Discussion

Ankylosing spondylitis and rheumatoid arthritis are assumed to be T cell mediated inflammatory autoimmune diseases (114-116), their pathogenesis has been regarded as a consequence of the activation of T cells by yet unknown antigens and the co-stimulatory molecules CD4, CD8 and CD28. Several potential antigens have been proposed to initiate the immune response or be a target of it including autoantigens such as type II collagen, aggrecan G1 domain, glycoprotein (gp)39 and heat shock proteins, but also viral and bacterial antigens. Following activation, T cells initiate the inflammatory cascade through secretion of either interleukin 2 or interferon gamma, or through direct cellular interaction with macrophages and synoviocytes.

Quantification and visualization of cellular immune responses have recently become more sophisticated by flow cytometry (106,107,117) which allows determination not only of the cellular subtypes under investigation but also, at the same time, the measurement of the intracellular cytokines secreted by the same cells after non-specific or antigen-specific in vitro stimulation of T cells (106). This technique is capable to detect antigen-specific T cell frequencies as low as $1x10^{-5}$ (107,117). Such an improvement in sensitivity is essential if one wants to look for T cell responses to autoantigens which are normally difficult to detect because of low frequencies. In our study, I concentrated on $CD4^+$ T cell responses because I investigated whole recombinant proteins which are normally processed via the pathway II of antigen presentation and the epitopes created are normally only presented to $CD4^+$ T cells. In the context of the strong HLA-B27-association the identification of epitopes presented to CD8+ T cells by HLA-B27 are also of great interest but are technically more demanding and were not a major subject of my thesis (118).

T cell response triggered by h-hsp60 and y-19KD in AS and RA

Stress or heat shock proteins are a family of approximately 25 highly conserved proteins which are upregulated in response to various forms of stress and facilitate the biosynthesis and maturation of proteins within cells (protein folding) (119). They also promote assembly and disassembly of polypeptides and play a

major role in cellular function, not only during the stress response but also at a basal state (120).

As hsp are immunogenic molecules and can be expressed on cellular membranes, their role in auto-immune and inflammatory diseases, particularly in rheumatoid arthritis, has been studied (89, 121). Cellular immune responses of T cells and a humoral response by antibody production against hsp occurring in the course of some diseases have been observed (120,122). In contrast, some studies suggested that preimmunization with microbial proteins belonging to different hsp families protects from subsequent arthritis induction (123-125).

Up to date, most of these studies mainly concentrated on animal model of arthritis. Little is known about the effect of hsp on the pathogenesis of human autoimmuno-diseases. There are especially only few information on the role of human hsp60 in AS and RA. Prakken et.al (126) described that there was significant T lymphocyte proliferative responses to human hsp60 in peripheral blood mononuclear cells and/or synovial fluid mononuclear cells from oligoarticular juvenile rheumatoid arthritis within 3 months after the onset of arthritis, only a small percentage of a control group showed such positivity. These authors also found a correlation between improvement of disease activity and a decrease in the hsp60-specific T cell response, in some cases the T cell response became even negative when patients went into remission. Sato et al.(127) reported that hsp60 were expressed on the surface of PB and SF lymphocytes from patients with RA, but these expression happens only in the active stage, not in the improvement stage of the disease. Macht and his coworkers(128) showed that PBMC from some patients with established RA gave proliferating responses to human hsp60 that were above the normal range and/or peaked earlier than PBMC from normal individuals. Furthermore, disease activity and severity did not differ between those RA patients whose hsp 60 were stimulatory for cells and those who did not react upon stimulation with hsp60. But a significant negative correlations were found between IL10 production by hsp 60 stimulated cells and disease activity. Our group have reported before that some level of crossreactivity between Yernisia-specific hsp60 and human-specific hsp60 is detectable in patients with Yersinia-induced reactive arthritis (110), but T cell response to h-hsp60 has not been investigated in AS.

In this study, I quantified the production of T cell cytokines induced by h-hsp60 at the single cell level in AS and RA patients and compared it to that of healthy controls. Of all samples inverstigated, the frequency of IFNγ^+ CD4 T cells responsive to h-hsp60 was highest in normal persons (45%) and similar in the AS and RA patients (36%, 36.5%, respectively). The percentage of TNFα^+ CD4 T cells response to h-hsp60 was also highest in the healthy controls (70%), and again, a similar frequency of 60% and 59% was observed in AS and RA patients. These results suggested that T cell responses to h-hsp60 are also present in healthy people and does therefore not seem to be pathogenetic for RA or AS.

The development of many autoimmune diseases has been etiologically linked to exposure to infectious agents (129). Models proposed to account for the relationship between infection and autoimmunity include inflammation-induced presentation of cryptic self-epitopes, antigen persistence and molecular mimicry. Yersinia is one of the triggers for reactive arthritis, its antigenic fragments have been found in joint structures of patients with reactive arthritis (130). Among the Yersinia derived antigens, the Y-19KD plays a dominant role as a target molecule for the cellular immune system (110). But so far, no evidence supported that Y-19KD is involved in the pathogenesis of AS or RA. Our results indicate that the T cell response to Y-19KD is present at a low level in some patients with RA and AS but also in healthy controls, probably indicating a previous contact with Yersinia or with cross-reacting bacteria.

It cannot be excluded that T cells specific for one of the two antigens are present in the synovium and just are not detected in peripheral blood. However, the absence of clear differences between antigen-specific T cells in SF of RA- and AS-patients argues against this, unless a similar pathogenesis for these two diseases is assumed, what is unlikely.

T cell response to antigens derived from cartilage in AS and RA patients

Human cartilage glycoprotein-39 (HC gp39) is a major secretory product of articular chondrocytes and synovial cells. The complete complementary DNA sequence contains an open reading frame encoding a protein that is 383 amino acids in length. There is evidence that HC gp-39 synthesis is induced under inflammatory or degenerative condition (86), and its messenger RNA was found in cartilage

obtained from RA patients, whereas healthy adult cartilage obtained at surgery did not contain a significant amount of this transcript (86). Verheijden et al (131) described that HC-gp-39 derived, motif-based peptides were indeed selectively recognized by peripheral blood mononuclear cells from RA patients and no responders were found in the healthy donor group. Of great interest was the observation that injection of the intact protein in BALB/c mice resulted in immunity to HC gp-39, which was found to be associated with the development of a chronic, relapsing arthritis. Moreover, inhalation of the protein led to tolerization of antigen-specific T cells and to suppression of HC gp-39-induced arthritis (131). Vos, et al. reported (132) that the cellular immune response to HC gp-39 derived peptides was observed not only in RA patients but also in patients with systemic lupus erythematosus and inflammatory bowel disease as well as in osteoarthritis. These observations make a role for HC gp-39 in joint destruction. Possibly, however, it does not seem to be specific for the joint.

Collagen II and aggrecan are the 2 major proteins of cartilage, endowing the tissue with tensile strength and compressive stiffness, respectively. Degradation of either protein could lead to significant loss of cartilage function. Collagen-induced arthritis in susceptible strains of mice was found to be an animal model of T cell-mediated inflammatory polyarthritis and there are some evidence that collagen II is degraded in cartilage from patients with rheumatoid arthritis or osteoarthritis (133). Immunization of laboratory animals with collagen II can induce an erosive arthritis with some similarities to RA (134). However, previous studies suggest that only a minority of patients with RA (5-15%) have anti-collagen antibodies (134) and only a weak proliferative T cell responses have been reported in a minority of patients (135).

In the context of the pathogenesis of SpA it is of interest to stress that aggrecan is present in fibrocartilaginous entheseal regions of the tendon, which insert at the bone, but not in the human midtendon (136). Furthermore, the G1-domain of the aggrecan molecule is the major degradation product of intervertebral discs (83,137). These are all sites which are primarily affected in SpA but not in RA. Aggrecan is the large aggregating proteoglycan from cartilage containing chondroitin sulphate and keratan sulphate is attached to a multidomain protein core. It aggregates by binding to hyaluronic acid and this is further stabilised by a separate globular link protein. The N-terminal globular G1 domain, an approximately 60 kD molecule, is involved in this aggregation process. Aggrecan cleavage sites have

been identified which indicate that the G1 domain is indeed present in vivo (138,139). From osteoarthritis patients it is known that G1-containing fragments are abundantly found in synovial specimens (140). The main aims of this study was to answer the question whether one of the cartilage derived putative autoantigens G1, HC gp39 or collagen II might be involved in the pathogenesis of AS. These data suggest that the G1 domain of aggrecan but not HC gp39 and collagen II is a target of the immune response in AS. Indeed, the results show, on the basis of the relative frequency of IFNγ^+ cells among CD4$^+$ T cells, that the G1 domain of aggrecan is recognized by almost two thirds of patients with AS (61.7%) and half of the investigated patients with RA (54.5%). In contrast, normal healthy individuals showed a reactivity only in a few cases (10%). Again, using the same technique, no T cell response to other cartilage-derived antigens was detected. The T cell response to HC gp39 and collagen have not been investigated in AS so far, but were reported in RA. Importantly, in this study the response of synovial fluid (SF) CD4+ T cells to the G1-domain was examined in AS patients for the first time. The data clearly show that a significantly higher number of antigen-specific T cells is present in SF compared to PB. Taken together, these results indicate that the G1 domain of aggrecan might play a role in the cellular autoimmune response in AS and RA. The question of the pathogenetic relevance of these findings has to be answered in future studies.

Based on the results presented here, it can not be decided whether the G1-specific T cell response plays a primary role in causing the immunopathology or whether it is rather a secondary event after cartilage destruction caused by other mechanisms. The fact that the T cell response to this autoantigen does not seem to be specific for one rheumatic disease is compatible with (i) aggrecan being a major component of human cartilage which is affected by various rheumatic diseases such AS, RA but also osteoarthritis, (ii) the physiological role, cleavage and breakdown of aggrecan and also (iii) previous results describing immune reactivity to the G1 on both the cellular and the humoral level in different rheumatic diseases (84,140,141). Nonetheless, the demonstration of such a cellular response in both PB and SF of AS- and RA-patients is encouraging enough to pursue this question in future experiments. G1 can be found in the cartilage of all inflammatory sites which are affected in SpA such as the entheses, the vertebral disc, articular cartilage, and the eye which could be taken as an argument that G1 might be the primary target

antigen in AS. Furthermore, the G1-domain of versican, which has a high homology to the aggrecan G1, is present in the aortic wall (83).

The identification of T cell epitopes within the G1 protein

The identification of T cell epitopes is crucial for the understanding of the host response in autoimmune diseases. MHC molecules on the surface of antigen presenting cells present peptide fragments derived from proteins to T lymphocytes. Once a target protein is defined for the T cell response, the antigenic epitope can be mapped with synthetic peptides (101). In our study, two T cell epitopes within the G1-protein (AA residues 292-309 and 252-269) were identified. This suggests that they might play a role in the pathogenesis of AS. Furthermore, the T cell response both to the whole G1 protein and to G1-derived single peptides confirms the presence of a G1-specific immune response in AS and, importantly, it also excludes a false positive response due to a contamination in the protein/peptide preparations. It has been reported before (82) that aggrecan can induce erosive polyarthritis and spondylitis in BALB/c mice, and that the G1-domain of the proteoglycan aggrecan contains the arthritogenic region. In this animal model, two T cell epitopes residing on G1 within residues 70-84 and 150-169 could be shown to be immunodominant. Furthermore, adoptive transfer of T cells specific for these peptides induced arthritis in BALB/c mice (82). The immunodominant epitopes found in mice and men were not identical. However, this finding is not surprising since mice and men have a largely different MHC-background. Whether the weak but significant association of AS with the class II molecule HLA DR2 (33) is involved in the human CD4 response to the G1 found in this study has not been investigated to date.

A final proof for a critical role of the G1-molecule in the pathogenesis of AS will come from the detection of antigen-specific T cells in cartilage (114,142), possibly through tetramer technology (143), or by induction of G1-specific T cell tolerance, possibly through mucosal tolerance (144). Due to the strong HLA-B27 association in AS, it will be also important to investigate the G1-specific CD8+ T cell response. Furthermore, it will be very interesting to look for G1-directed immune responses also in other SpA such as reactive arthritis and psoriatic arthritis in which, clinically, the same anatomic structures are involved.

Sensitiveness and specificility of IFNγ and TNFα secretion by antigens

Except the IFNγ-response triggered by h-hsp60, 19kd or G1, I also detected antigen-specific TNFα-response in this study. The combined analysis indicated that antigen-induced cytokine positive samples could be subdivided into three classes: IFNγ or TNFα single positive, and IFNγ and TNFα double positive, which suggested that IFNγ and TNFα can not only independently exert their effect but have also synergistic effecs. Single IFNγ$^+$ T cells induced by h-hsp60, y-19KD or G1-domain are lower than single TNFα$^+$ or IFNγ/TNFα double positive cases both in patients and healthy controls. The number of TNFα$^+$ (including TNFα single positive and TNFα/IFNγ double positive) cases was higher than IFNγ positive cases (IFNγ single positive and IFNγ/TNFα double positive). These data indicate that antigen-triggered TNFα-secretion by T cells is more sensitive but less specific compared to IFNγ-secretion. It remains to be determined whether T cell responses to candidate antigens, for example autoantigens, can also be assessed by TNFα-secretion. But the investigation of the TNFα response is of special interest in consideration of (i) the high amount of TNFα present in inflamed sacroiliac joints of AS patients (142), (ii) the reportedly lower amount of TNFα secreted in peripheral blood of AS patients (36) and (iii) the efficacy of anti-TNFα treatment in AS and other SpA patients (145).

Analysis of the relationship between h-hsp60 and y 19KD

For AS and RA patients, a surprising result of my study was that almost all IFNγ$^+$ samples specific for Y-19KD were simultaneously also h-hsp60 specific except one AS patient who was only Y-19KD specific; What is more, all TNFα$^+$ samples specific for Y-19KD were accompanied by h-hsp60 specific TNFα$^+$. This phenomenon indicates that h-hsp60 probably shares a similar epitope with Y-19KD, another possibility is that hsp60 expression upregulated during stress conditions such as inflammation, and Y-19KD might be one of those triggers that induce hsp60 expression. The exact mechanisms remain to be investigated in further studies.

Cytokine patterns in ankylosing spondylitis

It is now generally accepted that a balance between Th1 and Th2 cells determines the phenotype and progression of a lot of diseases, such as inflammatory and autoimmune diseases (64,146). Th1 cells, which produce IFNγ and TNFα,

predominantly mediate cellular immune responses and are involved in the expression of chronic inflammatory diseases, whereas Th2 cells, which produce large amounts of IL4, IL10 and IL5, are responsible for induction of the humoral response. Th1/Th2 imbalance is considered as one of the causes for auto-immune disease. Previous studies suggested that RA is a Th1 disease, with a shift toward a Th1-mediated immune response (64).

An antigen-specific T cell secretion of IL-4 or IL-10 could not be detected in this study. The production of the Th1-cytokines IFNγ and TNFα upon antigen contact but of no Th2 (IL-4) or Th-regulatory (IL-10) cytokines might indicate that the G1-response might play a role in the immunopatholgy of AS and RA and that this does not appear to be counteracted by suppressive cytokines. For the non-specific cytokine secretion, a lower IFNγ production but not higher Th2 cytokine level triggered by SEB were observed in AS patients compared to RA. It seems that AS is neither a simple Th1 disease nor Th2 one, which indicates that the theory of imbalance of Th1/Th2 is not enough to explain the pathogenesis of AS. Recently, two types of regulatory $CD4^+$ T cell subsets have been reported, one of them is Th3 exclusively producing active TGFβ and no IL10, IL4, IL2, or tumor necrosis factor α. This cell type has been shown to suppress T cell-mediated diseases (147). The other one is Tr (regulatory)1 that produce high levels of IL10 and low TGFβ, which also down regulates Th1 response (148), Our findings indicate that a low Th1 cytokine but not a high Th2 cytokine occured in AS patients. It could be interesting to charaterize whether Tr1 or Th3 CD4 cells are involved in pathogenesis of AS.

Application of magnet activated cell sorting for the isolation of autoantigen-specific T cells

Recently, a new technique was developed to examine antigen-derived immunodominant epitope by MACS and flow cytometry (107,117), which makes a more detailed analysis of the immune response possible. Its brief procedure (for more detail see methods) includes: firstly, antigen-specific cells are separated by Magnet Activated Cell Sorting after short term stimulation and quantification analysis of positive T cells are performed by flow cytometry; subsequently, antigen-specific cells were expanded in vitro; finally, the cells are restimulated with the antigen-derived recombinant protein and peptides, and T cell epitope are determinated by flow cytometry.

This method is of high sensitivity for the determination of antigen-derived T cell epitopes. The frequency of antigen-specific T cells by flow cytometry was shown to be much higher than previously estimated based on T cell clones or limiting-dilution techniques (106,149). After enrichment by MACS, the sensitivity can be further enhanced (fig.16). Although not all surface IFNγ^+ T cells are antigen-specific ones (compared to intracellular staining), to a great extent, antigen-specific T cells were separated by MACS, and antigen-specificity of T cells was confirmed by restimulation after being for several days in culture (fig.17). This method can be used to screen and determinate T cell epitopes, although the tetramer technology has also to be considered as a good method for the detection of antigen-specific T cell frequency and T cell epitopes. However, this method is confined to a specific major histocompatibility complex (MHC)-peptide complex which has to be constructed separately for each MHC-peptide complex examined, and can't be used to screen T cell epitope derived from antigens.

Our group has successfully used this method of the cytokine secretion assay for detecting T cell epitope derived from bacterial antigen (data not shown), which indicates that this technique is sensitive and reliable. I have also successfully separated G1-specific T cell (fig.16) and expanded them in culture. But, there exist some challenges about this method. For example, G1-specific T cell expanded not so quickly because autoantigens are a relative weak stimulus for T cells, which means that it will take longer culture time to get enough cells for the determination of T cell epitopes in comparison to bacteria-derived antigen, and long time culture could lead to a loss of antigen specificity of T cells (fig.17). These problems remain to be solved in future studies

Downregulation of non-specific and antigen-specific cytokine production by anti-TNFα antibody infliximab

In this study I could show for the first time that treatment of patients with active AS with the anti-TNFα monoclonal antibody infliximab induces a reduction not only of TNFα secreted by T cells but also of T cell-secreted IFNγ. In contrast, an analysis of cytokine secretion by T cells in patients who were treated by placebo did not show any change in the cytokine secretion pattern indicating that the observed effect in the infliximab treated group was due to treatment and not by chance. Furthermore, when patients in the placebo group were switched to infliximab a similar drop in the TNFα-

and IFNγ- production by T cells was observed. Interestingly, when PB MNC were stimulated by LPS in vitro which stimulates preferentially monocytes/macrophages no change in cytokine secretion was detectable during this study.

While IFNγ-secretion by T cells was investigated before during infliximab treatment TNFα secreted by T cells was investigated for the first time. There was a further decrease in TNFα-production during treatment with the lowest value at the end of this 12 week study. Of interest, this low TNFα-production by T cells was observed 6 weeks after the last infliximab infusion. IFNγ-production by T cells declined in parallel to the change in TNFα-production indicating that not only TNFα secretion but Th1-capacitiy of T cells in general is affected by this treatment. I also investigated the antigen-specific response to the G1-domain of cartilage-derived aggrecan. This has been implicated as a possible T cell autoantigen in AS and other rheumatic diseases based both on results from animal models and in studies in patients (150,151). I have shown in this thesis that peripheral blood T cells from about 60% of AS patients respond antigen-specifically with IFNγ- and TNFα-secretion after in vitro stimulation with the G1-protein (152). In this study I found a decrease in the antigen-specific TNFα- and IFNγ-secretion both in the CD4- and in the CD8-T cell subpopulation after in vitro stimulation with overlapping peptides from the whole G1-protein derived from aggrecan, however, this difference was only significant for the CD8-subpopulation. In context with the strong association of ankylosing spondylitis with the MHC class I antigen HLA-B27 it has been suggested that HLA-B27 presents an arthritogenic peptide to CD8+ T cells which then are causing the local immunopathology (118). Whether the relative strong response of CD8+ T cells to the G1-peptides and its clear reduction during infliximab can be interpreted in this context has to be shown in future studies. Interestingly, such a good antigen-specific CD8+ T cell response was present although I did not use nonameric peptides but overlapping eighteen amino acid long peptides. A similar good CD8-response to longer peptides has been described before and might be explained by processing of longer peptides before binding to the MHC class I molecule (153).

Previous studies suggested that TNFα has an inhibitory effect on T cell function which can be restored by TNFα-blockade (154). One report with a similar study design as ours treating patients with various forms of spondyloarthropathies including AS patients with the same dosis of infliximab at the same time intervals

reported no change in the IFNγ-production by CD4 + T cells after 6 weeks, however, a significant increase of IFNγ-positive CD4+ T cells after 12 weeks (155). The reasons for the difference of these results compared to ours are not clear. However, the facts that I included a placebo group which showed no change, that the placebo group showed a similar drop in the cytokine production after these patients were treated with infliximab, that I observed a similar change in the IFNγ- and TNFα production, and that I found a reduction after non-specific and antigen-specific stimulation in vitro are argueing in favor of our results.

In Crohn's disease treatment with infliximab pointed more clearly to a reduction of IFNγ-production by T cells. It induced a sharp reduction in the number of IFNγ producing lamina propria mononuclear cells in gut biopsies (156) and in colonic T cell cultures derived from patients with Crohn's disease (157). Furthermore, it had been shown that TNFα increases the production of IFNγ by lamina propria MNC suggesting a direct link between the presence of TNFα and IFNγ-production (158). In this study such an association seemed to be specific for lamina propria MNC but not for PB MNC. Our study indicates that such a link is not specific for the gut.

I did not investigate T cell cytokine secretion after the first days following infusion and I can therefore not comment on this time point. It has been reported earlier that the number of IFNγ-secreting CD4+ T cells increases during the first 3 days in rheumatoid arthritis patients treated with infliximab (159). Nonetheless, during treatment over 3 months both the number of CD4- and CD8-positive T cells producing TNFα and IFNγ was significantly reduced in patients with AS as shown in our present study.

Rather surprisingly, I did not observe a change in the production of TNFα after in vitro stimulation of MNC with LPS 6 weeks after start of treatment. One previous study conducted in Crohn's disease reported that TNFα secretion by monocytes decreased drastically in the first days after infusion of infliximab but increased steadily over the following 4 weeks (160). Thus, an inhibition of the TNFα-producing capacitiy of monocytes does not to be long lasting and does not correlate with the excellent clinical response I see after 6 weeks.

During treatment of RA patients with the soluble TNFα receptor etanercept a transient increase of the number of IFNγ+ cells using the ELISPOT assay was reported after 4 weeks but no change compared to baseline after 8 weeks (161). I

also investigated cytokine secretion during a placebo-controlled study with etanercept in patients with ankylosing spondylitis. Although I observed a good clinical response I observed a significant increase in the number of IFNγ- and TNFα-positive T cells which was in clear contrast to our here presented results during infliximab treatment (unpublished observations; manuscripts in preparation).

The exact mechanism how infliximab works is not clear. Our results indicate that just neutralisation of TNFα in the fluid phase cannot be the only explanation because I found, in contrast to a treatment with etanercept, a long lasting suppression of T cell function. It has been proposed that infliximab could act by binding to membrane-associated TNFα, mediating lysis of activated macrophages and polymorphonuclear leucocytes via complement fixation or antibody-dependent cell cytotoxicity (162). However, I did not observe a significant change in the relative number of CD4+, CD8+, CD14+ and CD19+ cells in this study, which suggests that cytotoxicity is probably not involved in the therapeutic effect of infliximab.

In summary, our data show that infliximab downregulates preferentially the T cell capacitiy in the production not only of TNFα but also of IFNγ, an effect which is still present at least 6 weeks after the last infusion. This lasting effect on the immunoregulation could explain not only its good clinical effect but also some side effects. The observed reduction of the Th1-response is in line with the increased frequency of tuberculosis cases in patients treated with infliximab because a Th1-response is crucial for fighting these intracellular microbes (163).