Review

Proteinase 3, protease-activated receptor-2 and interleukin-32: linking innate and autoimmunity in Wegener's granulomatosis

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ABSTRACT

Proteinase 3 (PR3) is a multifunctional neutrophil-derived serine protease influencing cell cycle, differentiation, and cell death. This molecule is the main target antigen of autoantibodies in Wegener's granulomatosis (WG) known as antineutrophil cytoplasmic antibodies (PR3-ANCA). WG usually starts as granulomatous inflammation of the upper respiratory tract (localized phase) and progress to systemic disease with PR3-ANCA-associated vasculitis (generalized phase). PR3-ANCA is thought to play a critical role in the pathogenesis of vascular damage in WG. 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. Recent findings provide evidence that PR3 might function as endogenous "danger/alarm" signal that communicates the presence of tissue injury to dendritic cells (DC) via protease-activated receptor-2 (PAR-2), triggers their maturation and instructs DC to induce Th1-type cell responses in WG. Furthermore, PR3 has the capacity to bind and activate IL-32, a recently discovered proinflammatory cytokine that has emerged as an important player in innate and adaptive immune response.

Collectively, these results delineate new pathogenic pathways at the molecular level and provide insights into the mechanisms by which PR3 may contribute to the early pathogenesis of WG supporting the pivotal role of the interaction of Wegener's autoantigen with the "gateway" receptor PAR-2 in mediating both innate and adaptive immune response in WG.

Introduction

Proteinase 3 (PR3) (EC.3.4.21.76) belongs to family of neutrophil serine proteases, as well as leukocyte elastase (HLE) and cathepsin G (CG), and the catalytically inactive azurocidin. Although there are strong structural and functional similarities among these serine proteases, PR3 has unique properties in many respects. In particular, PR3 is the major target antigen of antineutrophil cytoplasmic antibodies (PR3-ANCA) in Wegener's granulomatosis (WG), so-called "Wegener's autoantigen" (1). Furthermore, PR3 (synonyme myeloblastin) is involved in controlling the growth and differentiation of myeloid cells and has antibiotic activity. PR3 has also been shown to regulate the activity of cytokines and chemokines by proteolysis and to modulate their release through cellular activation by interaction with specific cell-surface receptors (for review 2). However, the 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.

The spectrum of WG

WG is a life threatening disease characterised by upper/lower respiratory necrotizing granulomatous inflammation, glomerulonephritis and necrotizing small-vessel vasculitis. The etiology of this disease is still unknown, but a genetic predisposition has been described, in particular the influence of distinct HLA- allele DPB1*0401 (3). In addition, environmental factors, including infections and/or chemicals, have been postulated as disease-driving agents. However, it is unclear how

genetic susceptibility in concert with other factors lead to the development of disease. Interestingly, epigenetic mechanisms appear to play a pivotal role in modulating pathogenetic events in genetically predisposed individuals with autoimmune diseases and further studies are needed to investigate the epigenetic alterations and their potential role in modulating pathogenetic events in WG (4).

The pathogenesis of WG involves at least two pathomechanisms – granulomatous inflammation and (ANCA-associated) vasculitis – that operate together to produce vessel and tissue damage specific for this disease. The involvement of upper airways with pathogenic granulomatous inflammation defines localized (initial) phase of disease and suggests that this disorder may be initiated by an aberrant cell-mediated immune response to an exogenous or even endogeneous antigen. Interestingly, the granulomatous histopathology occurs in the absence of any identifiable infecting organisms in WG, supporting the hypothesis that a tissue autoantigen may initiate the immune response. The characteristic expansion of a subset of T cells – effector memory T-cells (Th1-type $CD4^+CD28^-$ T cells) – in pathological specimens supports the idea that patients with WG have immunoregulatory defects that may initiate and perpetuate the granulomatous inflammatory lesions (5, 6). However, the nature of the antigen which induces granulomatous inflammation and the reason why immunoregulatory pathways fail the control of inflammation once initiated remain unclear. The autoantigen PR3 is abundantly displayed in affected tissue and recently, we showed that the granulomatous lesions of endonasal tissue of WG contain B-lymphocyte-rich areas in the vicinity of PR3+ neutrophils and plasma cells (7). However, the role of PR3 and/or autoreactivity to PR3 in the induction of the granulomatous inflammation is far less understood.

Generalized WG is characterised by small vessel vasculitis involving different organs and nearly all patients with active disease are positive for autoantibodies targeting "Wegener's autoantigen" PR3 (PR3-ANCA) (1, 8). Apart from their diagnostic value (specificity >95%) and correlation with disease activity, PR3-ANCA appear to play a direct pathogenic role in inducing systemic vasculitis by interacting with neutrophil granulocytes as *in vitro* and *in vivo* studies suggest (9).

The causative agent(s) and the molecular pathways leading to the development of ANCA, and from the granulomatous phase of disease to generalised systemic vasculitis during the course of WG are largely unknown. Most likely, in each stage of the disease, a cascade of events is required in addition to a distinct genetic background and epigenetic alterations (3, 4) to induce a progression to full-blown life threatening stage (generalised systemic vasculitis). The mechanisms driving the development and the persistence of granuloma or triggering the ANCA-mediated vasculitic phase have to be determined.

PR3-mediated DC maturation

One of the key questions with respect to the pathophysiology of human autoimmune diseases is how autoreactivity to the particular autoantigen(s) is initiated and maintained and whether the autoantigen itself influences the induction of autoreactivity. As the repertoire of human autoantigens is surprisingly limited, there have to be certain structural, biochemical or immunological properties which make these molecules immunogenic in predisposed subjects. Evidence from animal models suggests that DC not only play a crucial role in initiating and maintaining immune responses to foreign antigen, but also to self-antigen in autoimmune disease (10). DC recognize antigen through distinct pattern recognition receptors. Data from recently published animal models show that immune responses mediated by such pattern recognition receptors are important in the transition from autoreactivity to a self-antigen to autoimmune disease (11-13).

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) (14). 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 (15-17). 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, were the first clinical symptoms of disease occur- and possibly, where autoimmunity is generated (7). 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 (7). 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 (19, 20). As PR3 can be mobilized upon apoptosis independent from degranulation, expression of PR3 on the surface of apoptotic blebs and ectosomes may render PR3 as an antigenic target.

Recently, 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 peridontitis (21). In addition, Fields and colleagues demonstrated that murine serine proteases can act as danger signals, serving as maturation stimulus for DC via PAR-2 (22). Finally, our group has recently shown, that PAR-2 is expressed on alveolar macrophages in granulomatous lesions of the lung in WG (23). Thus, PARs provide a system that detects tissue injury and triggers a set of cellular responses that contribute to various responses including inflammation (24, 25).

Therefore, we tested the hypothesis whether PR3 possess the capacity to interact and activate protease-activated receptor-2- (PAR-2)-expressing antigen

presenting cells (APC) and thereby potentially links this inflammatory activity to the initiation of an adaptive immune response (induction of PR3-specific T cells).

In detail, 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 $CD4^+$ T cells, which produce INF-γ and drive the polarization towards a Th1 phenotype. Notably, we showed that PR3-maturated DCs derived from WG patients induce a statistically significant higher response of PR3-specific $CD4^+$ T cells as compared to Crohn's disease patients and normal donors (26).

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.

Since -its discovery in 1995, numerous *in vitro* and *in vivo* studies have demonstrated that PAR-2 is involved in the mediation of inflammation and immunity. However, its role is controversial as there is evidence of both pro- and anti-inflammatory activities (for review 27). PAR-2 is widely distributed through the body, especially in the epithelium, endothelium, fibroblasts, neutrophils, T cells etc., and displays a unique activation process: specific proteases of diverse classes and from different sources (host cells or pathogens) cleave within the N-terminal extracellular domain at a particular site, unmasking a new aminoterminus starting with the sequence SLIGKV, which binds intramoleculary and activates the receptor. The proteases can display opposite effects, either activating or disabling PAR-2 (for review 24). There is contradictory evidence about the activating or disarming effect of neutrophil serine protease PR3, HLE and CG on PAR-2 expressed on different type of cells (for review 27, 28). One possible explanation is that the pattern of glycosylation of PAR-2 depends on the cell type by which PAR-2 is produced (29). For PR3: both activation of PAR-2 in oral epithelial cells through cleavage at Arg^{36}-Ser^{37} (21) and potentially disarming cleavage sites downstream from Ser^{37} (30) have been reported. The "terminator" or "activator" activity of proteases such those released by neutrophils (*e.g.*, PR3, HLE) or pathogens (*e.g.*, *Pseudomonas aeruginosa* elastase) may have important biological consequences in the development of granuloma in WG.

Studies show that PAR-2 activation evokes the synthesis of cytokines and prostaglandines and activates signalling pathways such as those involving nuclear factor-κB and mitogen-activated protein kinase, which underpin inflammatory response. Recent studies both *in vitro* and *in vivo* demonstrate a potential role for PAR-2 in neurogenetic inflammatory pain, inflammatory bowel disease and rheumatoid arthritis (for review 27, 28). Other studies have implicated PAR-2 in the process of tissue protection and repair. For example, PAR-2 activation in airways evokes epithelium- and prostanoid- dependent relaxation in isolated bronchi from several species and activation of PAR-2 has a protective effect on motility impairment and tissue damage induced by intestinal ischemia-reperfusion (for review 27, 28). What defines the duality of function for PAR-2 has yet to be determined.

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 (Fig. 1). Thus, PR3 has the potential to cleave the peptide on the opposite site of the thetered ligand (SLIGKV). In contrast, Uehara *et al*. reported that PR3 cleaves the PAR-2 peptide at the site R^{36}-S^{37} (21). Differences in purity of the proteases may account for the divergent findings regarding the cleavage site of PR3.

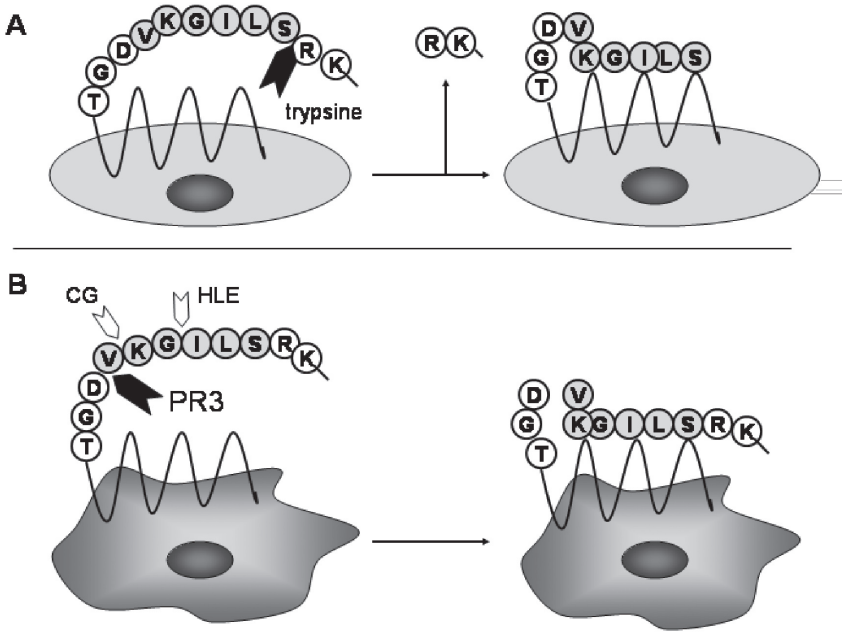

Fig. 1. *Cleavage of human PAR-2 peptide by serine proteases:* the cleavage product of PR3 ended with a valine residue at position 42 (V^{42}- D^{43}), CG cleaved between lysine and valine (K^{41}-V^{42}) and HLE between isoleucine and glycine (I^{39}-G^{40}). Trypsin, a known activator of PAR-2, was used as a positive control. Modified from (26).

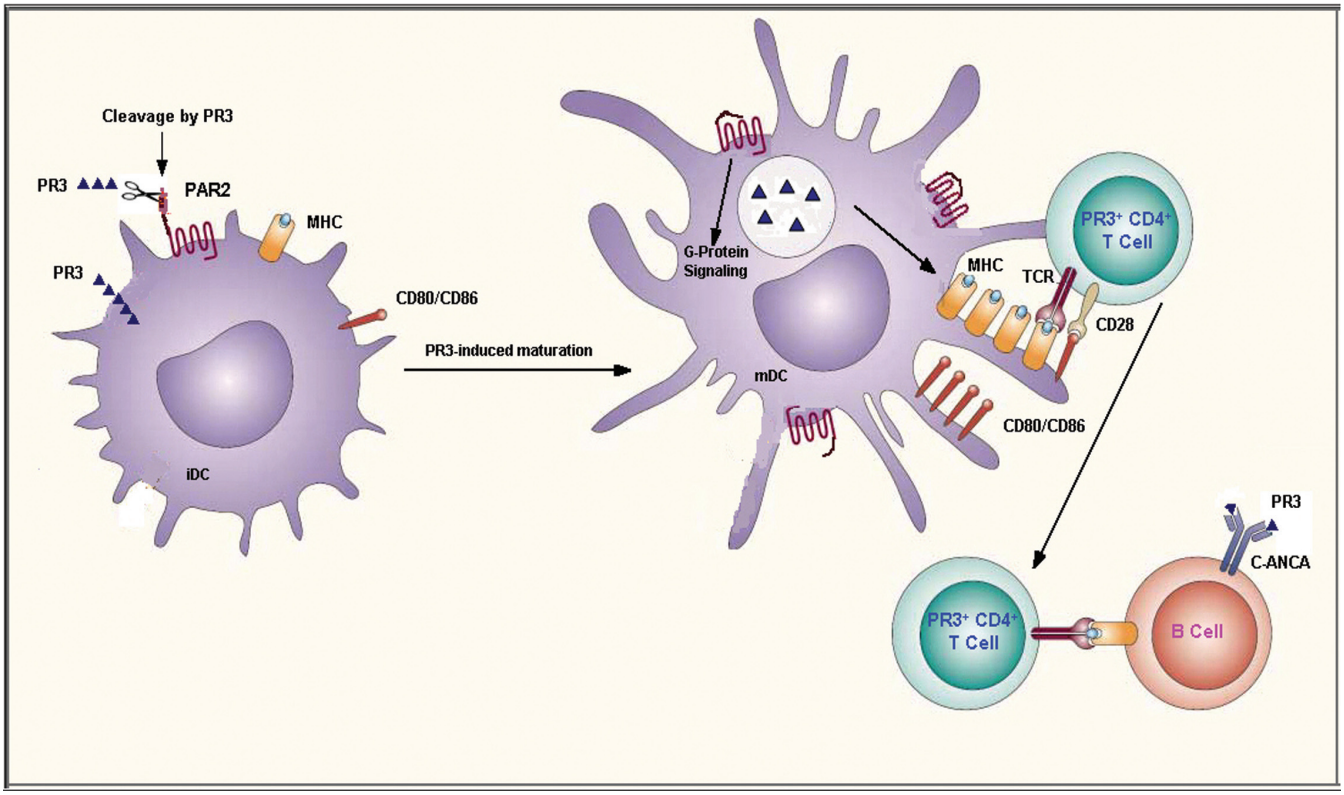

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Fig. 2. *The gate-way receptor model:* In WG expression of PR3 in the extracellular space is increased. PR3 stimulates the expression of PAR-2 on DC and activates PAR-2 resulting in maturation of DC, as indicated by expression of CD80, CD83, CD86 and HLA-DR and these PR3-maturated DCs stimulate $CD4^+$ T cells to generate increased expression of IFN-γ. Hypothetically, T-cell activation by PR3-maturated DCs may finally promote the development of B-cells towards ANCA-producing plasma cells. Modified from (14).

We assume that cleavage at site V^{42}-D^{43} may lead to release of the tethered ligand which can possibly act as an unbound agonist at the activation site of PAR-2, as it has been shown for other PAR-2 agonists like SLIGRL-NH_2 or tc-LIGRLO-NH_2.

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. This effect would not be expected if PR3 would disarm PAR-2 rather than activating the receptor; *(2)* the principal mechanism of PAR-mediated activation is through Gαq 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 Gαq 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 and can not be generalized to other serine proteases; *(4)* the PAR-2 agonist peptide SLIGKV-NH2, 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. Actually, our data show that both serine proteases (HLE, cleavage after position 39-40 and CG, after position 41) destroy the activating peptide by cutting inside its ligand sequence. Elastase and cathepsin G do not activate PAR-2 and this is in agreement with previous reports (86).

The receptor and the mechanism by which PR3 initiates PAR-2 up-regulation in DC is still unknown. The involvement of cell surface PAR-2 seems unlikely, since in our study the expression of PAR-2 on the immature DC surface was below the detectable limits, and at the moment we can only speculate that other receptor(s) may be targeted by PR3. DC express the broadest repertoire of Toll-like receptors (TLR), which can recognise a plethora of microbial compounds and endogenerous "danger/alarm" signals from injured cells, which will then can initiate immune responses by activating DC. Therefore, it has been demonstrated that HLE may activate TLR-4. This finding raises important questions: Is PR3 able to modulate the expression of TLRs on DC? How does this process affect PAR-2 expression? The answers to these questions may elucidate, as yet poorly defined, the role of PAR-2 and PR3 in the complex inflammatory network of WG.

Our data suggest that proteolytic activation of PAR2 on DC represent a new avenue in the direction to understand the early pathogenic events in WG:

DC can become stimulated to mature by PR3 released by damaged cells through the PAR-2 signaling pathway. These PR3 maturated DC become fully competent APC for the stimulation of autoreactive Th1-type $CD4^+$ T cells and DCs derived from WG patients induce a significant higher response of PR3-specific $CD4^+$ T cells as compared to Crohn's disease patients and normal donors. The gateway-receptor interaction model proposed by Plotz fits well with our data and hypothetically, chronic T-cell activation by PR3-maturated DCs finally promote the development of B-cells towards ANCA-producing plasma cells (Fig. 2) (14, 26). Further studies should prove the validity of this concept.

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 injury (*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. Evidence in support of a role of autoantigens in these processes include the finding that several other autoantigens (*i.e.*, histidyl-transfer RNA synthetase, retinal S-Antigen, etc.) can initiate an innate immune response and in sensitive individuals, adaptive immune response by attracting DC, T- and B-cells expressing chemokine receptors (31). The failure of adequate immunoregulatory mechanisms might promote conversion of this physiological autoreactivity to autoimmune disease (32).

Taken together, our studies have revealed a novel molecular pathway to clarify the effects of PR3 in early pathogenesis of WG and support the pivotal role of the interaction of Wegener's autoantigen with the "gateway" receptor (PAR-2) in mediating both innate and adaptive immune response in WG.

PR3 and IL-32-alpha

At sites of granulomatous inflammation in WG, elevated levels of extracellular released, neutrophil-derived serine proteases (PR3 and HLE) temporally coincide with high concentrations of bioactive cytokines (*i.e.*, TNF-α). This led to the hypothesis that neutrophil-derived serine proteases may control inflammatory processes through the proteolytic modification and release of proinflammatory cytokines. Cumulative data indicate that serine protease (*i.e.*, HLE, PR3) are directly involved in the modulation of the cytokine network (for review 2).

Interestingly, PR3 exhibits a unique property regarding the interaction with interleukin-32, a recently discovered proinflammatory cytokine that induces TNF-α, IL-1β, IL-6 and 2 CXC chemokine family members involved in several autoimmune diseases (33). PR3 is a specific IL-32α 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 (33). However, at the moment it is unclear whether PR3 functions primarily as binding protein for endogenous IL-32α or cleaves IL-32α, resulting in biologically active fragments.

Cytokine-induced inflammation is also linked to PR3 and PAR-2 expression and activation. Recently, it was demonstrated that IFN-γ is a potent inducer of PR3 expression in epithelial cells; this IFN-γ induced PR3 then cleaves PAR-2 and transforms the inactive IL-18 precursor into an active cytokine (34). Furthermore, several studies demonstrated that PAR-2 acts as an upstream regulator of cytokine in a variety of synovial leukocytes, including macrophages and mast cells (25). This is a significant finding in respect to WG, since it is known that this disease is driven by proinflammatory cytokines.

Interestingly, we are currently investigating IL-32 expression on circulating blood leukocytes and we detected a high IL-32-alpha intracellular and a small amount on the cell surface on human neutrophils. IL-32 is partially co-localized with PR3 on the plasma membrane (unpublished data).

Together, these 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-γ production by $CD4^+$T cells. Since the IL-32 production is caspase1/IL-18/IFN-γ dependent (35, 36), it is possible that the cleavage and activation of IL-32 by PR3 takes also place on the cell membrane of 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 (37), could trigger new activity in previously induced lesions.

Today, immunosuppressants (*e.g.*, cyclophosphamide) in combination with

glucocorticoids are still the standard therapies in WG. Biological agents (*i.e.*, TNF-α blocker, rituximab) offer new and promising therapeutic options. However, both therapies are far from ideal, since around 25% of patients have severe adverse events (38, 39) and remission can not sustained efficaciously. Consequently, WG has become a chronic, relapsing disease, with sometimes irreversible organ damage. Therefore, more efficacious and safer alternative treatments are needed. Agents intended to block PAR-2 activity or to neutralise IL32 are available and may be novel and important therapeutic biologicals (40). These agents are welcome alternative in view of the limitation of current therapies, and further investigations on the therapeutic potentials of these biologicals are warranted.

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