Chapter 7

Summary and General discussion
Summary
Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovial inflammation. Many cells of the immune system are involved in this chronic inflammatory process (Fig. 1). The primary target organs are the joints although as the disease progresses systemic manifestations often also appear. As a consequence of the inflammatory process, RA patients generally develop a progressive loss of cartilage and bone in joints, resulting in painful joints and impaired functional status. Understanding the immune-pathogenic development of RA is a crucial step toward improving its management. In this thesis we focused on two major points: gaining more insight into the immune-pathogenic mechanisms underlying RA and investigating the possible anti-inflammatory role of HSP70.

Figure 1. This schematic figure summarizes the most important participants in RA pathogenesis. The left side illustrates the interaction between antigen presenting cell (dendritic cell) and T cell in the lymph node, which then can activate other T and B cells, and cause proliferation of a T cell population. These cells migrate to the synovial tissue, where further activation, and perpetuation of additional T cells occur simultaneously, leading to the activation of B cells. These B cells then produce (auto)antibodies such as rheumatoid factor (RF) and/or anti-cyclic citrullinated peptide (CCP) antibodies. In addition, the T cells activate fibroblasts and macrophages, which then secrete a variety of different proinflammatory cytokines such as TNFα, IL-1, and other mediators including matrix metalloproteinases (MMPs), prostaglandins, and nitric oxide. This leads to inflammation of the synovium, swelling of the joints, and severe damage of articular cartilage. DC=dendritic cell; T=T cell; B=B cell; MΦ=macrophage; FLS=fibroblast-like synoviocytes. Adapted from Moreland LW (63).

In order to study arthritis, we made use of the proteoglycan-induced arthritis (PGIA) model in BALB/c mice, which is a T cell-dependent, autoantibody-mediated progressive relapsing autoimmune model resembling many characteristics of RA. Originally PGIA was induced by four PG immunizations of which the first and fourth injection were in complete Freund’s adjuvant (CFA) and the second and third PG injection in incomplete Freund’s adjuvant (IFA). In chapter 2 we described that by replacing the Freund’s adjuvants by dimethyldioctadecylammonium bromide (DDA), arthritis can be induced by only two PG immunizations. Although the clinical and immunological features of the “classic” form of PGIA are preserved, the onset of the disease is faster and the severity is increased. An additional advantage is...
that hereby all undesired side effects of the Freund’s adjuvants, such as the role of interfering immune responses induced by microbial HSPs present in CFA, are eliminated. DDA exerted a strong stimulatory effect via the activation of innate immunity and forced the immune response toward Th1 dominance (chapter 2). In the PGIA model using DDA as adjuvant, we explored the anti-inflammatory capacity of immune responses to HSP70 in arthritis and searched for the mechanisms involved (chapter 3). HSP70 pretreatment dramatically suppressed the development of inflammation and subsequent tissue damage in PGIA. Moreover, HSP70 pretreatment resulted in a regulatory immune response not only to HSP70 but also to PG. To better understand the role of T cells in arthritis we generated a T cell receptor transgenic (TCR-Tg) mouse (chapter 4 and 5). The T cells of this mouse are specific for an epitope (designated 5/4E8) in the G1 domain of human cartilage PG (amino acids 70-84). These TCR-5/4E8-Tg mice are a unique source of naïve antigen-specific T cells that are capable of inducing progressive chronic arthritis, both spontaneously (<15%), and after immunization with PG (100%) and upon adoptive transfer to BALB/c.SCID mice. Based upon the clinical and histopathological features and the autoimmune aspects shared between PGIA and RA, we believe that these TCR-5/4E8-Tg mice are a valuable tool for further analysis of the mechanisms associated with the initiation and pathogenesis of autoimmune arthritis; and more specifically, for the analysis of the role of antigen specific T cells in disease development.

As in PGIA, T cell responses against cartilage PG do also occur in RA, at least in a subset of patients. In chapter 6 we tested for responses to several PG epitopes in longstanding RA patients compared to osteoarthritis (OA) patients and healthy controls. In particular, two epitopes (amino acids 16-39 and 263-282; both located in the G1 domain of human PG) appeared to be frequently recognized RA and OA patients as shown by proliferation and preferential Th1 cytokine production. This indicates that PG-specific responses might be involved in RA immune pathogenesis either primarily or, more likely, as a consequence of cartilage destruction.

Taken together, PG-specific immune responses were studied in PGIA as well as in RA and showed that the PGIA model provides a unique opportunity to study possible autoimmune mechanisms. Pretreatment with HSP70 showed the potential to modulate (suppress) the arthritogenic effect of PG via IL-10 mediated response and thus to treat arthritis.
General discussion

1. Relevance of the PGIA model and insights in the pathogenesis

Inflammatory diseases such as RA are complex and have a polygenic origin. To fully understand its pathogenesis remains a huge challenge. RA is probably not one disease but, rather, a syndrome caused by several different pathological processes (1). The widely variable responses to virtually any treatment modality (besides corticosteroids) in RA also underlines that the disease is quite heterogeneous. In order to understand the molecular pathogenesis of inflammatory arthritis and for the development of new therapies, animal models are essential tools to study specific mechanisms. Pathways leading to destructive inflammation may be shared, to a large extent, between humans and animals. There are many different experimental models for RA and they most likely represent several different pathways leading to the disease or reflect variants of the human disease and there are arguments in support of the existence of each of them for studies of RA (1,2). On the basis of the clinical, immunological, and genetic hallmarks, among the most relevant animal models of RA appear to be those induced by cartilage matrix components such as type II collagen and PG (3).

**PGIA**

The PGIA model is clearly helpful in enabling the investigation of a complex system involving chronic inflammation and autoimmune responses. Immunization of BALB/c mice with human cartilage PG depleted of both chondroitin sulfate (CS) and keratan sulfate (KS) side chains in the adjuvant DDA leads to the development of progressive polyarthritis (3,4). This development of the disease is based upon T and B cell responses cross-reactive between the immunizing human and self (mouse) cartilage PG. While no model perfectly matches the pathogenesis or etiology of human RA, PGIA has a number of features which make it very useful. It is a well-defined and reproducible model showing many similarities to RA in clinical appearance, histopathology, immune regulation, inflammatory cell migration and genetics (complex susceptibility i.e. involvement of MHC and non-MHC alleles) (5). The progressive character and high incidence of PGIA make this model optimal for testing immunomodulatory agents (5-7). It is a BALB/c mouse model which facilitates the use of transgenics and knockouts. In addition, it can be efficiently induced by adoptive transfer of T cells (8). A limitation so far has been the need to use CFA and the relatively long arthritis induction time.

The use of the synthetic lipophilic quaternary amine adjuvant DDA accelerated the development of a more severe arthritis via a more potent activation of the innate immunity. DDA acts as an adjuvant through enhancing interactions with both antigen and components of the host immune system (9). In PGIA induced with PG in DDA the overall immune responses (antibody production and antigen-specific T cell responses) were highly comparable with arthritis induced with PG in CFA. However, smaller doses of cartilage PG (2x instead of 3-4x) and use of crude cartilage extract from osteoarthritic cartilage (instead of highly purified PG), which has only a suboptimal arthritogenic effect when injected in CFA, were very effective in provoking arthritis when mixed with DDA. While the overall effects of DDA and CFA on the T cell response were highly comparable, PG-specific IFNγ:IL-4 ratios in spleen cells or peripheral lymph node cells were significantly different and clearly shifted toward Th1 in the DDA/PGIA model. In the
peritoneal cavity, at least a 2-4-fold increase in macrophage influx, accompanied by more activated (CD11c+) dendritic cells was observed in DDA-injected mice compared to CFA-injected mice. A critical role for innate immunity in arthritis induction is consistent with the findings of studies that used only adjuvants (nonspecific stimulators) in genetically susceptible strains of rodents (10,11). Thus, nonspecific activation of the immune system is probably an important component of the disease pathological mechanism. Our observations using DDA as adjuvant support this important role of innate immunity, in addition to the inducing adaptive immune responses (PG-specific Th1 T cells). Macrophages and dendritic cells involved in innate immune responses were stimulated more potently by DDA compared to CFA resulting in an accelerated onset of arthritis and more severe arthritis. In PGIA, a rapid accumulation of PG-specific Th1 cells in the synovium appears to be the most critical component of the development of arthritis (5). Autoantibodies to PG contribute to the severity of disease possibly by specifically binding to the cartilage surface, thus inducing more extensive cartilage damage (12). It seems that DDA has stimulated both the adaptive immunity (significantly higher ratios of PG-specific IFNγ to IL-4 were found in PG/DDA immunized mice) and the innate immunity. Most components of innate immunity play an important role in perpetuating inflammation by their interactions with the cognate immune cells, and they also can induce subsequent tissue damage (13). This could have contributed to the loss of cartilage, resulting in the increased release of mouse PG fragments which may have further stimulated the PG-specific immune responses and amplified the inflammatory process.

The need for an innate stimulus in addition to a specific stimulus for the development of arthritis is clearly demonstrated in various animal models (14). In RA, the destructive lesions probably also result from both adaptive immune responses and non-antigen-specific inflammatory processes. Although there is no consensus on what initiates RA and on the major players in the pathogenesis stemming also from the fact that RA is quite heterogeneous, it is believed that perpetuation into a severe chronic arthritis occurs only when both the adaptive and the innate immune systems are involved (15). This is underlined by the efficacy of therapeutic interventions that can target either unique etiologic pathways related to adaptive immune responses (CTLA4-Ig (16), anti-CD20 (17)) or shared terminal effector mechanisms (anti-TNFα (18), IL-1R antagonist (19)), however only in a subset of patients, which again shows that many mechanisms may be involved.

**TCR-5/4E8-Tg**

Several lines of evidence indicate that the effector mechanism, which initially attacks small joints, is T cell driven. One way of gaining more insight into the role T cells in PGIA was by establishing transgenic mice expressing a TCR specific for one of the immunodominant epitopes of human PG, namely, the epitope recognized by T cell hybridoma 5/4E8. This specific epitope of human PG differs only in 2 amino acids from the homologous mouse PG sequence. TCR-5/4E8-Tg mice supply us with a unique source of naive antigen (arthritogenic epitope)-specific CD4+ T cells that are capable of inducing progressive chronic arthritis. Only 15% of the TCR-Tg mice after several backcrossings to the arthritis susceptible BALB/c background developed spontaneous arthritis, but severe arthritis was quickly induced after immunization with PG in DDA. Interestingly, also immunization with PG in the absence of adjuvant resulted in arthritis indicating the importance of T cells in the initiation of arthritis in this model.
In addition to arthritis development in the TCR-5/4E8-Tg mice, adoptive transfer of spleen cells from arthritic TCR-Tg induced arthritis in BALB.SCID mice. Arthritis was induced by this transfer without adding exogenous PG or specific peptide, which was not possible using splenocytes from arthritic wild-type donor BALB/c mice (20). We hypothesize that T cells from arthritic TCR-5/4E8-Tg mice migrate to the joints upon adoptive transfer and become further reactivated (or maintain their activated shape) by mouse PG in the mouse joint where self-peptides are released during the normal turnover of the cartilage matrix. As a consequence of the inflammatory processes in the joint the production of mouse PG peptides might be increased, which could amplify the autoimmune response.

Remarkably, even naïve TCR-5/4E8-Tg spleen cells together with PG (without DDA) could induce arthritis in BALB/c.SCID and BALB/c.RAG2-/- mice upon transfer. The critical role of T cells in arthritic processes in PGIA thus seems unquestionable. PG-specific T cells can help to investigate the role of joint antigen-specific cells in inflammation and T cell migration. Recently, it has been demonstrated that thymic production of arthritogenic T cells, due to a mutation of the ZAP-70 gene (a key signal transduction molecule in T cells) leading to a selection shift of the T cell repertoire towards high self-reactivity, causes autoimmune arthritis in mice (21). This SKG mouse model of arthritis showed that the clinical and pathologic abnormalities are clearly T cell-dependent, while macrophage and fibroblast cytokines such as TNFα and IL-1 are required for full expression of the disease. This again underscores the importance of the interplay between adaptive and innate immunity in the pathogenesis of arthritis. Indeed, experience with anti-CD4, -CD5, and -CD52 antibody therapeutic interventions in RA have demonstrated that merely killing T cells is not enough to treat RA (22). As found in PGIA, the cell-cell interactions that occur in RA synovium are multiple, complex and fundamentally important to the pathogenesis and outcome of this disease (23). Further elucidation of the critical cell-cell interactions in RA synovium should provide additional therapeutic targets and will provide a rational basis for safe and effective combinations of biologic interventions (23). TCR-5/4E8-Tg mice offer a unique opportunity to further study these autoimmune mechanisms as well as therapeutic interventions.

2. Insights in the pathogenesis of RA; PG recognition in RA

During the progression of arthritis, cartilage has been shown to be damaged by the invasion of pannus. The degradation of the cartilage matrix by various metalloproteinases upregulated by TNFα and/or IL-1, and by apoptosis of chondrocytes, dominates the clinical and histopathological picture of RA (24-26). The cartilage degradation releases matrix components, such as type II collagen (CII) and PG fragments, which then may be exposed to the immune system. PG (aggrecan) or CII within healthy cartilage normally is not subjected to immune surveillance, due to the avascular structure of hyaline cartilage. However, potential antigenic determinants might be generated during degenerative or inflammatory processes, and then these (auto)antigenic components can trigger immune reactions. Although the target organ of RA is the synovial joint, there is no clear evidence that any macromolecule of cartilaginous tissues, bone, or synovium, is a preferential disease-inducing autoantigen (27). However, T cell epitopes on matrix molecules may be critical in the pathogenesis of arthritis. Several studies have demonstrated that patients with joint diseases show immune responses to various cartilage matrix molecules, such as to CII (28,29), human cartilage glycoprotein 39 (HC gp-39) (30) and PG (31-34).
Recently, the epitope repertoire of the human cartilage PG was determined in HLA-DR4-humanized and HLA-DQ8-humanized mice and it was shown that these transgenic mice immunized with human cartilage PG developed arthritis, but only when these class II MHC molecules were present on the arthritis-susceptible (BALB/c) genetic background (35). Two highly immunogenic T cell epitopes, both in the G1 domain of human PG could be identified, and were both associated with arthritis. We tested these human PG-epitopes recognized in HLA-Tg mice for T cell recognition in patients with RA and OA, and healthy controls. In this study we demonstrated that T cell responses to cartilage PG epitopes occur in RA with a pro-inflammatory cytokine profile. This might highlight that PG is a relevant joint-specific autoantigen in the pathogenesis of RA. The reasons for the loss of self tolerance to this major cartilage component still remain elusive. In addition, it has raised questions as to whether the the PG autoreactivity is causal, an early disease-driving event, mediates the joint pathology or merely is a consequence of cartilage destruction. (36).

Recently, in this respect Burkhardt et al. have been provided first evidence that citrullinated derivatives of well conserved immunodominant native CII epitope might be targets of an autoantibody response at very early stages of RA (37). They suggested that as B cell tolerance is more easily broken, this could result in breakdown of tolerance of the better controlled T cells. Autoimmunity to cartilage-specific modified self components might be a critical intermediate step that helps to lower the threshold for the final breakage of tolerance to other joint proteins in chronic inflammatory joint disease (37). Thus, PG (or CII) autoimmunity in RA may be a secondary phenomenon, induced following inflammation in the joints and might play a role in the persistence of the disease, rather than in the actual induction of arthritis.

Whether PG-specific immune responses are causal or a consequence of cartilage destruction, a selective down-regulation of the immune response to PG may be a challenging task for treatment of RA patients. Mucosal tolerance is a natural mechanism that can prevent harmful immunological reactions to antigens by altering the activity of immune cells through the induction of antigen-specific tolerance without modulating the entire immune system. Oral treatment with CII has been shown to prevent and suppress disease in collagen-induced arthritis (CIA) (38) and antigen-induced arthritis (39). Its efficacy in RA however has not been convincingly demonstrated (40-42). The reason for discrepancy in the results of the different studies could be explained by differences in the dose, the formulation of CII or patient selection and suggests that the therapeutic window is narrow (42). The responses to oral CII in JIA have so far been encouraging (43,44). For PG, it has been demonstrated that nasal administration of PG exerted a strong suppressive effect on both the incidence and severity of the PGIA, most probably by reducing responsiveness towards the immunizing PG antigen (45).

In summary, PG of the articular cartilage is one of the candidate autoantigens which may be the target of pathogenic autoimmune responses in the induction or perpetuation of joint inflammation in RA. Indeed most of the RA patients demonstrated immunity against PG epitopes and this might relate to the underlying pathogenic mechanisms. This should encourage further work on the implications joint-specific autoimmunity in the pathogenesis of RA.

Animal models of arthritis can be used to understand elements of the arthritic process in patients, although final proof of concept must come from clinical studies (46). In order to benefit from using an animal model, we must understand and utilize the common (shared) immune-pathogenic components of the human disease (RA) and a corresponding
animal model (PGIA). Our findings support the use of the PGIA model to extrapolate to human diseases; as T cell epitopes of PG that were defined in HLA-DR4 and HLA-DQ8 transgenic mice stimulated T cells from human RA and OA patients.

3. Heat shock proteins and their possible use as therapeutic interventions

Traditional disease modifying and biological response modifying agents are very useful in controlling disease activity, limiting disease progression, and improving function in patients with RA. However, very few patients achieve full remission from treatment with these medications either when given alone or in combination. A better understanding of RA will provide the basis for new treatment approaches in RA. Over the last few years, it has been hypothesized that susceptibility to autoimmunity is associated with the absence or a failure of regulatory T cells (Tregs) to downregulate the inflammatory process (47). Increasing evidence suggests that T cells which react against heat shock proteins (HSPs) can serve as a feedback loop to inhibit immune responses and can be used to restore such imminent failure of regulation (48,49).

HSPs function as chaperones in intracellular protein folding, assembly and transport. They are classified into families on the basis of their molecular weight. HSPs might be candidates as immunomodulatory agents in arthritis for several reasons: 1) HSP families are present in all organisms, both in prokaryotic and eukaryotic cells. 2) HSPs are highly conserved; e.g. some mammalian family members have highly conserved microbial homologues which could result in immunological cross-recognition. 3) HSPs are immunodominant antigens. 4) They are present at the target site; as they are over-expressed by cells in the synovium during inflammation (50-53).

Here, we studied the capacity of immune responses to HSP70 to prevent or arrest inflammatory damage in PGIA and searched for the anti-inflammatory mechanism. HSP70 pretreatment resulted in a significant delay of the arthritis onset and dramatically reduced disease severity; less inflammation and almost no synovium cell infiltration, cartilage damage or bone erosions were detectable. The \textit{in vivo} protective effect of HSP70, however, was accompanied not only with increased HSP70-specific proliferation but also with increased \textit{in vitro} T cell proliferation to PG. This increased PG-specific T cell proliferation seems to be contradictory to \textit{in vivo} results (suppressed PGIA). On the other hand, HSP70 has an adjuvant effect as well, known from work from Srivastava and others (54,55). As this is the first time the anti-inflammatory effect of HSP70 is investigated in an (self)-antigen-induced arthritis model, this increased PG-specific response could not have been noticed so far, thus this observation will need further attention. This effect might be part of the regulatory mechanism, because, not only after \textit{in vitro} restimulation with HSP70, but also restimulation with PG showed enhanced IL-10 and IFN\(\gamma\) production in mice that were otherwise protected by the administration of HSP70. A simultaneous production of IFN\(\gamma\) and IL-10 is a characteristic of T regulatory 1 (Tr1) cells (56). In JIA patients with a remitting form of disease (oligoarticular JIA) responses to HSP60 are consistent with a benign clinical course and these HSP60-specific T cells have the phenotype of human Tr1 cells (57). On the other hand, IFN\(\gamma\) has also been described as a prototype Th1 proinflammatory cytokine (58) and the production of IFN\(\gamma\) in response to immunization with PG is an important contributor to the induction of PGIA (5). The IL-10:IFN\(\gamma\) ratio, however, can be an indicator of the balance of Tregs and Th1 cells. We recorded an increased IL-10:IFN\(\gamma\) ratio in
response to PG in HSP70-pretreated mice. IL-10 plays an important role in the immunoregulatory mechanisms that control inflammatory responses (59,60). IL-10 has the capacity to down-regulate monocyte-derived proinflammatory cytokines such as IL-1, IL-6, and TNFα (61). IL-10 production seems to be protective in RA patients, especially against progression of joint destruction (62). HSP70-specific T cells as well as PG-reactive T cells, displaying a regulatory phenotype, may have been influenced by the neighboring harmful autoreactive T cells. Thus, these Tr1 cells can switch the proinflammatory cytokine profile of PG-specific cells into an anti-inflammatory (regulatory) IL-10 producing phenotype, thereby modulating the development of inflammation in PGIA.

The exact mechanism involved in HSP-targeted immunoregulation needs to be investigated further. We propose that increased exposure to microbial HSP70 (in this case by immunization) leads to the activation of self-HSP70-reactive T cells. Such self-reactive immune responses might have anti-inflammatory potential, i.e. the upregulation of self-HSP70 in inflamed synovial tissues might be the natural feedback reaction. Thus, HSP70 production may target the anti-inflammatory immune repertoire at the site of inflammation, where they can exert their immunoregulatory activities (48,49). As the HSP70 mapping studies showed T cell responses towards highly conserved or even identical sequences of HSP70, we can assume that following HSP70 immunization cross-reactive responses to self-HSP70 were induced. Thus, boosting this anti-inflammatory repertoire by artificial HSP immunization, may offer attractive immunotherapeutic possibilities. However, further research is necessary to reveal which epitope(s) of HSP70 can induce protective immune responses. In addition, it would be important to test whether these HSP70-specific cells can exhibit a suppressive effect upon adoptive transfer to arthritic mice and whether the PG-specific IL-10 producing cells exert a protective effect on arthritis. The PGIA model offers many possibilities to answer these questions.

Conclusion

RA is a complex autoimmune disease controlled by various cells of innate and adaptive immunities, environmental factors and genetic components (13). A successful treatment of RA will depend on the early diagnosis and a better understanding of pathological processes. The PGIA model enabled the investigation of several in vivo mechanisms in a complex system of inflammation and autoimmunity (5). The analysis of PGs and HSPs, both present at the site of inflammation, provided insight into the pathogenesis of autoimmunity and arthritis and suggests that both proteins may be targets of immunomodulation by different mechanisms.
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