The role of mucosal inflammation in initiation of rheumatoid arthritis
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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION
In ancient times, Hippocrates (400 BC) described a patient whose “rheumatism” was cured by the extraction of a tooth (1). In modern times, mucosal inflammation, including periodontitis, has been linked to the development of rheumatoid arthritis (RA) since the 1820s (2). In the early 20th century, tooth extraction was used in the treatment of RA. However, in the 1950s it was concluded that tooth extraction to reduce RA disease activity was not evidence-based and tooth extraction in the treatment of RA was abandoned (3).

The exact combination of factors and order of events triggering RA development are not known, but genetic and environmental risk factors have been identified (4), including HLA-Shared Epitope (HLA-SE) and smoking. Identifying additional risk factors that could play a role in initiation of RA is important. At high risk for developing RA are individuals who are seropositive for autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). Generation of these autoantibodies might be initiated by microbial infections at mucosal sites. In the studies described in this thesis we assessed whether mucosal inflammation plays a role in the development of autoantibodies which are a hallmark for RA.

In chapter 2, the current literature on the link between periodontitis and RA is summarized and discussed. It has been shown that RA and periodontitis share common genetic (HLA-SE) (5) and environmental (smoking) risk factors (6, 7), presuming that periodontitis and RA are connected. Next, periodontitis was linked to RA via the periodontal pathogen Porphyromonas gingivalis. This microorganism is unique in expressing its own peptidylarginine deiminase (PAD) enzyme, named PPAD (8). PPAD has been hypothesized to contribute to the pathogenesis of RA by breaking immune tolerance through formation of (bacterial and/or human) citrullinated proteins (9). Experimental evidence for PPAD in the link between periodontitis and RA comes from the exacerbation of collagen induced arthritis (CIA) in a mouse model after P. gingivalis strain W83 infection, whereas a PPAD-null mutant did not augment CIA in this mouse model (10). Furthermore, epidemiological studies have indicated that prevalence of periodontitis is higher in RA patients than in healthy subjects (11-15). Finally, some evidence exists that non-surgical treatment of periodontal disease may reduce the severity of RA in RA patients with moderate and severe chronic periodontitis (16, 17).

Besides periodontitis, mucosal inflammation in the lungs and intestine has been mentioned as a potential trigger for RA (18). This presumption is supported by the high prevalence of lung disease in RA patients (19). RA-related autoantibodies may (partly) originate in the lungs (20, 21) The latter suggests that autoantibody initiation in RA does not start in the joints but in the lungs. With regard to the gastrointestinal mucosa, this mucosa site is presumably involved in the development of RA too, since murine studies (22, 23) have demonstrated that specific gut bacteria can enhance or attenuate the susceptibility to experimentally induced arthritis. In humans, there is
less evidence for involvement of gastrointestinal mucosa, although several studies (24-26) identified differences in relative abundance of several gut-residing microbes (such as *Prevotella* and *Lactobacillus* species) between untreated early RA patients and controls.

Summarizing, a variety of mucosal sites has been presumed to be involved in autoimmunity and the initiation of autoantibodies such as RF and ACPA in particular. Therefore, the overall goal of the studies described in this thesis was whether RA specific autoimmunity is indeed initiated during mucosal inflammation.

**Does mucosal inflammation initiate development of RA associated autoantibodies?**

RA associated autoantibodies can be present in serum years before clinical onset of RA (27). As mentioned before, initiation of RA associated autoantibodies generation may occur at inflamed mucosal tissues, e.g., in the oral cavity, gut or lungs. The aim of the study described in chapter 3 was to assess systemic presence of RA associated autoantibodies in non-RA patients with oral or lung mucosal inflammation. Therefore, presence of RA associated autoantibodies (IgA and IgG anti-cyclic citrullinated peptide 2 antibodies (anti-CCP), IgM and IgA RF, IgG anti-carbamylated protein antibodies (anti-CarP) and IgG and IgA anti-citrullinated peptide antibodies against fibrinogen, vimentin and α-enolase) was determined in serum of non-RA patients with periodontitis (n=114), bronchiectasis (n=80) or cystic fibrosis (n=41). RA associated autoantibody levels in serum were compared to those of periodontally healthy controls (n=36) and established RA patients (n=86). Association of the diseases with RA associated autoantibody seropositivity was assessed with a logistic regression model, adjusted for age, sex and smoking. It was found that IgG anti-CCP seropositivity was associated with bronchiectasis (p<0.05), whereas the association with periodontitis was borderline significant (p=0.05). IgA anti-CCP seropositivity was associated with cystic fibrosis (p<0.05). The association of IgM RF seropositivity with bronchiectasis was borderline significant (p=0.05) and IgA RF seropositivity was associated with cystic fibrosis (p<0.05). Apart from the influence of smoking on the association of IgA RF in RA patients, there was no influence of age, gender and smoking on the association of RA associated autoantibody seropositivity with either of the diseases studied. Anti-CarP levels and IgG reactivity against citrullinated fibrinogen, α-enolase and vimentin were only increased in RA patients (p<0.0001). From this study it was concluded that, although overall levels were low, RA associated autoantibody seropositivity was associated with lung mucosal inflammation and may be associated with oral mucosal inflammation. To further determine whether mucosal inflammation functions as a site for induction of RA-autoantibodies and precedes RA, longitudinal studies are necessary in which RA-autoantibodies of specifically the IgA isotype should be
assessed in inflamed mucosal tissues and/or in their inflammatory exudates (e.g., gingival crevicular fluid or sputum) in patients who are at high risk to develop RA.

As mentioned, periodontitis has been hypothesized to play a role in the initiation of RA via excessive citrullination in the periodontium. This excessive citrullination could induce ACPA formation (28). It has been shown that neutrophils are abundantly present in inflamed periodontal tissues (29). These neutrophils express PAD4, the enzyme involved in citrullination (30). Besides phagocytosis and degranulation, neutrophils can form neutrophil extracellular traps (NETs), whereby a network of extracellular fibers is formed which traps pathogens. The trapped pathogens are killed with anti-microbial peptides such as neutrophil elastase and cathepsin G present in the NETs. Histones are the major components of NETs that have a high affinity for DNA and are able to bind microbes. Besides that, histone citrullination is an important step in NET formation (31). In the study described in chapter 4 we assessed presence of citrullinated histones in inflamed periodontal tissue, and whether sera from periodontitis and RA-patients contain autoantibodies against citrullinated histones. Citrullinated histones H2A, H2B (32) and H4 (33) have been described as targets of autoantibodies in RA patients. We assessed whether histone H3 is also a target of autoantibodies in RA patients, since histone H3 was found to be citrullinated by PAD4 in neutrophils after an inflammatory response (34).

The presence of citrullinated histone H3 and PAD4 was determined in periodontal tissue from 15 non-RA periodontitis patients by immunohistochemistry. Sera from 36 healthy controls, 113 periodontitis- and 84 RA-patients were assessed on the presence of autoantibodies against citrullinated histones by Western blot and against citrullinated histone H3 by ELISA. The results showed that seropositivity for anti-citrullinated histone H3 was found significantly more often in RA-patients compared to healthy controls and periodontitis patients (p<0.0001), while anti-citrullinated histone H3 levels were similar between healthy controls and periodontitis patients. Furthermore, anti-citrullinated histone H3 levels were higher in anti-CCP positive RA-patients compared to anti-CCP negative RA-patients (p=0.0008) and correlated moderately with anti-CCP levels (p=0.22, p=0.0462). No associations were found between anti-citrullinated histone H3 levels and the periodontal status or smoking behavior of RA patients, however. The presence of citrullinated histone H3 in inflamed periodontal tissue supports a role for periodontitis in the generation of antigens that are targeted by ACPA.

The intestine represents the largest mucosal surface of the human body. It is known that the intestine contains citrullinated proteins under conditions of inflammation (35). Therefore, we assessed in the study described in chapter 5 whether RA associated autoantibody levels are elevated in patients with inflammatory bowel disease (IBD) without RA, this because 5-20% of IBD patients report joint complaints during the disease course (36).
The studied IBD patient cohort consisted of 391 patients diagnosed with either ulcerative colitis \((n=226)\) or Crohn’s disease \((n=165)\). IgA and IgG anti-CCP, and RF (IgM and IgA) levels were measured in sera from IBD patients, and as reference groups 36 healthy controls and 86 RA-patients were included. The results showed that anti-CCP levels, especially IgA, were increased in IBD-patients compared to healthy controls. As expected, the levels of anti-CCP were lower than in RA-patients. Furthermore, none of the IBD-patients had IgG anti-CCP levels above the cut off used for diagnosis of RA as well as that IgM and IgA RF seropositivity was comparable to that of healthy controls. Arthralgia was the main reported arthropathy by IBD patients, but arthralgia was not more often reported in IBD patients who were seropositive for arthritis associated autoantibodies. In other words, the development of arthralgias in IBD patients is independent of the presence of these autoantibodies.

The studies described in chapters 3-5 support the hypothesis that mucosal inflammation can induce development of ACPAs. Our studies varied from those reported in literature \((37-40)\) in that anti-CCP levels were measured at a lower sample dilution \((1:10\) instead of \(1:50)\). Therefore, we were able to determine anti-CCP levels below the diagnostic cut off, with a higher accuracy. This meant we could measure minor but detectable presence of anti-CCP in patients with mucosal inflammation compared to healthy controls. Furthermore, we determined that the inflamed periodontium expresses citrullinated histones that are targeted by ACPA. The presence of citrullinated histones in the inflamed periodontium supports the hypothesis that the inflamed periodontium may be indeed a site where ACPA initiate. Moreover, RF seropositivity was increased in non-RA patients with mucosal inflammation compared to healthy controls, but RF is less specific for RA than ACPAs and actual RF levels do not allow for drawing firm conclusions yet with regard to arthritis development. The presence of RF is likely to be protective for the host in non-RA patients with conditions such as infections \((41)\). The protective role of RF might be contributed to the ability of low-affinity RF to clear IgG anti-pathogen antibodies \((41)\).

In our studies we did not assess the mechanism that drives the development of ACPA. It has been suggested that environmental factors \(\text{e.g., smoking}\) play a role the initiation of ACPA, while genetic factors \(\text{e.g., HLA-SE}\) are responsible for further increasing ACPA levels \((42)\). In our studies we showed that non-RA patients with mucosal inflammation have higher ACPA levels than healthy controls. However, a limitation of our studies in these non-RA patient cohorts was that the prevalence of genetic risk factors for RA was not assessed. Therefore, it remains to be determined whether the increase of ACPA levels that was observed in non-RA patients with mucosal inflammation is attributed to environmental factors only or also has to attributed to genetic factors.
Recently, a mechanism was proposed that links the development of ACPA in HLA-SE positive individuals to T-cell mediated cross-reactivity between human- and pathogen-derived peptides (43). Thus, longitudinal studies that measure the ACPA response in non-RA cohorts with mucosal inflammation in combination with HLA-DRB1 genotyping are likely to provide additional insight into the development of ACPA. Furthermore, future studies that investigate the human gut and/or oral microbiome in patients at risk for developing RA could identify microbes which are involved in ACPA development via cross-reactivity between microbial and human peptides.

**Prediction of RA development in seropositive arthralgia patients**

As early therapeutic intervention in RA-patients has been shown to result in a better disease outcome, it is of paramount importance to identify patients who are at high risk of developing RA. Seropositive arthralgia patients (SAP)(44) are defined as individuals with (a history of) arthralgia who are seropositive for RF and/or ACPA. It has been reported that around 35% of these patients develop RA after a median of 12 months (45). Therefore, SAP were investigated to search for immune factors which could add to the already described risk factors in identifying patients that progress to RA during follow-up. Besides autoantibodies, also the expression of cytokines, cytokine-related factors and chemokines indicative for an activated adaptive immune system is increased in the pre-clinical phase of RA (46).

In the study described in chapter 6 we assessed whether infection with *P. gingivalis* is prognostic for the development of RA in SAP. Infection with *P. gingivalis* was determined by measuring the antibody response against *P. gingivalis* in a prospective follow-up cohort of 289 adult SAP. During follow-up (median 30 months), 94 (33%) of the SAP developed RA. However, SAP who developed RA did not have elevated anti-*P. gingivalis* antibody levels at baseline compared to SAP who did not develop RA within the follow-up period. When using cut off values for anti-*P. gingivalis* seropositivity, the proportion of IgA and IgG anti-*P. gingivalis*-positive patients was even higher in SAP who did not develop RA. A weak correlation of IgM anti-*P. gingivalis* with ACPA was found in SAP who developed RA. Multivariate analysis showed also no added influence of anti-*P. gingivalis* antibody levels, C reactive protein (CRP) levels, age, gender and smoking on RA development. Within the limitations of this study, it can be concluded that anti-*P. gingivalis* antibody levels are not prognostic for RA development. However, we determined this antibody response using whole *P. gingivalis* lysate as antigen. Future studies in SAP should focus on measuring the antibody response against specific bacterial peptides/proteins that could be cross-reactive with human proteins that are targeted by ACPA. Such studies will demonstrate whether initiation of ACPA is indeed a result of cross-reactivity between bacterial and human antigens.
In healthy conditions, maintaining tolerance to self is essential for protection against autoimmune diseases. Besides the apoptosis of immature self-reactive lymphocytes in the thymus and activation-induced cell death of mature T-cells, suppression of immune responses against self-proteins is maintained by regulatory T-cells (Tregs). The function of Tregs is to suppress immune activation by modulating diverse cellular functions such as T-cell proliferation and cytokine production. Tregs are defined by the expression of CD4 and CD25 on the cell surface and on the expression of the nuclear suppression factor forkhead box protein 3 (Foxp3). Further subdivision into separate functional Treg subsets can be performed on the basis of CD45RA and CD45RO expression (47).

In the study described in chapter 7 we assessed whether altered peripheral Tregs and defined subsets, besides a broadened ACPA response, may qualify as prognostic biomarkers for RA development in SAP. We followed a cohort of 34 SAP who were prospectively followed on the development of arthritis every 6 months for at least 2 years. Every visit, peripheral blood mononuclear cells (PBMCs) were isolated and stored. At inclusion, peripheral Treg (CD4+CD25+FoxP3+) numbers and subsets, defined as CD45RA+FoxP3low naive Tregs (Fr I), CD45RA FoxP3high activated Tregs (Fr II) and CD45RA FoxP3low non-Tregs (Fr III), were compared to age- and sex-matched healthy controls and treatment-naive RA patients. SAP that developed RA were compared to non-switchers and analyzed for Treg numbers and functional Treg subsets at inclusion. Also, peripheral Treg and Treg subset numbers were compared in switched SAP before and at diagnosis. Finally, to assess the ACPA repertoire, IgG and IgA reactivity was measured against citrullinated peptides from fibrinogen, α-enolase and vimentin.

The results of this study showed that Treg numbers did not differ between healthy controls, SAP and RA patients. Whereas the numbers of functional Treg subsets Fr I and Fr II were comparable between groups, Fr III was increased in SAP compared to healthy controls (p=0.01). Fourteen (41%) SAP developed RA during follow-up. Treg numbers and subsets of switched SAP were comparable to non-switched SAP. At RA diagnosis, Treg numbers in switched SAP were not changed compared to 6 months before arthritis development. Hence, there is presumably no contribution of altered Treg numbers leading to the loss in suppression of autoimmunity in RA pathology. Switched SAP displayed a more diverse IgG ACPA repertoire compared to non-switched SAP. A major limitation of our study was the relatively small patient group (n=34). Another Treg study in a larger patient cohort (n=100) did, however, find decreased Treg levels to be predictive in SAP (48). A difference in follow-up periods between the studies (3 months (48) versus 6 months (our study)) may explain the discrepancy in results.

Future studies should determine whether the functional capacity of Tregs is affected in SAP during RA development. We already found that an increased and broadened ACPA response was indicative for RA development in SAP. Therefore,
measuring the ACPA repertoire might allow for identifying high-risk patients, but the test applied for these measurements is not yet suitable for routine use. Also, other parameters such as IgM RF status, CRP levels, tender joint count, morning stiffness and family history are probably more appropriate to use to predict RA development. Nevertheless, it would be interesting to assess in future studies whether (a history of) periodontitis or the presence of mucosal inflammatory diseases in the lung and gut is predictive for RA development in SAP cohorts in addition to known factors such as HLA-SE and family history (45). Furthermore, a longer follow-up period than used in the studies performed in this thesis might be required to determine which SAP will ultimately develop RA.

**Future perspectives and concluding remarks**

Future studies should address whether RA-associated autoantibodies indeed originate from mucosal tissues. For example, autoantibody levels could be determined in sputum or gingival crevicular fluid from autoantibody seropositive individuals without RA with, respectively, bronchiectasis or periodontitis. Also, the origin of ACPAs at mucosal sites could be determined by assessing the presence of ACPA producing B-cells/plasma cells in lungs, gum tissue or intestinal tissue using single B-cell based cloning technology (49). Furthermore, the reactivity against citrullinated autoantigens could be determined by isolating and expressing immunoglobulin genes from mucosa derived B-cells of RA patients or SAP. The use of this technique could provide more insight whether besides B-cells in joints (50) and circulation (51), also mucosa derived B-cells can express ACPAs.

Future research should also be directed towards the assessment whether cross-reactivity between microbial and human antigens adds to ACPA development. Whether specific microbes that are involved in periodontal, lung or intestinal mucosal inflammatory diseases contribute to this process remains to be investigated. Amongst others, it needs to be resolved whether RA-associated autoantibodies are just biomarkers or also exert a pathogenic effect. For example, in a number of mice models it was shown that ACPA indeed can enhance arthritis (52, 53), but other models failed to show such an association (54). Possibly, ACPA can be pathogenic or therapeutic (55) depending on the target of a specific ACPA.

In summary, the results of the various studies described in this thesis indicate that levels of RA associated autoantibodies are increased in non-RA patients with mucosal inflammation. Whether these increased levels indeed trigger the development of RA in (a subset of) these patients need to be proven in longitudinal studies. On the other hand, broadening of the ACPA levels and repertoire is indicative for imminent arthritis/RA in SAP, while anti-\(P.\ \text{gingivalis}\) antibody levels and Treg numbers SAP who developed RA are comparable to SAP who did not develop RA.
References


