Staphylococcus aureus and T cell activation in Wegener's granulomatosis

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Wegener's granulomatosis (WG) is a form of systemic vasculitis which, although well studied, still constitutes a black box with respect to its pathogenesis and –physiology. The complexity of the disease is partly due to the fact that autoimmune phenomena, among which the most characteristic is the presence of autoantibodies to neutrophil enzymes (ANCA), can be observed alongside clinical features such as necrotizing vasculitis, granuloma formation in the upper and lower respiratory tract and glomerulonephritis. However, evidence for the causal link between these phenomena is still circumstantial, despite efforts to demonstrate notably the contribution of ANCA to vascular and glomerular lesions.

When dissecting the mechanisms that result in the generation of the disorder WG it may be helpful to distinguish between an initiator or trigger phase of the disease and an effector phase, in which the actual vascular damage ensues. Obviously, due to our restricted insight into the course of the disease, the borders between these two phases are vague and mechanisms bridging the two phases are probably present.

The current thesis is focused on pathophysiologic factors and mechanisms involved in the trigger phase of the disease and the events following this phase. The starting point of this thesis is the current concept that, while genetic determinants create a background that may favor development of WG, environmental factors are needed to trigger pathological processes. Among these environmental factors, bacterial infections, in particular with *Staphylococcus aureus*, have received special attention. Chronic staphylococcal carriage in WG patients is not only significantly higher than in healthy individuals, but this bacterial agent has been shown to be a risk factor for disease relapses. Moreover, various clinical studies assessing the efficacy of the antibiotic co-trimoxazole in WG have reported on its capacity to reduce the frequency of disease relapses and to induce partial or complete remission, underscoring the potential role of a bacterial infection in this disease.

Central to this thesis is the postulate that *S. aureus* and its superantigens (SAg) are implicated in the pathophysiology of WG. We have focused on SAg because these molecules are known as strong immunostimulators, in particular of T cells. In view of the disturbed immune balance in WG and the presence of autoimmunity, it is conceivable that bacterial SAg may be responsible for exaggerated activation of T cells, resulting in the creation of an appropriate costimulatory milieu for the production of ANCA.

The thesis has been organized in two parts, the first being dedicated to *S. aureus* and its SAg as risk factors for disease relapse in WG, while the second part deals with aspects of T cell activation, expansion and cytokine production in the context of SAg or WG-associated autoantigens as stimulatory agents. Furthermore, the experimental chapters are preceded by extensive reviews of the implication of bacterial SAg in vasculitides in general, and in WG in particular (**chapter 2**) and of the various (and yet unexplored) mechanisms by which S. aureus may be involved in the pathophysiology of WG (**chapter 8**). Since these respective reviews also present detailed discussion of the experimental chapters, the present summary will present a synopsis of the studies carried out in the context of this thesis.

PART ONE: *S. aureus* and its SAg: Risk factors in WG

S. aureus carriage is not eradicated by co-trimoxazole: Possible stealth strategy

Various clinical studies have demonstrated a beneficial effect of treatment of WG patients with the antibiotic co-trimoxazole. This effect consisted in a reduction in the number of disease relapses associated with treatment and the attainment of partial or complete remission. However, a direct effect of co-trimoxazole on the eradication of *S. aureus* has not been shown. In **chapter 3** we addressed the hypothesis that co-trimoxazole eradicates *S. aureus* carriage in WG patients. We took advantage of the fact that frequently, after antibiotic treatment is discontinued, *S. aureus* is detected again in these patients. Thus, we postulated that staphylococcal strains acquired after antibiotic treatment are

genetically different from strains carried prior to treatment, indicating that existing bacteria had been eradicated by the antibiotic co-trimoxazole. Relatedness between staphylococcal strains isolated before, during and after treatment with co-trimoxazole was assessed by genetic fingerprinting using a random amplified polymorphic DNA (RAPD) method.

Our study shows that, in most of the patients analyzed, staphylococcal strains isolated at various time points before, during and after treatment with co-trimoxazole are genetically identical, suggesting that the antibiotic was inefficacious in eradicating carriage of the bacterium. One of the interpretations that may be relevant for further investigation is that antibiotic treatment may induce the formation of staphylococcal small colony variants, which are able to survive in endothelial cells. This stealth strategy may enable *S. aureus* to elude detection as well as assaults by the immune system or antibiotics. Moreover, we suggest that the concept of co-trimoxazole efficacy in WG being due to its immunosuppressive properties rather than its antibiotic action, may deserve reevaluation.

Staphylococcal toxic-shock-syndrome-toxin 1 (TSST-1): Risk factor in WG

Staphylococcal superantigens (SAg), among which the enterotoxins A-E (SEA-SEE), the toxic-shock-syndrome-toxin-1 (TSST-1) and the exfoliative toxins A and B (ETA, ETB) have been postulated to be implicated in the pathogenesis of various autoimmune disorders on account of their strong immunostimulatory capacity. In WG we have previously shown that *S. aureus* strains carrying genes for SEA, SEC, TSST-1 and ETA are present. In **chapter 4** we have assessed the relative risk of WG patients carrying SAg-positive *S. aureus* to develop disease exacerbations.

This retrospective, longitudinal study, in which we detected and typed SAg genes by PCR, documents for the first time patterns of SAg carriage in a large cohort of patients and a defined clinical setting. We show that the staphylococcal SAg genes *sea*, *sec*, *eta* and *tsst-1* are particularly frequent in WG. Furthermore, we show that the presence of a SAg-positive *S. aureus* strain, irrespective of the type of SAg, is not a risk factor for the development of disease exacerbations. However, a subset of *S. aureus* strains, namely those carrying the *tsst-1* gene, constitute a significant risk factor for relapse.

Previous studies strongly suggest that TSST-1 is involved in the pathogenesis of another form of vasculitis, Kawasaki disease. In this disorder, the association between the presence of TSST-1 and activation of T cells, resulting in expansion of TSST-1 responsive T cell subsets has been demonstrated. Thus, this SAg may deserve further attention in the context of WG.

PART TWO: T cell activation in WG: Association with super- ant autoantigens

T cells in WG are chronically activated

In WG the immune balance is disturbed resulting in autoimmunity. Cellular, i.e. T cell immunity is thought to play a role in the pathology of the disorder, one of its important tasks being to provide costimulatory help for the production of ANCA. It is conceivable that excessive stimulation of T cells, for example by external factors such as bacterial agents, may promote increased production of ANCA, thus contributing to disease exacerbations. In **chapter 5** we have investigated the activation status of peripheral blood T cells from WG patients in relation to disease activity. Cellular and soluble activation markers (CD25, sCD25 and HLA-DR) were assessed by FACS or ELISA.

We show that T cell activation, expressed as the percentage of T cells expressing activation markers, is significantly increased in WG patients during both active disease and complete remission, as compared to healthy individuals.

Our findings suggest that a persisting stimulatory factor may be responsible for the chronic activation of T cells. We postulate that this factor may be a bacterial SAg.

The presence of SAg-positive S. aureus in WG is not associated with T cell expansions

Taking the postulate of the previous chapter as a starting point, namely that staphylococcal SAg may be responsible for persistent activation of T cells in WG, in **chapter 6** we investigated the

association between the presence of SAg-positive *S. aureus* and T cell expansions in WG. The interaction of a SAg with a T cell is mediated by T cell receptor V-beta chains encoded by particular V-beta gene families, and results in proliferation of the respective T cell subset. Thus, detection of T cell expansions, in conjunction with the concomitant detection of the corresponding SAg constitutes a strong indication of T-cell stimulation by that specific SAg. In a cross-sectional and a longitudinal study we measured expansions of T cell subsets expressing SAg-reactive T cell receptor V-beta chains by FACS and detected SAg genes in *S. aureus* strains by PCR.

Although present significantly more frequently than in healthy individuals, T cell expansions were not associated with the presence of the corresponding SAg. This finding suggests that a factor other than SAg may cause persistent T cell activation, observed in the previous chapter. Moreover, this finding is consistent with that of chapter 4, in which we showed that, except for TSST-1, SAg were not a risk factor for disease exacerbation in WG. Unfortunately, in the present study only few staphylococcal strains were positive for TSST-1 and thus it was not possible to investigate the relation between this particular SAg, T cell expansions and disease activity in more detail. This study suggests that a causal link between staphylococcal SAg and T cell activation in WG is either not present or not detectable.

The autoantigens proteinase 3 and myeloperoxidase induce a Th2 cytokine milieu and T cell proliferation in vitro

Whereas in the previous chapters we addressed the role of SAg, whose immunostimulatory potential virtually equals that of mitogens, in WG, in **chapter 7** we focused our attention on the potential of the WG-associated autoantigens proteinase 3 (PR3) and myeloperoxidase (MPO) to elicit T cell proliferation and cytokine production *in vitro*. T cell proliferation was used as an indicator for the capacity of these autoantigens to detect a population of autoreactive T cells in the peripheral blood. Cytokine production *in vivo* after stimulation with the autoantigens was investigated for the first time in this study with the aim of obtaining insight into the possible effects of these autoantigens *in vivo*. Although both autoantigens are localized in granules of polymorphonuclear cells, they can be shed as a consequence of activation or apoptosis of these cells. Apart from their proteolytic and toxic activity, and from their ability to induce production of autoantibodies (ANCA), it is conceivable that PR3 and MPO may as well elicit cytokine production by other cell types from the peripheral blood.

We stimulated peripheral blood mononuclear cells from vasculitis patients with either PR3 or MPO, as well as control stimuli. Both autoantigens provoked proliferation of CD4 T cells from individual patients, but also from healthy individuals, suggesting that, apart from potential activation of autospecific T cells, cross-reaction with a-specific T cell subsets takes place. Both autoantigens, but especially PR3, induced a TH2 cytokine milieu, characterized by high production of IL-10 and low production of IFN-γ. Moreover, we also detected high production of IL-6.

Building on *in vivo* and *in vitro* data available on the relation between T cell function, cytokine patterns and disease activity in WG, we speculate that PR3 can act as a modulator of cytokine milieu in vasculitis patients and healthy individuals. Under non-pathologic conditions (i.e. in healthy individuals and quiescent vasculitis patients), the effects of the pro-inflammatory cytokine IL-6, which is triggered by PR3, are counter-acted by IL-10, likewise produced upon stimulation with PR3. In this context PR3 is released in low amounts by occasional activation of PMN. Under pathologic conditions (patients with active PR3-ANCA associated vasculitis), strong activation of PMN by a pro-inflammatory cytokine milieu, in conjunction with the presence of ANCA, results in high release of PR3. Moreover, increased production of IL-6 by endothelial cells has been documented during active disease. Furthermore, IL-10 production in active generalized vasculitic disease was shown to be significantly lower than in localized vasculitic disease. Thus, it could be speculated that autoimmune and pro-inflammatory mechanisms, triggered by PR3 and

supported by ANCA, cannot be sufficiently counteracted by IL-10.

Outlook

Although we were not able to confirm a link between staphylococcal SAg, T cell activation and disease activity in WG, we have raised a number of questions with regard to the role of SAg and T cells in this form of vasculitis, which may deserve further investigation. We have shown that among staphylococcal SAg, TSST-1 is a risk factor for disease exacerbation in WG (chapter 4). Since treatment of WG patients with co-trimoxazole does not lead to eradication of *S. aureus* (chapter 3), new means may need to sought for monitoring *S. aureus* strains producing TSST-1. The mechanism by which this particular SAg may affect the course of disease remains unknown, since we found that the presence of SAg is not associated with activation and proliferation of peripheral blood T cells *in vivo* (chapter 6). However, persistent T cell activation is present in WG patients with WG, even during complete remission (chapter 5). It remains to be established which triggers are responsible for persistent T cell activation. The autoantigens PR3 and MPO, which are present in the circulation of WG patients, elicit moderate T cell stimulation and proliferation *in vitro* (chapter 7), suggesting that the high numbers of activated T cells that are detectable *in vivo* cannot be exclusively accounted for by autoantigenic stimulation. Cytokines that are produced by PBL *in vitro*, upon stimulation with the autoantigens PR3 or MPO (chapter 7) may be partly responsible for "bystander" T cell activation *in vivo*. Moreover, it is conceivable that *in vivo*, complex interaction of various stimuli may skew the immunologic balance towards T cell activation. In this scenario, *S. aureus* may play an important, though yet unidentified role. **Chapter 8** offers an extensive overview over the possible directions that studies into the role of *S. aureus* in WG may take. However, as has been suggested at various points and places in this thesis, a major challenge for future research will be to find out in how far studies in patients will be able to provide the answers we seek concerning pathophysiologic mechanisms.