinical value and hence they are often relegated to a category of “esoteric” Aab. However, the clinical value of some of these Aab becomes clearer if the perspective is changed from that of viewing them in the context of a clinical cohort to the context of a serological cohort. Simply stated, the clinical value of many Aab is based on the notion that if they are relatively sensitive and/or specific markers for a given SARD, that they have diagnostic and prognostic value. However, the clin-

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ical value of many esoteric Aab that are not sensitive markers of SARD becomes more apparent if disease associations are examined and certain diseases emerge as common constituents of serological cohorts. Hence, this contrast of disease associations between disease cohorts and serological cohorts can be regarded as a serological paradox when considering the clinical value of the broad spectrum of Aab that have been described to date.

## Introduction

Systemic autoimmune rheumatic diseases (SARD) are characterized by the presence of circulating autoantibodies (Aab) directed to a variety of intra- and extracellular components. Historically, Aab have been used primarily to assist the clinician in detecting, diagnosing, classifying and following the clinical course of SARD. Not long after the discovery of the LE cell and antinuclear antibodies (ANA), studies were designed to determine if Aab were also involved in pathogenesis of their associated diseases. In part, these investigations were sparked by observations that Aab in organ specific autoimmune disease such as Grave's disease, Addison's disease, pernicious anemia and myasthenia gravis could be linked to the pathogenesis of these conditions [1]. A half century of extensive studies of the pathogenic role of Aab in SARD has been marked by progress but, in many cases, a direct pathogenic role of most Aab in SARD remains unknown or controversial.

As studies of Aab progressed, it became clear that they were not an exclusive feature of established disease because they were also seen in first degree relatives of patients, individuals with *forme fruste* disease, patients with apparent unrelated conditions such as infections and malignancy, and even in normal blood donors. This picture became more complex when it became known that Aab antedated the onset of clinical disease [1, 2].

Most SARD are characterized by a spectrum of Aab directed to a wide range of nuclear, cytoplasmic, cell membrane and extracellular components. The Aab targets include proteins, nucleic acids, nucleoproteins, phospholipids, glycoproteins, and glycolipids. In systemic lupus erythematosus (SLE) there are now over 150 [3, 4], in systemic sclerosis over 50 [5, 6] and in antiphospholipid syndrome over 30 [7] Aab described and the list continues to grow. The focus of this review will be the challenges encountered in understanding the clinical importance of Aab, particularly "esoteric" Aab that are uncommon or not considered to be specific for certain diseases (Table 1).

## Autoantibody profiles: a challenge for diagnostic platforms

Very early in the study of SLE, it was obvious that an individual serum at any time during the clinical course of the disease contained multiple Aab that are typically directed to components of the same macromolecular complex [8, 9]. At

**Table 1.** Targets of esoteric autoantibodies\*.

Localization	Autoantigens
Golgi complex	golgins-67, -97, 95/gm130, golgins-160, 245, giantin
Endosomes	early endosome antigen 1 (EEA1), cytoplasmic linker protein (CLIP170), lysobisphosphatidic acid (LBPA), GRASP-1
GW Bodies (processing bodies)	GW proteins (GW182, GW2, GW3), hAgo2, Ge-1/Hedls, RAP55/LSm15, LSm4
Centrosome	pericentrin, PCM-1, -2, ninein, mob-1
Proteasome	A3-HC9, Ki~p28g
Assemblyosome — SMN complex	Sm, RNA helicase (Gu), fibrillarin, p80 coilin
Intracellular Exosome**	PM/Scl-75, -100, hCsl4, hRrp4, 40, 41, 42
Extracellular Exosomes***	
Cell Membrane	Aquaporin 4****

\* reviewed in [22, 24], \*\* see [97], \*\*\* see [70–74], \*\*\*\* see [70–74]

the same time, it was thought that sera from patients with polymyositis, systemic sclerosis (SSc), Sjögren's syndrome (SjS) and rheumatoid arthritis had a rather monospecific, if not narrow, autoantibody profile [10]. However, with the advent of multiplexed and multianalyte immunoassays, it has been clearly demonstrated that sera from these patients also commonly contain multiple Aab [11]. Support for the concept that multiple Aab are found in individual serum is supported by experimental and observational studies showing that Aab develop along a pathway described as intra- and inter-molecular epitope spreading [12–14].

The observation that SARD sera often contain multiple Aab has raised the question as to their clinical and/or pathological significance. In other words, does knowing that a patient with Raynaud's phenomenon has antibodies to RNA polymerase III and mitochondria add value to the clinical management of the patient? Or does knowing that a patient with a photosensitive skin rash, alopecia and arthritis has antibodies to Sm, chromatin and cyclic citrullinated peptides have any clinical relevance? The answers to these kinds of questions are, for the most part, unknown and this is due to several factors. First, the antinuclear antibody (ANA) test remains the screening assay of choice when physicians evaluate patients for a diagnosis of SARD. While the ANA screening test has many positive features, it is not the method of choice to identify multiple autoantibodies in an individual serum with high precision. Given the tremendous strides in identifying the molecular biology of many autoantigens described to date, the ability to identify multiple Aab in a single serum is now made possible by using multiplexed diagnostic plat-

forms [15, 16]. An important question is whether the indirect immunofluorescence (IIF) screening test has sufficient sensitivity to detect all clinically relevant Aab. To address this question we evaluated a cohort of sequential and unselected sera that were tested at a serum dilution of 1/160 as recommended by an expert committee [17] and that were negative in the HEp-2 IIF screening test and retested them by ALBIA (INOVA: ENA8) and found that 18 % had a positive result (unpublished data). Among the autoantibodies in this presumed Aab/ANA negative cohort, some were directed to ribosomal P protein, Jo-1 and Ro52. In recognition of this shortcoming of IIF screening tests, some laboratories have inverted the diagnostic algorithm by first screening sera with multiplexed technologies and then testing negative sera with an IIF assay. The cost-effectiveness of either approach requires thorough analysis.

To complicate the clinical diagnostic scenario further, it is widely known that many patients with one autoimmune condition often have, or develop, one or more additional autoimmune diseases (reviewed in [18]). A recent study that highlighted the importance of testing for multiple Aab found that approximately 50 % of patients with pernicious anemia had concurrent autoimmune thyroid disease [19]. In a recent study of SLE patients, clinically overt disease was found in only six percent but subclinical thyroid disease was identified in twelve percent and positive thyroid autoantibodies in the absence of thyroid disease in seventeen percent [20]. Further, thyroid Aab preceded the occurrence of clinical autoimmune thyroid disease in 70 % of these SLE patients. Thus, a major clinical challenge is the knowledge that in many cases the second autoimmune disease, whether it is Hashimoto's thyroiditis, celiac disease, or pernicious anemia, is undiagnosed. These observations and many others like them, point to the importance of detecting multiple Aab in a single serum sample.

In considering some of these challenges and the ideal diagnostic platform of the future, Bizzaro and his colleagues proposed that the ideal diagnostic platform would include the simultaneous detection of 25–30 Aab coupled with the detection of 2 or more immunoglobulin isotypes; a highly automated, high throughput system that had high analytical accuracy; and all being performed at a cost of 5–10 fold lower than that of conventional tests for these multiple Aab [21]. There is healthy skepticism in the industry that these parameters (particularly cost containment and kit pricing) can be met but in the face of rapidly escalating health care costs, economical and cost effective diagnostics will likely emerge the winners.

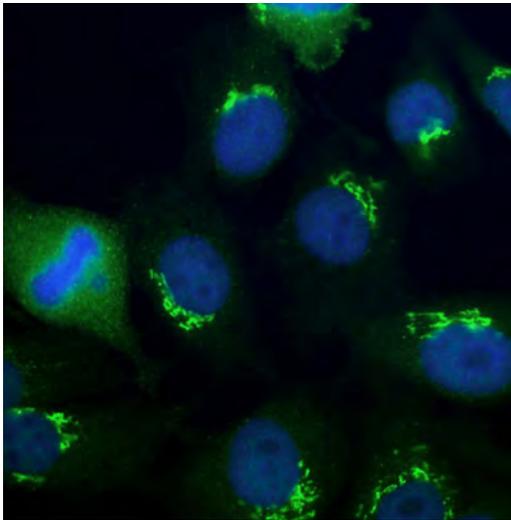
## **The autoantibody paradox**

In the past, most prospective and retrospective Aab analyses have focused on the most common Aab such as dsDNA and anti-Sm in SLE; anti-topoisomerase I and anti-centromere in systemic sclerosis; anti-SSA/Ro and anti-SSB/La in Sjögren's syndrome; anti-Jo-1 and anti-PM/Scl in polymyositis; anti-CCP and rheumatoid

factor in RA; anti-cardiolipin and anti- $\beta$ 2 glycoprotein I in anti-phospholipid syndrome. Such studies are typically based on the perspective that only the most common and relatively specific Aab in disease cohorts are clinically relevant. Admittedly, certain Aab are rarely encountered in cohorts with established diagnoses and for that reason they have been referred to as 'esoteric' Aab (i.e. seen in <5 % of disease cohorts) (Table 1) [22, 23]. However, it may not be widely appreciated that in a diagnostic laboratory setting esoteric Aab are detected as commonly as many other more widely studied Aab [24]. To explore and elucidate the concept of the Aab paradox represented by the studies of esoteric Aab, we will highlight four different esoteric Aab, anti-Golgi, anti-CENP-F, anti-GW bodies and anti-aquaporin 4 (AQP4), and by examining the disease associations of serological cohorts rather than disease cohorts, shed light on the value of identifying these Aab in a clinical setting.

### **Anti-Golgi antibodies (AGA)**

The Golgi complex is localized in the perinuclear region of most mammalian cells (Fig. 1) and is characterized by membranous stacks organized as distinct cis-, medial- and trans-Golgi networks [25–27]. The Golgi complex has a prominent



**Figure 1.** IIF of human autoantibodies to golgins in the Golgi complex are characterized as intense IIF lamellar and nearby speckled staining in region on one side and adjacent to the nucleus of HEP-2 cells. Original magnification  $\times 600$ .

function in the processing, transporting, and sorting of newly synthesized proteins from the rough endoplasmic reticulum.

In the last two decades the identity of Golgi complex autoantigens has been elucidated and led to the identification of a unique family of proteins, referred to as golgins [28]. The golgin autoantigens include giantin/macrogolgin/GCP372, golgin-245/p230, golgin-160/GCP170, golgin-95/gm130, golgin-97, 115, and golgin-67 [25–27, 29–33]. All of the golgins, except giantin/macrogolgin and perhaps golgin-67, are peripheral Golgi components bound on the cytoplasmic face of Golgi membranes. Giantin/macrogolgin has a single trans-membrane domain in the C-terminus but the majority of the molecule projects into the cytosol [34]. Golgin-245 and golgin-97 were localized to the *trans*-Golgi compartment [35] and gm130/golgin-95 was reported in the *cis*-Golgi compartment [36]. Golgin-245 and golgin-97/GM130 attach to Golgi membranes through a GRIP domain in their C-termini [37].

Unlike many human autoantigens that are found in cell surface blebs during apoptosis [38], the Golgi complex and other cytoplasmic organelles (i.e. mitochondria, lysosomes, endosomes, peroxisomes) co-clustered to a crescentic region of a misshaped “half-moon” nucleus [39]. In addition, a viral etiology in the generation of AGA was implied in studies showing that mice infected with a certain strain of the lactate dehydrogenase-elevating virus produce AGA [40].

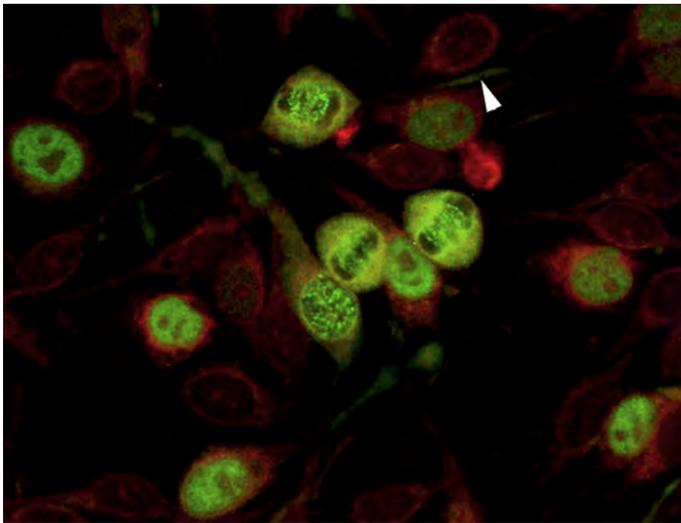
Anti-Golgi complex autoantibodies (AGA) were initially identified in the serum of a SjS patient with lymphoma [41] and this was followed by other reports that described AGAs in SjS [42], SLE [43], rheumatoid arthritis [44], mixed connective tissue disease [45], Wegener’s granulomatosis [46] and HIV infection [29, 47]. Immunoblotting and immunoprecipitation studies have shown that the proteins recognized by human AGA are remarkably heterogeneous [48] and suggests that other Golgi antigen targets are yet to be identified. In a study of 80 sera, the frequency of AGA was correlated with the molecular masses of the golgins [49]. For example, Aab to giantin/macrogolgin, the highest molecular weight golgin, were the most frequent, being found in 50 % of the AGA sera. By contrast, antibodies to golgin 97 were the least common, being found in only approximately 4 % of the AGA sera. The most reactive of the giantin/macrogolgin epitopes were those that encompass the C-terminal trans-membrane domain [49].

Although AGA are generally considered to be rare, at the Advanced Diagnostics Laboratory at the University of Calgary, they were found to be at least as common as antibodies to Sm [22]. The importance of AGA in clinical practices highlights the paradox discussed above. First, AGA are quite rare (<1 %) in unselected SARD sera when serological cohorts are studied, but up to 30 % of AGA positive sera are from SjS and patients with other SARD (reviewed in [24, 50]). Evidence indicating that AGA associate with a subset of SjS or other diseases has yet to be proven. However, it is of interest that high titer AGA have been suggested to constitute an

early sign of systemic autoimmune diseases even in the absence of clear clinical manifestations [51].

### Anti-CENP-F antibodies

Historically, we first became interested in Aab to centromere protein (CENP)-F when we published our studies of centromere related patterns of IIF produced by a subset of SSc patients with the CREST or limited cutaneous variant of the disease [52–54]. During the course of those studies we became aware that several IIF patterns resembled the typical CENP pattern but had remarkable differences [55]. One of these patterns we tentatively named NSP-II, which at first glance resembled anti-CENPs, but the staining was different from antibodies to CENP-A or CENP-B, which typically stain both interphase nuclei and mitotic chromatin. The NSP-II pattern did not have staining of interphase cells but gave a fairly typical CENP pattern in metaphase cells (Fig. 2). In addition, there was often staining of cells in anaphase, telophase (stem body) and cells in prometaphase (G2-G3) [56]. Eventually, the target autoantigen was identified as the CENP-F protein [57, 58]. CENP-F (also called mitotin) is a large (~400 kDa) coiled-coil, nuclear matrix



**Figure 2.** Typical indirect immunofluorescence staining pattern of CENP-F autoantibodies characterized by speckled staining of metaphase cells. Unlike antibodies to CENP-A/CENP-B, there is no staining of the majority of interphase cells. Staining of the midbody (white arrow) of telophase cells is seen in some sera but is not a universal feature of all CENP-F positive sera. Cells are counterstained red with Evan's blue. Photograph courtesy of Wendy Pollock, University of Melbourne & Gribbles Pathology, Australia.

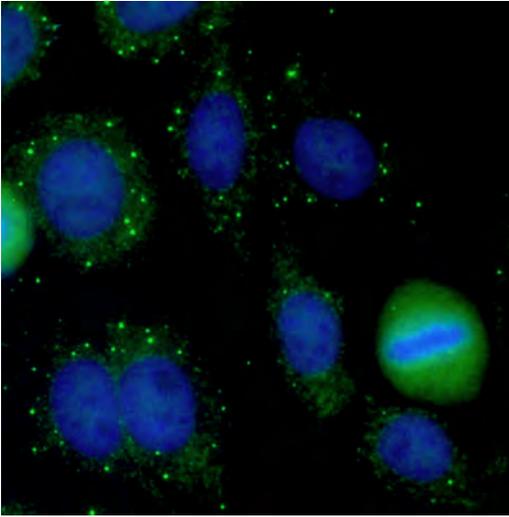
protein that plays a role in the kinetochore-mediated mitotic functions, participates in the regulation of cell division, and is used as a proliferation marker of malignant cell growth in clinical and research laboratories (reviewed in [59]).

CENP-F Aab can be detected by special studies that utilize immunodominant peptides [60], which we have adapted to the ALBIA platform. Initial clinical studies indicated that approximately 50 % of patients that harbor this Aab have a malignancy [57, 60] but more recent studies in our laboratory indicate that the prevalence of malignancy in this serological cohort is much higher, around 80 % (unpublished data). In a 2005 publication, Bencimon and colleagues reported the prevalence and specificity of anti-CENP-F Aab in 347 non-Hodgkin's lymphoma (NHL) patients before they received any therapeutic intervention. Using a radioimmune assay (RIA) they found that 7.2 % of NHL patients and 1.3 % control patients had anti-CENP-F Aab as determined by RIA. By IIF, 2.9 % of NHL patients displayed the CENP-F or CENP-F-like pattern, whereas none in the control group did. These data demonstrate that a significant incidence of anti-CENP-F Aab was observed in NHL before any treatment and that RIA has much higher sensitivity but lower specificity than IIF. We have similar experience in our laboratory: an analysis of a cohort of various malignancies (lymphoma, breast and prostate cancer, melanoma) in an ALBIA that used the two immunodominant fragments of CENP-F [60] found that the prevalence of anti-CENP-F was 20 % but there was no association with any one malignancy or stage of the disease. In addition, only ~50 % of sera with reactivity as detected by ALBIA had detectable CENP-F IIF staining (unpublished results). The key issue in utilizing and understanding CENP-F is that when cohorts of individual malignancies are surveyed for CENP-F Aab, the frequency is generally low (< 20 %) but when a cohort of anti-CENP-F patients is surveyed, at least 50 % have a malignancy.

### **Anti-GWB antibodies**

GW bodies (GWBs) are unique cytoplasmic structures involved in messenger RNA (mRNA) processing and RNA interference (RNAi). GWBs contain mRNA, components of the RNA-induced silencing complex (RISC), microRNA (miRNA), Argonaute proteins, the Ge-1/Hedls protein and other enzymes involving mRNA degradation [61, 62], many of which are autoantibody targets [63–66]. Sera with anti-GWB produce a typical cytoplasmic discrete speckled IIF pattern on HEP-2 and most other mammalian tissue culture cells (Fig. 3). A study to identify the GWB autoantigens targeted by 55 anti-GWB sera by ALBIA and immunoprecipitation of recombinant proteins indicated that Aab in this cohort of anti-GWB sera were directed against Ge-1/Hedls (58 %), GW182 (40 %) and Ago2 (16 %) [66]. Clinical data indicated that the most common clinical presentations were neurological symptoms (i.e. ataxia, motor and sensory neuropathy) (33 %), SjS (31 %) and the remainder had a variety of other diagnoses that included SLE, RA and

primary biliary cirrhosis (PBC). Although these studies of an anti-GWB serology cohort indicated that Sjögren's syndrome was one of the common diagnostic categories, a study of a cohort of a clinically-defined SjS cohort failed to identify a single patient with anti-GWB (unpublished data). Similarly, IIF studies of SLE and PBC cohorts indicated that less than 10 % of sera have anti-GWB antibodies (unpublished data and [67]).



**Figure 3.** Autoantibodies to GW bodies are characterized as numerous cytoplasmic discrete speckles that are distributed throughout the cytoplasm of HEp-2 cell substrates (Immuno-Concepts). GWBs mark the cellular sites for mRNA processing via the microRNA and other pathways. Original magnification  $\times 400$ .

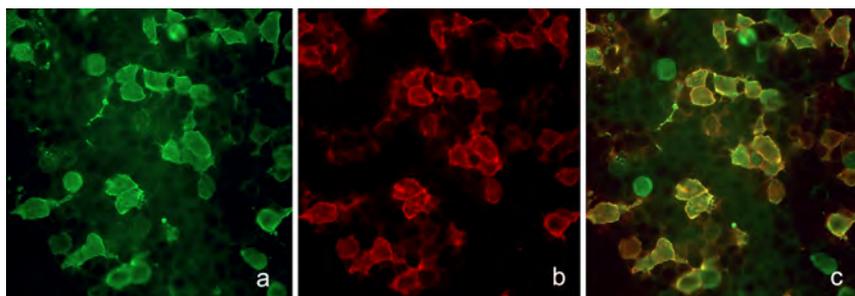
### Anti-Aquaporin 4 antibodies

The discovery of a specific autoantibody response directed against aquaporin-4 (AQP-4) in Devic's disease, a disease also known as opticospinal multiple sclerosis and, most commonly, neuromyelitis optica (NMO), [68–70] has been a significant step forward in defining, understanding the pathogenesis and giving a rational basis for therapeutic intervention of this condition (reviewed in [70–74]). NMO is a neurologic disease characterized by severe optic neuritis and transverse myelitis and attended by high morbidity and mortality. Of note, as implied in the name as 'opticospinal multiple sclerosis', NMO has features that overlap with multiple sclerosis (MS). Thus, an early and accurate diagnosis of NMO is extremely important because the optimum treatment for MS and NMO can differ considerably.

Aquaporin-4 (AQP4) is a water channel protein that is predominantly expressed in brain and spinal cord and evidence from clinical and pathological observations strongly supports the notion that AQP4 autoantibodies play a major role in the pathogenesis of NMO. For example, the pathological hallmark of NMO is a selective and characteristic deposition of immunoglobulins and complement on astrocytes at the *glia limitans*, which is accompanied by destruction and loss of glial fibrillary acidic protein and AQP-4 positive astrocytes followed by demyelination and eventually global tissue destruction [71, 75]. Of note, the distribution of NMO lesions in the brain and spinal cord correlates with the tissue distribution of AQP-4 expression. A recent immunogenetic study of Japanese patients showed that the frequency of the HLA-DPB1\*0501 allele was significantly increased in anti-AQP4 antibody-positive patients (89.5 %, odds ratio = 4.8; 95 % confidence interval = 1.6–14.3,  $n = 38$ ,  $P = 0.032$ ) compared with controls (64.0 %,  $n = 125$  T) [76]. Other evidence supporting an Aab-mediated disease is that clinical therapies designed to reduce the Aab load through plasmapheresis [75, 77], and/or targeting B lymphocytes [78], seem to be effective in alleviating some signs and symptoms of NMO. Taken together, this evidence supports the concept that NMO is an Aab-mediated autoimmune disease, although direct proof of the pathogenic role of AQP-4 antibodies or their temporal relationship to the disease has yet to be demonstrated.

In 2004, Lennon et al described an NMO IgG antibody using IIF on mouse cerebellum sections that showed a characteristic pattern of staining around microvessels, the pia, and Virchow-Robin spaces [68]. This assay was 58 % to 73 % sensitive and 91 % to 100 % specific for NMO. Since then, and following the discovery that AQP4 was the target antigen, a number of immunoassays have been developed to detect AQP4 antibodies: radioimmunoassay (RIA) [70], radioimmunoassay (RIA) [70], fluoroimmunoassay (FIPA) [79, 80], ELISA [81] and immunofluorescence utilizing cell based substrates transfected with the AQP4 cDNA [82, 83]. A study that compared the performance of some of these assays concluded that a cell based assay had higher sensitivity than the other assays [80]. Since the native form of the protein in western blots or recombinant full length or truncated proteins are poorly reactive, it is thought that the reactive AQP4 epitope is conformational or requires tertiary structure expression such as orthogonal arrays [84, 85]. Accordingly, the reactive portion of the protein has been localized to the third extracellular loop [85]. In an unpublished study, we used SPOT technology and previously published approaches [66, 86] that synthesize overlapping 15 mer peptides representing the full length AQP4 but no significant reactivity to these short peptides could be identified in four human NMO sera. We have also used a cell based assay wherein tissue culture cells are transfected with AQP4 (a gift of Euroimmun, Luebeck, Germany) (Fig. 4) and found high sensitivity (80 %) and specificity (90 %) in a small cohort of NMO and MS sera, results that are consistent with observations on cell based assays in other laboratories [83]. The Aab

titers on these substrates were  $> 1/640$  and we also found that this cell based assay had higher sensitivity than other immunofluorescence based assays using human optic nerve or cerebellum (unpublished). A particular challenge in using organ or tissue sections and cell based assays is to discriminate AQP4 antibodies from other autoantibodies that can coexist in the same sera, particularly in SLE and SjS patients. However, we found that by using a rabbit anti-AQP4 antibody in a co-localization study, that the AQP4 reactivity can be distinguished from other Aab (Fig. 4).



**Figure 4.** Autoantibodies to aquaporin 4 (AQP4) can be detected by indirect immunofluorescence on a cell line transfected with the corresponding cDNA (Euroimmun). A serum from a SLE patient with neuromyelitis optica reacts (panel a) with the AQP4 transfected cells that are specifically marked by rabbit antibodies to AQP4 (panel b). The two patterns of staining overlap as shown in the merged panel c. Cells stained by the SLE serum but not the rabbit anti-AQP4 represent cells reacting with other autoantibodies in the patient's serum. Original magnification  $\times 400$ .

While most attention has focused on anti-AQP4 in NMO, these antibodies have been anecdotally described in other conditions such as SjS [87, 88], SLE [89] and SLE associated with anti-phospholipid antibodies [90], myasthenia gravis [91, 92], gluten enteropathy [93] and following herpes zoster infection [94]. However, as with other esoteric Aab, it is important to note that in disease cohorts of conditions like SLE, SjS and MS this Aab is rare but when serological anti-AQP4 cohorts are examined, the antibody is remarkably specific and sensitive for longitudinal (multi-segmental) transverse myelitis and/or optic neuritis.

In summary, antibodies to AQP4 represent one of the more important recent breakthroughs in identifying a target autoantigen in NMO and allow a more accurate diagnosis of transverse myelitis seen in the setting of SLE [89, 95], SjS [88, 96]. The importance of this discovery is that this Aab is likely pathogenic and although the frequency of anti-AQP4 is remarkably low in SLE, SjS, MS and other diseases, a serological cohort of anti-AQP4 patients have a very high ( $> 80\%$ ) frequency of NMO, multisegmental neuromyelitis optica or related neurological problems.

## Conclusion

In conclusion, studies focused on Aab that are commonly seen in disease cohorts could overlook potentially important biomarkers for SARD and other autoimmune diseases. Equally important, a clear understanding of the clinical associations of esoteric Aab is of critical importance because the diagnostic laboratory must be able to comment on their clinical relevance. Prospective and retrospective studies are urgently needed to determine the association of diseases with these serological cohorts. Such studies must be attended by the continued development of Aab assays in multiplexed platforms that facilitate their detection in SARD and other autoimmune sera.

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## Abbreviations

**Aab**, autoantibodies; **ALBIA**, addressable laser bead immunoassay; **AGA**, anti-Golgi antibodies; **ANA**, antinuclear antibody; **AQP4**, aquaporin 4; **CCP**, cyclic citrullinated peptide; **CENP**, centromere protein; **dsDNA**, double stranded DNA; **ELISA**, enzyme linked immunoassay; **IIF**, indirect immunofluorescence; **LIA**, line immunoassay; **MS**, multiple sclerosis; **NHL**, non-Hodgkin's lymphoma; **NMO**, neuromyelitis optica; **OSAD**, organ specific autoimmune diseases; **RA**, rheumatoid arthritis; **RIA**, radioimmunoassay; **RNP**, ribonucleoprotein; **SARD**, systemic autoimmune rheumatic diseases; **SjS**, Sjögren's syndrome; **SLE**, systemic lupus erythematosus; **SSc**, systemic sclerosis; **Sm**, Smith antigen.

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