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T cell response to putative antigens and downregulation of cytokine production by infliximab in ankylosing spondylitis patients

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Introduction

The spondyloarthropathies (SpA) are frequently occurring inflammatory rheumatic disease (1), in part leading to significant burden of disease with pain and disability probably not so much different from rheumatoid arthritis (RA, 2). Ankylosing spondylitis (AS) and undifferentiated SpA (uSpA) are the most frequent subtypes (3). The pathogenesis of the SpA has remained obscure although the extensive association with HLA B27 was reported already more than 25 years ago (4). Antigens derived from bacteria such as klebsiella (5) and autoantigens derived from the cartilage such as proteoglycan (6) have been tested but no convincing evidence for their involvement has been obtained to date.

1. Ankylosing spondylitis

Ankylosing spondylitis is a chronic systemic inflammatory disorder that primarily affects the axial skeleton (spine and sacroiliac), sacroiliac joint involvement is its hallmark (7). Hip and shoulder joints may be involved in some patients, but the involvement of the more peripheral limb joints occurs in only 20%. Ankylosing spondylitis may occur in association with reactive arthritis (Reiter's syndrome), psoriasis, ulcerative colitis or Crohn's disease, these forms of AS are called secondary AS. But most patients have no evidence of these associated diseases and are best classified as suffering from primary AS. The major clinical features of AS can be divided into skeletal and extraskeletal manifestations. Its typical presentation is with low back pain of insidious onset, arthritis of hips and enthesopathies are common. Extraskeletal complications include acute anterior uveitis, aortic valvular disease, and the cauda equina syndrome. Of which, acute anterior uveitis is the most common extraskeletal involvement in patients with AS. It occurs in 25-30% of patients at some time in the course of their disease (8), and is relatively more common in HLA-B27^{+} than HLA-B27^{-} patients with AS (9). Clinical manifestations of AS usually begin in late adolescence or early adulthood, onset after age 40 is uncommon. The disease is three times more common in men than women (9), and the clinical and radiographic features of the disease probably evolve more slowly in women. Recent studies have clearly shown that the demographic and clinical spectrum of AS is much wider. The disease affects women not infrequently and is of higher prevalence than previously appreciated (10). The course of AS is highly

variable and can be characterized by spontaneous remissions and exacerbations, particularly in early disease. The outcome is generally favorable because the disease is often relatively mild or self-limited, and the majority of the patients remain fully employed (11). Only rarely does AS show persistent disease activity that results in early and severe disability.

2. Genes and AS

2.1 Association of AS with HLA-B27

In AS, the aetiology is a mystery. The joint destruction is considered to be the result of the interaction between the host's immune response and enviromental factors, but we do not know what triggered this self-destructive process and why it persists. AS occurs mostly in immunogenetically susceptible hosts, its most important feature is an association with the histocompatibility antigen HLA-B27 (12) which is demonstrated in about 90-95% of cases. The prevalence of HLA-B27 in the general population, however, shows considerable geographic variation, occurring in 50% of Haida Indians of northern Canada (13), but being virtually absent among Black Africans (14) and Cuatemalan Indians (15). At present, there exists no satisfactory explanation of the great variation in the frequency of this genetic marker amongst various ethnic groups.

Class I antigens of the major histocompatibility complex (MHC) are 44kDa polymorphic molecules that are noncovalently associated with a monomorphic protein, β2 microglobulin, and expressed as a heterodimer on the surface of many cell types. Allelic variations in the amino acids of class I MHC molecules have classically been identified by antisera. HLA-B27 is a serologically defined allele of the HLA-B locus, one of the three classical loci encoding class I MHC molecules. Examination of HLA-B gene products that react with HLA-B27 typing alloantisera has revealed a family of allelic subtypes, twenty of these have been designated by the World Health Organization HLA Nomenclature Committee as HLA-B2701 through to HLA-B2720, of which, HLA-B2701 has been found in only a few individuals (16), and thus has not been amenable to population studies. However, association of ankylosing spondylitis with four of the other subtypes has been observed (17). By contrast, the study about African Blacks from Gambia indicated that there does not seem to be an association between HLA-B 2703 and ankylosing spondylitis (18). HLA-B2703 is a very unusual HLA-B molecule, containing a histidine at position 59

rather than the tyrosine that is present in all other HLA class I molecules, this feature might explain its lack of association with ankylosing spondylitis. There is also no evidence supporting an association of HLA-B2706 and B2709 with AS. However, it is likely that ankylosing spondylitis is associated with each of the other HLA-B27 alleles. Whether HLA-B27 alleles are also associated with the other spondyloarthropathies is currently unkown, but appears to be a good possibility.

An important question is whether HLA-B27 itself is a direct disease susceptibility factor, or alternatively, merely a marker for a disease susceptibility gene in close linkage disequilibrium with HLA-B27. Indirect evidence from clinical epidemiology strongly suggests that HLA-B27 itself is the disease susceptibility gene (19). More recently, direct evidence that the HLA-B27 molecule itself can predispose to the spondyloarthropathies has come from studies of transgenic rats expressing HLA-B2705. These animals spontaneously develop a broad spectrum of disease manifestations closely resembling human HLA-B27 associated disease (20).

It was observed that infectious agents can trigger reactive arthritis in HLA-$B27^+$ individuals (21), One hypothesis has been constructed to account for that a small region of a human antigen is identical to amino acid sequences of proteins encoded by the triggering microorganism (22). This hypothesis has been termed molecular mimicry, which suggested that a structure in the triggering bacterium with a homology to HLA-B27 incited an antibody response which was then perpetuated by the presence of HLA-B27. Mimicry may also be of a structural gene product or the HLA-B27 molecule itself. Although it is not clear how mimicry of a class I MHC molecule would lead to an anatomically localized disease as is the case in the spondyloarthropathies, there is considerable evidence for the sharing of antigenic determinants between HLA-B27 and different bacterial products.

Monoclonal antibodies against HLA-B27 have been observed to react with bacterial cell envelope glycoproteins from shigella flexneri, Klebsiella pneumoniae, and Yersinia enterocolitica (23). Similarly, one of these HLA-B27 reactive antibodies (B27M2) has identified two cross-reative proteins of 80kDa and 60kDa from a K.pneumoniae isolate (24). Using computer search for bacterial proteins with amino acid sequence homology to HLA-B27 has yielded positive results. The nitrogenase enzyme in K.pneumoniae was found to contains a six amino-acid region that is homologous to HLA-B2705 residues 72-77 (25), and what is more, a elevated levels of antibodies against an HLA-B27-derived peptide containing the region of homology

with K.pneumoniae was shown in 54% of reactive arthritis patients and 30% of ankylosing spondylitis patients (26). These results clearly indicate that there is an amino acid sequence homology between HLA-B27 and the K.pneumoniae nitrogenase gene product. But, its relevance to disease pathogenesis is unclear.

2.2 Involvement of other genes

For most autoimmune disorders, the pattern of inheritance is very complex. The major histocompatibility complex (MHC) gene complex has been implicated as the major genetic component in the predisposition to these diseases but backcross studies in animal models of SpA suggest that multiple genes contribute to disease susceptibility (27). Furthermore, findings of human family studies of twins and sibpairs support the notion that genetic factors other than B27 determine which B27-positive individuals develop arthritis (27). Association of AS with HLA-B27 has been known for 25 years, however, there has been little progress in establishing whether other genes, particularly non-HLA genes, may play a role. The frequency of ankylosing spondylitis in randomly selected HLA-$B27^+$ individuals is only about 8%, but that in HLA-$B27^+$ family members of an HLA-$B27^+$ patient with AS approaches 20% (28). One explanation for these results is that other genes may play a role in determining disease susceptibility. The T cell receptor (TCR) was considered as one of the candidate gene product, since the specificity of the TCR involved in recognition of HLA-B27 and HLA-B27-bound peptides is likely to be an important determinant in the pathogenesis of the spondyloarthropathies (29). It is likely that specific residues of the HLA-B27 molecule recognized by T cells or binding peptides that are recognized by specific T cells play an essential role in determining the specific TCRs involved (29). Non-random usage of the various segments of the β chain of the TCR has been found in human T cell clones recognizing HLA-B27 (30), supporting the conclusion that limited numbers of TCR gene products may be involved in recognizing HLA-B27 and, perhaps, HLA-B27-bound peptides.

The class II region contains 17 HLA class II genes and pseudogenes (31). It has been speculated that other MHC genes in the class II region might also be involved in AS susceptibility. The preliminary data derived from a genome-wide screening for susceptibility loci in AS confirm the strong linkage disequilibrium of the MHC region with AS but extends the MHC allelic association to about 8 cM across the MHC region, between markers D6S276 and DRB1 (32). This may be exclusively

due to linkage disequilibrium with HLA-B27, or alternatively, it may be due to extended haplotypes that may contain 2 or more genes relevant to AS susceptibility. Different studies suggest a weak effect of DRB1 on the susceptibility to AS in different populations (33). Further studies are needed to validate these observations and to elucidate whether this association is due to linkage disequilibrium with the relevant susceptibility genes.

TNF genes play an important role in inflammation, infection, and immune response (34). TNFα is a proinflammatory cytokine that plays a pivotal role in the inflammatory pathway and may have beneficial or deleterious effects depending on the extent of its release. Evidence exists indicating that individual TNFα responsiveness is genetically determined. Putative disease-causing TNFα alleles may either contribute to disease susceptibility or may only be in linkage disequilibrium with the causative gene. A suggestive idea is that differential TNFα production may contribute to the development of AS in B27 positive individuals, and an explanation for these differences could be due to an involvement of a TNFα promoter polymorphism (35,36).

Recently, a new molecule, tapasin, has been identified in the class II region (37), which plays a critical role in HLA class I expression by acting as a physical intermediary between TAP (transporters associated with antigen processing) and the class I heavy chain. Especially, the B2705 molecule achieves high levels of surface expression in the absence of tapasin (38). There are no data concerning the role of tapasin in AS susceptibility, but its properties, which are related to antigen processing, might contribute to the role of B27 in conferring susceptibility to SpA.

3. The role of T cells in rheumatic diseases

A central event in the etiology of rheumatic diseases appears to be the induction of immune reactivity to an antigen (foreign or self) by T cells. After activation by antigen, T cells can proliferate to serve as helper cells for B-cell antibody production or the generation of cytotoxic T cells. In addition, activated T cells can produce cytokines leading to functional changes, such as synovial cell proliferation in rheumatoid arthritis (RA) or collagen synthesis by fibroblasts in scleroderma (39).

T cells have been directly associated with rheumatic diseases because they represent the largest cell population infiltrating the affected tissue and interacting with

other blood-derived and resident cells. An important role for T cells in the pathogenesis of reactive arthritis is underlined by the finding that the synovial lining of affected joints is infiltrated with activated $CD4^+$ T cells in reactive arthritis (40).

Some T cells may contribute to disease through the secretion of cytokines acting as a bone and cartilage destructive factor, for instance, interleukin 17 (41). In addition, it increases production of proinflammatory cytokines by monocytes and further enhance their effects on extracellular matrix destruction.

The association of HLA-B27 with ankylosing spondylitis implies a role for CD8 T cells in its pathogenesis since the only known functions of the polymorphic portions of class I MHC molecules are selection of the TCR repertoire of CD8 T cells in the thymus and presentation of antigen to CD8 T cells in the periphery. Thus the principal function of MHC molecules is the presentation of antigenic peptides to T cells. While MHC class II molecules present peptides to $CD4^+$ T cells, CD8 T cells respond to the MHC class I/peptide complex. One attractive explanation for the HLA-B27 association is that presentation of a peptide by HLA-B27 to $CD8^+$ T cells might be an important step in the pathogenesis of the disease (4239). Direct evidence that CD8 T cells are likely to be involved in the pathogenesis of the spondyloarthropathies has come from observations made in individuals with acquired immune deficiency syndrome (AIDS). Despite suppression of CD4 T cells, these individuals are capable of developing reactive arthritis (43). AS has not been reported, although this may relate to the extended length of time required to develop symptomatic AS. Reactive arthritis developing in patients with AIDS frequently following a gastrointestinal or genitourinary infection, is usually associated with HLA-B27 and can be quite aggressive. CD8 T cell function is usually normal, although the number and function of CD4 T cells can be severely depleted. Moreover, the finding that reactive arthritis is extremely aggressive in AIDS patients, although perhaps not occurring at increased frequency, suggests that CD8 T cells may be involved in the development of reactive arthritis.

There is also some evidence that CD4 T cells play an important role in AS. Patients with ankylosing spondylitis exhibit $CD4^+$ T cells responses to the human cartilage proteoglycan (44), adoptive transfer could induce a $CD4^+$ T cell mediated spondylitis (45). Another evidence supporting a role of T cells in both induction and perpetuation of rheumatic diseases is that therapy against T cell and its receptors could lead to a significant immunomodulation with marked changes in clinical and

laboratory parameters. Many studies have shown that CD4-mAb can prevent and, in a treatment setting, suppress activity in these disease models, including collagen-induced arthritis (46). Administration of the anti-CD4 Mab was followed by an immediate transient clinical benefit accompanied by a significant decrease in C-reactive protein levels and clinical improvement in patients with RA (47). To evaluate the roles of $CD4^+$ and $CD8^+$ T cell subsets in vivo in the induction of AS by immunization, Banerjee and coworkers (48) treated PG-immunized mice with isotype-controlled rat IgG2β monoclonal anti-CD4 or anti-CD8 antibodies, some of the mice were left untreated. They found that $CD4^+$ T cell depletion resulted in total inhibition of the disease with markedly decreased anti-PG antibody responses. $CD8^+$ T cell depletion, however, significantly enhanced the severity of the disease without affecting peak anti-PG antibodies, as compared to the control mice. These results demonstrate a crucial role for $CD4^+$ T cells in the pathogenesis of this disease, at least in the animal model.

4. Role of cytokines in autoimmune arthritis

A wide variety of cytokines and growth factors are produced in tissue of autoimmune arthritis, including IL1α and β, IL6, M-CSF and TNFα (49). In the synovium, these cytokines can be detected at the mRNA level by blotting and by in situ hybrization (50,51). Immunohistological localization of these protein products has demonstrated predominant expression in macrophages. These proteins were also detected in the short term in ex vivo cultures of the entire mixture of cells derived by enzymatic disaggregation of the synovial membrance (50). Of importance was the observation that IL1 and TNFα could be detected by bioassay of synovial membrane cultures, and hence they were present in quantities able to signal effectively. T cell do not survive in the absence of stimulatory signals from the T cell receptor or cytokines. Cytokines present in the joint that may be important in sustaining T cell survival and function include IL2 (low amount), IL7, and IL15 (relatively abundant) (52). IL10 reportedly prevents apoptosis in B lymphocytes and T lymphocytes (53), and as it is abundant in RA joints it may have a role in sustaining the survival of T cells there, although its inhibitory effects are also expressed.

In autoimmune arthritis, chronic immune responses and inflammatory reactions often cause severe destruction of cartilage and bone. This destruction progressively invades the bone and spreads over the cartilage, occurring in two

forms, one highly cellular, expressing essentially the same mixture of cytokines as in the active synovium and associated with active erosion. Another subset is relatively acellular and expresses chiefly TGF, TNFα and IL1 which have been shown to play a pivotal role in the pathogenesis of the synovitis and are regulators of osteoclastic resorption. Direct evidence in support of a role for IL1 in the pathogenesis of bone erosions in inflammatory arthritis is provided by examination of the findings in animals in which there is overproduction of this cytokine. Analysis of the joint changes in rabbits that constitutively overexpress IL1α due to intraarticular gene transfer revealed severe inflammatory arthritis with evidence of bone and joint destruction (54).

Mice bearing a human TNFα transgene that leads to dysregulated expression of this cytokine also develop spontaneous and progressive polyarthritis at an early age, consistent with the view that TNFα is an important cytokine in the initiation and perpetuation of inflammatory arthritis (55). When these animals are backcrossed into the DBA/1 background, arthritis is accelerated and severe bone erosions develop in association with multinucleated cells in bone resorption lacunae (56). Treatment with a monoclonal antibody to murine TNFα/β after the onset of CIA in mice leads to amelioration of arthritis as well as a reduction in histologic features of joint damage (57). In humans with RA, there is also evidence that blockade of IL1 and TNFα can modulate inflammation and in some instances retard bone erosions. These data suggest that both IL1 and TNFα play a role in the formation and progression of bone erosions.

Like IL1 and TNFα, there is increasing evidence that IL6 may inhibit formation and induce bone resorption through its stimulatory effects on osteoclasts, for example IL6 gene knockout mice do not develop bone erosions (58). But it seems that interleukin-6 alone does not induce osteoclast formation, soluble interleukin-6 receptors (sIL-6R) triggered the formation in the presence of IL-6 in cocultures of murine osteoblastic cells and bone marrow cells (59).

The accumulating evidence suggests that TNFα is not only an inflammatory mediator in its own right but also is the key regulator of the production of other cytokines such as IL1, GM-CSF, IL6 and IL8. Other proinflammatory cytokines were also inhibited if TNFα was neutralised, leading to the new concept that the proinflammatory cytokines were linked in a network with TNFα at its apex. This led to

the hypothesis that TNFα is of major importance in autoimmune arthritis and as a therapeutic target. This hypothesis has been successfully tested in animal models of collagen induced arthritis, and these studies have provided the rationale for clinical trials of anti-TNFα therapy in patients with long-standing rheumatoid arthritis. Meanwhile, antagonists of IL1 and IL6 have also been tested in the clinic.

5. Cytokine pattern and rheumatic arthritis

Cytokines are involved in the regulation of growth, differentiation and function of hematopoietic and non-hematopoietic cells and play an important role in the regulation and outcome of an immune response. Subpopulation of human T cells (Th0, Th1 and Th2) can be distinguished by their cytokine-secretion pattern. Evidence is increasing from a lot of studies that the outcome of a human disease may depend on the subpopulation of T cells that predominates at the site of inflammation. It is now generally accepted that a balance between Th1 and Th2 cells determines the phenotype and progression of numerous diseases, such as inflammatory, allergic, or autoimmune diseases (60). The Th1 type cytokines interferon (IFN) γ and tumor necrosis factor (TNF) α are required for an effective cellular immune response and involved in the expression of chronic inflammatory diseases, while Th2 cells (secreting IL4 and IL5) are responsible for the induction of a humoral response. Cytokines of the Th1 spectrum are general elevated in successful responses to a variety of intracellular pathogens, and Th2 cytokines are elevated in allergic diseases and in helminth infections. The balance appears to be maintained not only by the cytokines considered originally to be of Th1/Th2 type but also by other inhibitory cytokines such as transforming growth factor β. Two other important cytokines which regulate the Th1 and Th2 responses are interleukin (IL)12 and IL10. IL12 selectively induces a Th1 cytokine pattern against which IL10 is an important negative regulator.

The immune responses driven by Th1 T cells and Th2 T cells are sometimes also influenced by a third T cell type whose main function is counterregulation or suppression of immune responses mediated by Th1 and Th2. It has been shown, for example, that the induction of oral tolerance by the feeding of relatively low amounts of myelin basic protein leads to the induction of immunoregulatory T cells, which prevent the development of experimental autoimmune encephalitis (61). Such T cells have a unique cytokine production pattern in that they produce high levels of TGFβ without necessarily producing either Th1 or Th2 cytokines, these subtype T cells have been

termed Th3 T cells. Th3 cells producing TGFβ have also been shown to occur in experimental models of colitis or diabetes or in $HgCl_2$ induced autoimmune disease (62). In these cases it is thought that such T cells play an important role in disease prevention or cure. Recently, another type of regulatory T cell has been identified that may be related to the aforementioned Th3 T cell. This cell, termed T regulatory cell 1(Tr1), is induced in vitro by stimulation of T cells in the presence of IL10 and is a T cell producing high levels of IL10 (63).

Cytokine profiles in RA patients have been characterized by a number of different approaches. The first studies investigated cytokine by enzyme-linked immunosorbent assay in synovial fluid or supernatant from T cells clones isolated from RA joint. Then, studies evaluated RA patients' joint cytokine mRNA by reverse transcription polymerase chain reaction or in situ hybridization. Rheumatoid arthritis is usually considered to be a Th1 disease, with the shift toward a Th1-mediated immune response (64) although there exist some contradictory results. It is now established that monocyte-derived pro-inflammatory cytokines such as TNFα and IL1 found in abundance in RA synovium play an important role in the pathogenesis of RA.

Reactive arthritis, which is induced by several intracellular bacteria and remnants of bacteria or even live bacteria can be found in the joint, serves as a useful model of chronic inflammatory diseases, because the triggering antigen can be identified, whereas for others, the triggering events are unknown. Our previous studies indicate that the key cytokine for a Th2 response, IL4, was found in synovial membrance of ReA patients more frequently than in rheumatoid arthritis patients using the technique of PCR and in situ-hybridisation (64). In another study the Th1/Th2 cytokine secretion pattern upon stimulation of synovial fluid mononuclear cells with the triggering bacterium was investigated in ReA patients, in whom low amounts of IFNγ and TNFα but high amounts of IL10 were found also hinting towards a Th2 pattern (40).

The concept of Th1/Th2 balance has attracted much interest recently in attempts to understand the pathogenesis of AS. In contrast to ReA, a pathogenic role of bacteria in AS is less clear and very little is known about cytokines in AS. A lot of researchers have been trying to assess whether the T cell cytokine pattern in AS might also be polarized towards a Th2 like pattern since ReA and AS are related clinical conditions. A cytokine pattern different from RA was recently observed in SpA (65), where low Th1 cytokines were found in AS patients. A similar situation was

demonstrated in another study (36): the T cell production of IFNγ and TNFα of AS patients was on average significantly decreased whereas the T cell production of IL4 did not differ from controls, which indicates that AS can be regarded as a low TNFα/IFNγ disease. However whether a typical Th2 type pattern characterised by the predominance of IL4 positive T cells is present remains to be further identified.

6. Putative antigens

Inflammatory responses provoked by pathogens are antigen-specific in their induction but are nonspecific in their effects. Consequently, they are potentially damaging to the host that produces them. In addition, the immune system can respond specifically to self antigens, thereby giving rise to autoimmune diseases. Ankylosing Spondylitis is a T cell dependent inflammatory possibly autoimmune disease, its pathogenesis is regarded as a consequence of the activation of T cells by yet unknown antigens and the co-stimulatory molecules CD3 and CD28. A lot of potential antigens have been proposed for this process, including type II collagen, aggrecan G1 domain, glycoprotein gp39 and heat shock proteins, and others. 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.

6.1 Antigens derived from cartilage

In joint diseases, there is a loss of the normal balance between synthesis and degradation of the macromolecules that provide articular cartilage with its biomechanical and functional properties. Articular cartilage is a multiphasic material with 2 major phases: a fluid phase composed of water and electrolytes, and a solid phase composed of collagen, proteoglycans, glycoproteins, other proteins, and the chondrocytes. Each phase contributes to its mechanical and physiologic properties.

Autoimmune reactions to auto-antigens may play a key role in the pathogenesis of inflammation in various rheumatic diseases (66). The major components of the matrix of articular cartilage and the intervertebral disc, namely type II collagen and the large aggregating proteoglycans, have both been incriminated as autoantigens in these diseases (66). Cartilage is normally secluded from immune surveillance due to its avascular structure (67); however, local

inflammatory processes (for example: from trauma or infections) may stimulate the production of various factors and enzymes which could degrade the cartilage matrix in sites which include diarthrodial joints and the intervertebral disc. This could then expose the cartilage components to the immune system leading to an autoimmune reaction to cartilage (66,67). The autoimmune attack on the joints could also be triggered by a cross reactive immune reaction in response to unrelated antigens by the mechanism of "molecular mimicry" (68). The net result of such autoimmune reactions could be further destruction of cartilage and release of more autoantigens. This could lead to a chronic, self-perpetuating inflammation in genetically predisposed individuals who are prone to develop these autoimmune reactions (67). Relevant to this is the finding that removal of all cartilage from an arthritic joint at arthroplasty leads to a loss of synovial inflammation in that joint (69). In addition, end-stage arthritis with a total destruction of cartilage leads to a gradual decrease in inflammation of the synovium in the affected joints (69).

Type II collagen is found in cartilage, as well as in the vitreous humor of the eye, which forms the basic fibrillar structure of the extracellular matrix. Like collagen type II, type IX and XI are also cartilage specific and are present together with type II collagen. There is evidence that many collagens, including type II, IX and XI in cartilage, exist as hybrid molecules. The induction of polyarthritis in rats, mice and primates on immunization with type II collagens has strongly suggested that this antigen possibly play an important role in the pathogenesis of various human rheumatic disease (70,71). The investigation of an immune response to type II collagen in rheumatic diseases have been investigated both at the cellular and humoral levels (72,73). Several lines of evidence point to type II collagen as a significant autoantigen, at least in RA. Autoantibodies directed against type II collagen are found in the serum and joint fluid-derived T cells of a significant proportion of RA patients (74), cells obtained from the affected synovium of RA patients recognize type II collagen (75). Furthermore, oral administration of chicken collagen to patients and human collagen to mice significantly improves RA and collagen-induced arthritis in some studies (76). When injected into selected strains of mice and rats and into nonhuman primates, type II collagen causes an inflammatory arthritis resembling rheumatoid arthritis (77).

Another cartilage-specific molecule is the large proteoglycan called aggrecan, which is arranged in 3 globular domains (G1, G2 and G3). The G1 domain binds to

hyaluronan and link protein in cartilage, resulting in the formation of macromolecular PG aggregates. Cartilage proteoglycan has an Mr of 1-3 x 10^6 with a protein core of Mr 210, 000 to which are attached numerous glycosaminoglycan chains, namely chondroitin sulfate (CS) and keratan sulfate (KS) (78). Numerous N-linked and O-linked oligosaccharides are present interspersed on the protein core. The N-terminal G1 globular domain binds non-covalently to hyaluronate (HA) in the cartilage matrix and is known as the hyaluronate binding region (HABR). This linkage is stabilized by link protein to form macromolecular aggregates of proteoglycan and link protein about an HA chain. There are two N-terminal globular domains called G1 and G2. The around 30 KS chains of proteoglycan are attached to a region C-terminal to G2. Up to 100 CS chains are attached to a CS region C-terminal to the KS rich region. The C-terminal end is characterized by another globular domain called G3 which has lectin-like properties. This region has also been shown to be alternatively spliced and may or may not contain additional regions with homologies to epidermal growth factor and complement proteins (79). Immune response to aggrecan have been detected in rabbits with experimentally induced synovitis (80). Patients with inflammatory arthritis exhibit cellular immunity to this molecule (81).

In AS, antigen derived from the cartilage such as proteoglycan have been tested as a possible autoantigen, but no convincing evidence for its involvement in pathogenesis has been obtained to date (18,44). While the animal model of HLA B27 transgenic rats has not been able to fulfil the initially high expectations derived from the suggestive clinical disease of these rats, a mouse model for AS has recently gained some interest. Injection of the G1 domain of the main proteoglycan aggrecan into BALB/c mice induces not only peripheral arthritis but also spondylitis (82). It could be shown that T cells play an important role in this model and G1 derived immunodominant T cell epitopes have been identified (82).Of some interest in the context of SpA, aggrecan is present in fibrocartilaginous entheseal regions of the tendon but not in the human midtendon (83). Versican, another proteoglycan has a similar G1 domain. Furthermore, G1 is the major degradation product of intervertebral discs. There are limited data in humans about the cellular immune response to the G1 to date, mainly based on lymphocyte proliferation. With these technique, a T cell response to the G1-protein has been reported in AS and RA patients (84).

One of the major secreted proteins of human articular chondrocytes in monolayers or explant cultures and of synovial fibroblasts is a glycoprotein with an apparent molecular weight of approximately 39,000, referred to as human cartilage glycoprotein-39 (HC gp-39). HC gp39 has gained interest recently. Multiple levels of evidence suggest that HC gp39 is a target of the immune response in the joints of RA patients: both HC gp39 messenger RNA and protein have been detected in synovial specimens and cartilage from RA patients but not from normal subjects (85); HC gp39 is the predominant secretory protein of synovial fibroblasts and is produced by articular chondrocytes (86); and serum levels of HC gp39 correlate with joint disease(87). But so far there is no report about the relationship between gp-39 and the pathogenesis of AS.

6.2 heat shock protein (hsp)

Based on high interspecies sequence homologies, inducible tissue expression and a strong immunogenicity, hsp have been repeatedly incriminated to be involved in various autoimmune disease. By now, various lines of evidence suggest that hsp play an active role in the development of autoimmune diseases in animals (88), and in human autoimmune diseases (89). However, Van der Zee and coworkers (90) reported that only passive transfer of a T cell clone responding to mycobacterial hsp60 evoked disease in naive recipient animals. The disease could not be induced by immunization with hsp60, instead protection was established. A similar situation was found in experimental models of arthritis in which immunization of mice with an immunodominant epitope (amino acid 261-271) from hsp 65 can protect from the development of pristane-induced arthritis by T helper type 2 pattern (91). Immunization with Mycobacterium tuberculosis heat shock protein 60 has been shown to protect rats from experimental arthritis (92). It has been documented that preimmunization with microbial proteins belonging to different hsp families(mycobacterial hsp70, hsp60, hsp10) protect from subsequent induction of arthritis (93,94). These different results indicate that hsps might play a major role as a target molecule or as a protective factor.

6.3 Antigens derived from bacteria

The development of many autoimmune diseases has been etiologically linked to exposure to infectious agents (95,96). Models proposed to account for the

relationship between infection and autoimmunity include inflammation-induced presentation of cryptic self-epitopes, antigen persistence and molecular mimicry (97,98). There is a clear relationship between bacteria and reactive arthritis, which usually occurs after a genitourinary tract infection due to Chlamydia trachomatis or an intestinal infection due to Yersinia, Salmonella, Shigella or Campylobacter jejuni. Persistence of bacteria or bacteria antigens may play a pivotal role in the immune process that finally leads to the inflammatory characteristics of reactive arthritis. Yersinia enterocolitica is a common pathogen of reactive arthritis, the synovial T cell from patients with yersinia-induced ReA response is primarily directed against bacterial components, of which the 19-KD urease beta subunit is regarded as one of the immunodominant proteins (99). By now, no sufficient evidence supported a relationship between bacteria infection and AS or RA. Especially for AS, it could be of some significance to test T cell responses to bacterial epitopes because AS and reactive arthritis belong to the group of spondylarthropathies which comprise a spectrum of diseases characterized by shared clinical features and a variably strong association with HLA-B27, with 20-40% of HLA-B27 positive patients with ReA developing the full picture of AS (100).

7. Identification of antigenic T cell epitopes

The identification of T cell epitopes is of significance for the understanding of the host response during autoimmune diseases. The characterization of relevant T cell epitopes is generally based on the analysis of the specificity of T cell lines propagated in vitro (101). Alternative approaches are the biochemical purification and sequencing of MHC-bound peptides or the T cell-screening of expression libraries prepared from the organism of interest. A more recent method is the identification of unknown T cell epitopes by the screening of combinatorial peptide libraries resulting in millions of different peptides (102). Epitope screening with synthetic peptides is expedited by the use of peptide spot libraries, which allow the automated and economic synthesis of multiple peptides (103).

The identification of MHC class I-restricted T cell epitopes is further facilitated by the existence of relatively strict peptide binding motifs of individual MHC class I molecules (104). If the CD8 T cell target protein is already identified, the knowledge of MHC class I binding motifs allows the educated guessing of epitopes and thus greatly reduces the number of synthetic peptides required for epitope identification.

But epitopes that do not exhibit the typical binding motif may be overlooked. Regarding the identification of CD4 T cell epitopes, the less strict binding requirements and thus the limited predictive value of MHC class II motifs (105) makes this approach less suitable.

Recently, a new technique has been introduced which allows the determination of antigen specific T cell frequency, the analysis of cytokine release after antigen specific T cell stimulation (106), and the identification of the cytokine secreting cells according to its surface marker(107). The advantages of flow cytometry are largely based on its ability to analyze very rapidly, even in small samples, multiple cell properties simultaneously, including size, granularity, surface antigens and intracellular cytokines. These features make identification of T cell epitope of antigens faster and more accurate, and it is even possible to further analyze the phenotype of the TCR of antigen-specific T cells after separating the specific cells by MACS (magnetic activated cell sorting) or FACS (fluorescence activated cell sorting).

Aims of the study

The pathogenesis of two important inflammatory rheumatic diseases, rheumatoid arthritis and ankylosing spondylitis, is not clear. In both diseases an immune response against an unknown autoantigen could play a crucial role. T cell responses to antigens derived from bacteria such as klebsiella or to autoantigens derived from the cartilage such as proteoglycan have been tested in AS, but no convincing evidence for their involvement has been obtained to date.

G1, gp39 and collagen II derived from proteoglycan aggrecan have been suggested as candidate autoantigen in AS over the last years. In this study, I applied the more sensitive and more specific technique of antigen-specific cytometry to investigate the T cell response to various cartilage-derived autoantigens. Taking IFNγ as primary outcome parameters I set out to quantify the antigen specific T cell response in peripheral blood (PB) and synovial fluid (SF) of patients with AS, RA and controls to answer the question whether T cells specific for the G1-domain of aggrecan and to single G1-derived peptides are detectable in AS-patients and controls, and compared this to the response after stimulation with GP39 and collagen II proteins.

AS is the final outcome in between 20-50% of HLA-B27 positive patients with reactive arthritis or inflammatory bowel disease. Therefore, the question has been raised whether the immunopathology in AS is caused by an antibacterial immune response. To search for possible bacterial antigen, I looked for T cell responses to Yersinia 19KD in AS patients by flow cytometry and compared this response with that in RA-patients and controls.

Based on high interspecies sequence homologies, inducible tissue expression and a strong immunogenicity, hsp have been repeatedly incriminated to be involved in various autoimmune diseases. To answer whether h-hsp60 is involved in the pathogenesis of AS, cytokine secretion triggered by h-hsp60 was also analysed at single cell level by flow cytometry in AS- and RA-patients as well as in controls.

To further analyse T cell response to G1-domain in AS patients, G1 specific T cell was separated by IFNγ secretion assay, its specificity was checked by restimulation with G1 protein.

Treatment of active AS with the monoclonal anti- TNFα antibody infliximab is clinically highly effective, the precise mechanism of action, however, is not known. Another aim of this study was to assess any change in the capacity of $CD4^+$ and $CD8^+$ T cells to produce cytokines during treatment. I conducted a double-blind, placebo-controlled, multicenter trial of infliximab in active AS. The antigen specific and non-specific cytokine production during treatment with infliximab was investigated in peripheral blood (PB) from 20 randomly selected patients (10 underwent infliximab treatment and 10 placebo as controls) to answer the question whether and how the cytokine pattern changed after treatment and whether this be related to the clinical effect.

Materials and methods

1 Materials

1.1 Equipment

Pipette	Eppendorf-pipette 0.5-10 µl, 10-100 µl, 50-200 µl, 50-250 µl,100-1000 µl, 1-10 ml (Eppendorf, Hamburg)
Sodium heparin tube	Becton Dickinson (Plymouth, UK)
96-well cell culture plate	NUNC (Denmark)
24-well cell culture plate	Costar (USA)
96-well immunoassay plate	Costar (Cambridge, MA)
Pipetting device	Pipetus akku (ICN Flow, Meckenheim)
Electropower supply	Typ BCN58-200L (Berlin Germany)
Centrifuges	Megafuge 1.0 R (Heraeus Sepatech) Biofuge pico, Heraeus (Osterode, Germany) Ttettich Rolanta (Osterode Germany) Eppendorf-Zentrifuge Model 3200, 5414 (Eppendort, Hamburg)
CO_2-incubator	BB16, heraeus (Osterode, Germany)
Microscope	Diavert, Leitz (Wetzlar, Germany) BX60F5, Olympus (Berlin, Germany)
Precision balances	Type 2662, Sartorius (Göttingen, Germany) Ohaus (CT600-S)
Digital balance	Basic (Satorius, München)
Magnetic stirrer	MR 3001, Heidolph (Kehlheim, Germany)
Mixer	Thermomixer comfort, Eppendorf (Hamburg, Germany) Vortex IKA VF2, Jahnke und Kunkel (Staufen I. Br.D) Vortex Genie 2^{TM} (Bender & Hobein, Zürich, Schweiz)

Waterbath	Haake GH/D8 (Bender & Hobein, Zürich, Schweiz)
Spectrophotometer	GeneQuant II (Parmacia, Freiburg)
Hood	Biological Safely Cabinets (Nu-425-600)
Magnetic cell separators	MiniMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany)
Columns	MS^+/RS^+ plus adaptor (Miltenyi Biotec, Bergisch-Gladbach, Germany)
FACSCalibur	Becton Dickinson (San Jose, CA)
Power Macintosh G3	(USA)
CellQuest™ software	Becton Dickinson (San Jose, CA)
ELISA reader	Dynatech MR5000 (Denkendorf, Germany)

1.2 General reagents

10 x PBS	GIBCO BRL (Eggenstein, Germany)
1 x PBS	Biochrom KG (Berlin, Germany)
Ficoll-paque™ Plus	Pharmacia (Uppsala, Sweden)
HBSS	GIBCO BRL (Eggenstein, Germany)
Trypan blue	Biochrom KG (Berlin, Germany)
Anti-CD28	Immunotech (Marseille, France)
SEB	Sigma (Munich, Germany)
PMA	Sigma (St. Louis, Mo)
Ionomycin	Sigma (St. Louis, Mo)
DMSO (Dimethyl Sulfoxide)	Serva Electrophoresis GmbH (Heidelberg, Germany) (used for cells freezing storage) Sigma Chemical CO. (Steinheim, Germany) (used for peptides solution)
Albumin Bovine Fraction V	Biomol Feinchemikalien (Hamburg)
Penicillin/Streptomycin	Biochrom KG (Berlin, Germany)
L-Glutamin	Biochrom KG (Berlin, Germany)
Fetal Calf Serum (FCS)	GIBCOBRL (Life Technologies, Paisley, Scotland)
RPM1 1640	GIBCOBRL (Life Technologies,

	Paisley, Scotland)
Infliximab	cA2 Remicade (Fa Essex/Centocor)
FACS flow	Becton Dickinson (Heidelberg, Germany)
FACSRinse	Becton Dickson (San Jose, CA)
FACSafe	Becton Dickson (San Jose, CA)
Cellwash	Becton Dickson (San Jose, CA)
Brefeldin A	Sigma (Munich, Germany)
EDTA	Merck KgaA (Darmstadt, Germany)
Ethanol	(BDH, Karlsruhe)
37% Formaldehyde Solution	J.T. Baker (Holland)
Sodium azid	Merck KgaA (Darmstadt, Germany)
FACS lysing solution	Becton Dickinson (San Jose, CA)
FACS Permebealizing Solution	Becton Dickinson (Heidelberg, Germany)
Beriglobin	Centeon Pharma (Berlin, Germany)
IFNγ Catch Reagen	Miltenyi Biotec (Bergisch Glodbach, Germany)
IFNγ Detection Antibody (PE)	Miltenyi Biotec (Bergisch Glodbach, Germany)
Anti-PE MicroBeads	Miltenyi Biotec (Bergisch Glodbach, Germany)
Propidium iodide (PI)	Becton Dickinson (Heidelberg, Germany)
CFDA-SE	Molecular Robes (Eugene, Oregon)
IL2	Boehringer Mannheim (Frankfurt, Germany)
ELISA kits	Pharmigen (Bergisch Gladback, Germany)

1.3 Special reagents and buffers

1). 10% Sodium Azid

10 g sodium azid

100 ml DH_2O

2). 2% Formaldehyde:

2.7ml 37% formaldehyde

47.3ml 1 x PBS

3). FACS buffer (PBS/BSA buffer)

0.5% BSA (2.5g/500ml Albumin Bovine Fraction V)

50 ml 10 x PBS

5 ml 10% Na azid

adding $DH_20\rightarrow$ total volume 500ml

4). MACS buffer (sterile)

phosphate buffer saline pH 7.2

0.5% bovine serum albumin (BSA)

2 mM EDTA

5). 10% DMSO

DMSO was diluted 1:10 with FCS

6). Medium for cell cuture (sterile)

A: for short term culture

RPMI 1640 containing 10% heat-inactivated fetal calf serum

2 mM L- Glutamine

Penicillin 100 units/ml

Streptomycin 100 µg/ml

B: for long term culture

RPMI 1640 containing 10% autologous serum

2 mM L- Glutamine

Penicillin 100 units/ml

Streptomycin 100 µg/ml

7). RCLS (Red cell lysing solution, sterile)

NH_4Cl 8.29g

KHCO3 1.0g

EDTA 0.372g

Adding DH_2O to volume 1 L

8). FACS flow

1000 ml 10 x PBS

9000 ml milliPore Water

20 ml 10% sodium azide (final concentration: 0.02%)

9). ELISA buffers

Coating buffer:	0.1 M Na_2HPO_4, pH>9.0
Blocking buffer:	10% inactivated fetal calf serum (FCS) dissolved in phosphate buffered saline (PBS)
Washing buffer:	PBS with 0.1% Tween 20
Subtrate buffer:	0.05M phosphate-citrate buffer with 0.03% sodium perborate capsules (Sigma, St.Louis, MO)
Stopping buffer:	2N HCl

1.4 Recombinant proteins as antigen:

Aggrecan G1 protein	(delivered by Y. Zhang from McGill University, Montreal, Canada.)
Glycoprotein 39	(kindly provided by M.Boots, Akzo Nobel, The Netherlands)
Collagen II	(kindly provided by Fibrogen, U.S.)
Human hsp60	(obtained from R. Lauster, DRFZ, Germany)
Yersenia 19KD	(obtained from R. Lauster, DRFZ, Germany)

1.5 Staining antibodies

Anti-CD3 PE	Becton Dickinson (San Jose, CA)
Anti-CD4 PerCP	Becton Dickinson (San Jose, CA)
Anti-CD4 Cy5	Pharmingen (San Jose, CA. Coupled in our lab.)
Anti-CD8 PerCP	Becton Dickinson (San Jose, CA)
Anti-CD8 PE	Becton Dickinson (San Jose, CA)
Anti-CD14 PE	Becton Dickinson (San Jose, CA)
Anti-CD19 FITC	Becton Dickinson (San Jose, CA)
Anti-CD69 PE	Becton Dickinson (San Jose, CA)
Anti-CD69 FITC	Becton Dickinson (San Jose, CA)
Anti-IFNγ	Pharmingen (San Jose, CA; Coupled to Cy5 in our Lab; 1:400 dilution)
Anti-IFNγ FITC	Pharmingen (San Jose, CA)
Anti-TNFα	Pharmingen (San Jose, CA; Coupled to FITC in our Lab. 1:800 dilution)
Anti-TNFα FITC	Pharmingen (San Jose, CA)
Anti-IL10 APC	Pharmingen (San Jose, CA)
Anti-IL4 PE	Pharmingen (San Jose, CA)
Anti-IL4 APC	Pharmingen (San Jose, CA)

2 Patients and donors

All patients were seen at the University Hospital Benjamin Franklin in Berlin, German. Aspiration of synovial fluid was performed for diagnostic or therapeutic reasons from knee joints, with approval from the Ethical Committee of the Benjamin Franklin

Hospital. AS patients fulfilled the modified 1984 New York criteria for a diagnosis of AS (108) and all RA patients the ACR-criteria for the diagnosis of RA (109).

Table 1. Characteristics of patients and controls

Diagnosis	Source	n	Age (years)	Disease duration
Ankylosing spondylitis*	PB	47	35.4 ± 8	6.7 ± 7
Ankylosing spondylitis	SF	7	43.3 ± 9	9.6 ± 1
Rheumatoid arthritis	PB	22	56.4 ± 5	6.2 ± 8
Rheumatoid arthritis	SF	4	51.2 ± 7	7.4 ± 9
Healthy control	PB	20	42.5 ± 7	

PB = peripheral blood *95% of patients were positive for HLA-B27.

SF = synovial fluid

The characteristics of the patients and donors are shown in Table 1. All patients were in an active state of disease which means that they had current inflammatory back pain, a joint effusion, an elevated CRP, with at least two of these three parameters being positive. However, the degree of activity varied and I did not attempt to quantify disease activity in more detail. Peripheral blood (PB) and synovial fluids (SF) of consecutive patients was collected.

For T cell response to candidate autoantigens G1, gp39 and collagen II, PB from 47 and SF from 7 AS-patients, PB from 22 and SF from 4 RA-patients and PB from 20 healthy controls (HC) were examined.

For analysis of T cell response to h-hsp60 and y-19KD, 45 of 47 AS patients, with mean age(years) ± SD: 35 ± 7.5, were tested; the case number of RA and HC, that were detected, was the same as that for analysis of T cell response to G1.

3 Methods

3.1 Preparation of G1 protein

Molecular production of the G1 domain of aggrecan contains 19 exons ranging in size from 77 to 4224 base pairs. It was recombinantly product by the following method:

Human aggrecan G1 domain (AG1) proteins were expressed and purifyed in an adenovirus expression system. Briefly, a cDNA fragment encoding the N-terminal 431 amino acids of human aggrecan with a His-tag at its C-terminus was generated from a human chondrocyte RNA preparation by RT-PCR (5'-GCAGATCTACTA TGGCCACTTTACTCTGGGTTTTCG -3 and 5'-CAGATCTCAATGGTGATGGTGAT GATGCTCAGCGAAGGCAGTGGC-3'). This PCR fragment was cloned into a pCR2.1 vector using a TA cloning kit (Invitrogen Inc., Carlsbad, CA). The construct, included human aggrecan G1 globular domain and a partial interglobular domain (IGD) plus 6 histidine residues at its C-terminal, was sub-cloned into pQBI-AdCMV5-IRES-GFP transfer vector from the ADENO-QUEST KIT (Quantum Biotechnologies Inc, Montreal, QC) at Bgl II site. After linearized by FseI restriction enzyme digestion, 1 mg of the recombinant transfer vector plasmid is co-transfected into 293 cells with 1mg of QBI-viral DNA from the same kit using Lipofectamine plus reagent (Lifetech Co. Burlington, ON). Screening and purification of recombinant adenovirus were performed according to Adeno-Quest application manual included in the kit. For recombinant AG1 production, 293 cells were splited onto 150mm dishes in 1 to 10 dilution in DMEM plus 5% FCS, grown for two days till cells were ~90% confluent, then the media were changed into 293 serum-free media (Lifetech Co. Burlington, ON), meanwhile recombinant virus were added at 50 MOI. On day 3 after infection, the supernatant containing recombinant AG1 protein was collected. The supernatant was applied to a Sephadex G-25 column equilibrated with PBS, pH7.4: the protein-containing fractions were collected and applied to a Ni-NTA agarose column (Qiagen Inc., Mississanga, ON). The column was washed with 40 mM imidazole in PBS containing 0.3 M NaCl, pH 7.4. Recombinant VG1 and AG1 were eluted with 100 mM imidazole in PBS, pH7.4, containing 0.3 M NaCl.

3.2 Generation of recombinant h-hsp60 and Y-19KD

The complete open reading frames of the Yersinia 19kd was amplified by PCR. The **human hsp60** gene was amplified from a complementary DNA preparation derived from a patient's cartilage, using Pwo I polymerase (Boehringer Mannheim, Mannheim, Germany) and commercial oligonucleotides (Tib-MolBiol, Berlin, Germany). The amplified fragments were isolated from agarose gels with a Jet Scorb kit (Genome, Bad Oeynhausen, Germany), digested with appropriate

restriction enzymes, and ligated into 1 of the pQE vectors (Qiagen, Hilden, Germany).

In the construction of the pQE 90 vector, the polycloning site of pQE60 was modified such that Nde I and Nsi I restriction sites were introduced. The RO vectors contained the lac repressor gene cloned into the Xba I site of the pQE series. These plasmids are independent from cotransformed pREP 4 strains. A positive clone was selected after transformation of the electrocompetent E coli strain M15 (pREP 4). Fluorescence sequencing was performed on an Alf sequencer (Pharmacia LKB) by Replicon (Berlin, Germany). Expression of the cloned gene was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (sigma). Cells were harvested, and purification of the recombinant proteins with a His tag was performed on Ni-NTA resin affinity columns according to the manufacturer's protocol (Qiagen).

The procedure for preparation of Yersinia 19KD and h-hsp60 is briefly described as follows (110): cultured bacteria were disrupted in a French press in the presence of a proteinase inhibitor cocktail. High speed ultracentrifugation (150,000g) was applied for 10 hours in order to separate cytoplasmic proteins from a pellet containing membrane proteins as well as nucleo- and ribonucleo- proteins. This pellet was acid extracted and the solute dialyzed t neutrality. Futher purification was performed on a fast protein liquid chromatography system (Pharmacia LKB, Freiburg, Germany) using a Mono-S cation exchange column with an appropriate salt gradient. The resulting peaks were then purified by reverse-phase high-performance liquid chromatography (HPLC) using a preparative C8 column (SuperPac Sephasil C8, 5 µm, 4 x 250 mm; Pharmacia LKB), with an acetonitrile gradient (0-80%) in 0.1% trifluoroacetic acid in water. The resulting peaks were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dialyzed against RPMI 1640 medium.

SDS-PAGE (T=12.6%, C=2.7%) was performed without boiling under reducing and nonreducing conditions. The gels were stained with Coomassie blue R 250.

For western blotting, Immobilon polyvinylidene difluoride (PVDF) membranes (Milipore, Bedford, MA) with Yersinia 19kd and human hsp60 bands were incubated with patients' sera in a 1 hour with peroxidase-labeled goat anti-human IgG and anti-human IgA (Dianova, Hamburg, Germany) diluted 1:5000.

3.3 Preparation of Collagen II

Collagen II, also recombinantly produced, was kindly provided by Fibro Gen, San Francisco, U.S. It was expressed in yeast and lacks hydroxylysine and the glycosylated forms of hydroxylsine.

3.4 Preparation of cartilage Gp 39

HC gp39 was isolated from MG-63 osteosarcoma cell line (111). MG-63 cells (human osteosarcoma CRL 1427; ATCC) were cultured in cell factories in serum-free medium. HC gp39 was purified from the culture supernatant by affinity chromatography on heparin-Sepharose followed by gel filtration on Superdex 75 (Pharmacia, Roosendaal, The Netherlands). Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and HPLC, the purity was >97%. N-terminal amino acid sequencing confirmed the identity of the protein.

3.5 G1 Peptides synthesis

Peptides were synthesized by a robotic multiple peptide synthesizer (SYRO, MultiSynTech, Bochum, Germany) using a Fmoc/tBu solid-phase synthesis strategy (112). Wang resin(p-benzyloxybenzylalcohol-polystyrene) (Novabiochem, Bad Soden, Germany) was used as solid support. Side chain protected Fmoc-amino acids were obtained from Senn Chemicals(Dielsdorf, Switzerland) and Novabiochem (Bad Soden, Germany). Peptides were characterized by reversed-phase HPLC (M480 pump, UVD-320 S diode-array UV-detector, GINA 160 autosampler, Gynkotek,

Table 2. Amino acid sequence of G1 protein

Position.	Amino acid sequence				
1-50	mttllwvfvt	lrvitaavtv	etsdhdnsls	vsipqpsplr	vllgtsltip
51-100	cyfidpmhpv	ttapstapla	prikwsrvsk	ekevvllvat	egrvrvnsay
101-150	qdkvslpnyp	aipsdatlev	qslrsndsgv	yrcevmhgie	dseatlevvv
151-200	kgivfhyrai	strytldfdr	aqraclqnsa	iiatpeqlqa	ayedgfhqcd
201-250	agwladqtvr	ypihtpregc	ygdkdefpgv	rtygirdtne	tydvycfaee
251-300	megevfyats	pedftfqeaa	necrrlgarl	attghvylaw	qagmdmcsag
301-309	wladrsvryp				

Germering/Munich, Germany) on Nucleosil C18, 100A, 5µm (Macherey-Nagel, Düren, Germany) and electrospray mass-spectrometry (ESI-Quattro II, Micromass Ltd., Altrincham, UK). 46 overlapping 18 mer peptides, which covered all 394 amino acid residues of G1 protein, were synthesized (table 2), and each peptide overlapped the next by 10 amino acid (table 3).

Table 3. Amino acid sequence for 46 overlapping 18 mer peptides

No.	Sequence
peptide 1	VETSD HDNSL SVSIP QPS
peptide 2	SLSVS IPQPS PLRVL LGT
peptide 3	PSPLR VLLGT SLTIP CYF
peptide 4	GTSLT IPCYF IDPMH PVT
peptide 5	YFIDP MHPVT TAPST APL
peptide 6	VTTAP STAPL APRIK WSR
peptide 7	PLAPR IKWSR VSKEK EVV
peptide 8	SRVSK EKEVV LLVAT EGR
peptide 9	VVLLV ATEGR VRVNS AYQ
peptide 10	GRVRV NSAYQ DKVSL PNY
peptide 11	YQDKV SLPNY PAIPS DAT
peptide 12	NYPAI PSDAT LEVQS LRS
peptide 13	ATLEV QSLRS NDSGV YRC
peptide 14	RSNDS GVYRC EVMHG IED
peptide 15	RCEVM HGIED SEATL EVV
peptide 16	EDSEA TLEVV VKGIV FHY
peptide 17	VVVKG IVFHY RAIST RYT
peptide 18	HYRAI STRYT LDFDR AQR
peptide 19	YTLDF DRAQR ACLQN SAI
peptide 20	QRACL QNSAI IATPE QLQ
peptide 21	AIIAT PEQLQ AAYED GFH
peptide 22	LQAAY EDGFH QCDAG WLA
peptide 23	FHQCD AGWLA DQTVR YPI

peptide 24	LADQT VRYPI HTPRE GCY

Table 3. continued.

peptide 25	PIHTP REGCY GDKDE FPG
peptide 26	CYGDK DEFPG VRTYG IRD
peptide 27	PGVRT YGIRD TNETY DVY
peptide 28	RDTNE TYDVY CFAEE MEG
peptide 29	VYCFA EEMEG EVFYA TSP
peptide 30	EGEVF YATSP EKFTF QEA
peptide 31	SPEKF TFQEA ANECR RLG
peptide 32	EAANE CRRLG ARLAT TGH
peptide 33	LGARL ATTGH VYLAW QAG
peptide 34	GHVYL AWQAG MDMCS AGW
peptide 35	AGMDM CSAGW LADRS VRY
peptide 36	GWLAD RSVRY PISKA RPN
peptide 37	RYPIS KARPN CGGNL LGV
peptide 38	PNCGG NLLGV RTVYV HAN
peptide 39	GVRTV YVHAN QTGYP DPS
peptide 40	ANQTG YPDPS SRYDA ICY
peptide 41	PSSRY DAICY TGEDF VDI
peptide 42	CYTGE DFVDI PENFF GVG
peptide 43	DIPEN FFGVG GEEDI TVQ
peptide 44	VGGEE DITVQ TVTWP DME
peptide 45	VQTVT WPDME LPLPR NIT
peptide 46	WPDME LPLPR NITEG EAR

3.6 Preparation of peptides solution

Peptides was first dissolved in Dimethyl Sulfoxide (DMSO), then diluted with DH_20. The quantity of DMSO have to be controlled to a final dilution of 1/ 500-1000 if a peptide solution is used to stimulate the cells because high concentrations could lead to cell damage. Usually, 50-150 µl DMSO was added into the tube containing the peptide, mixed well until the peptide was completely dissolved, then an appropriate volume of DH_20 was added to a total volume of 2 ml and stored at -20^0C.

3.7 Preparation of peripheral blood mononuclear (PBMC) and synovial fluid mononuclear (SFMC)

Peripheral blood mononuclear cells, consisting of lymphocytes and monocytes, were isolated from heparinized fresh blood samples from patients. Briefly, the samples were diluted 1:2 with HBSS, 35 ml of diluted blood were carefully layered over 15 ml Ficoll Paque (1.077 density) in a 50 ml conical tube and centrifuged at 2500rpm for 20 min at 20^0C (without brake). The upper layer, which contains diluted 25-50% autologous serum, was aspirated and the mononuclear cell layer was left undisturbed at the interphase. The interface cells (lymphocytes, monocytes and thrombocytes) were carefully transfered to a 50 ml conical tube and the conical tube was filled with PBS and mixed well. The supernatant was carefully removed completely after the sample was centrifuged at 1500 rpm for 10 minutes at 4^0C. The cell pellet was resuspended in 50 ml of HBSS and centrifuged at 1200 rpm (at such a slower speed to get rid of the platelets) for 10 minutes at 4^0C. Carefully, the supernatant was completely removed and the cell pellet was resuspended in an appropriate volume of PBS containing 2 mM EDTA. Subsequently the cells were counted. The PBMC were sometimes stored in a refrigerator overnight in PBS containing 2 mM EDTA supplemented with 10% autologous serum after the last washing step. In most case, cells resuspened in RPMI 1640 medium (with FCS, Glutamin, Penicilin and Streptomycin) and stimulated with antigen immediately. At this point, sample can also be frozen in PBS with 1% BSA and 10% DMSO.

For SFMC, the SF was firstly filtered against 70 µm filter to get rid of tissue pellet and then diluted 1:5 with HBSS, then washed at 1500 rpm for 10 min at 4^0C. The supernatant was decanted and the cells were resuspended with an appropriate volume of RPMI1640. 20-35 ml of resuspension was carefully layered over 15 ml Ficoll in a 50 ml conical tube and centrifuged at 2500rpm for 20 min at 20^0C (without brake); The following steps were the same as for PBMC (see above).

3.8 Freezing and thawing of PBMC

After having been separated by Ficoll-paque density centrifugation and washed two time, PBMC were suspended in cold approriate volume of FCS and mixed well with an equal volume of 20% DMSO. Then the cell solution was transferred into 2 ml Greiner tube (each tube contained 10-20 million cells in 1-2 ml

10% DMSO) and put on ice for 10-15 min. Finally, the cells were stored at –70^{0}C until use.

When frozen cells needed to be used, they were thawed rapidly in a 37^{0}C water bath, immediately transferred into RPMI or PBS or HBSS and washed two times (1500 rpm, 10 min, 40C). After thesupernatant was decanted, the cells were resuspended again in medium with 10% FCS, 2 mM L- Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

3.9 Antigen stimulation and cell culture

T cells were stimulated in vitro in the presence of brefeldin A which inhibits intracellular transport. Thus cytokines produced during the activation are retained inside the cell. The unstimulated control sample also contained brefeldin A. Protein antigen (G1, Gp39, Collagen II, h-hsp60, Yersinia-19KD) were tested at a optimal final concentration which was previously found. Anti-CD28 was acted as a costimulator.

Briefly (106), 1ml whole heparinized peripheral blood and antigens were put in the 15 ml polypropylene tubes (with caps), tubes were loosely caped and vortexed for 10 sec. The cells were stimulated with antigen (20µg/ml G1 protein, 20µg/ml Gp39, 10µg/ml h-hsp60, 20µg/ml Y-19KD, 20µg/ml Collagen II) in the presence of anti-CD28 (1µg/ml), in the presence of anti-CD28 alone as a negative control, or in the presence of SEB as a positive control for 6 hours. Brefeldin A (10µg/ml) was added for the last 4 hours of the stimulation. The culture tubes were left at 5^0 slant at 37^{0}C in a 5% CO_2 incubator. At 6 hours, 100 µl EDTA (2 mM final concentration) was added and vortexed for 10 sec, then incubated for 15 min at room temparature. Afterwards nine ml of 1 x FACS lysing solution (or RCLS) was added to 1 ml of blood and vortexed gently, incubated for 10 min at room temperature in the dark to lyse erythrocytes and fix cells (the cells must be fixed with 2% formaldehyde if erythrocytes were lysed with RCLS buffer). The pellet was washed again with 1 x PBS after having been centrifuged (1500rpm, 10 min, 4^{0}C). Finally, the cells were put in the PBS/BSA buffer and stored at 4^{0}C.

Synovial fluid was obtained by arthrocentesis which was necessary for therapeutic reasons. Whole synovial fluid (1ml containing 5 x 10^6 cells) were stimulated in the presence of anti-CD28 (1µg/ml) for 6 hours with antigens (negative and positive control were the same as those of PB, see above). Brefeldin A (10µg/ml)

was added after2 hours. The culture tubes were left at 5^0 slant at 37^0C in a 5% CO_2 incubator. At 6 hours, the sample were taken out of incubator, 100 µl EDTA (final concentration: 2 mM) was added, vortexed for 10 sec and incubated for 15 min at room temperature. The cells were fixed with 1ml 2% formaldehyde for 20 min at room temperature after having been washed with 1 x PBS (centrifuging at 1500rpm, 10 min, 4^0C), then the pellet was washed again with 1 x PBS and the cells were kept in the PBS/BSA buffer at 4^0C.

If there were less than 5 x 10^6 cells in 1 ml SF, the synovial fluid was diluted (1:5) with HBSS and washed (centrifuging 1500rpm, 10min, 4^0C). The cell pellet was resuspended with RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 2 mM L- Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin) to a final concentration of 5 x 10^6 cells per milliliter, then cells were stimulated with antigens in the incubator.

3.10 Cytokine staining and analysis by flow cytometry

Activated PB or SF cells were transferred into 12 x 75 mm polystyrene tubes, labelled, washed again with 0.5% PBS/BSA, and centrifuged (300g, 10 min, 4^0C); 500 µl of 1 x FACS permeabilizing solution was added into each tube, mixed well and incubated for 10 min at temperature in the dark, the cells were washed with 0.5% PBS/BSA at 1500 rpm for 10 min at 4^0C.

The cells were quadruple stained for CD4-, CD69-surface markers and two intracellular cytokines. To avoid nonspecific binding of antibodies to Fc-receptors, all the staining was done in the presence of Beriglobin (3mg/ml). The following antibodies were used: anti-CD4 PerCP (clone Leu-3a), anti-CD69 FITC, anti-CD69 PE (Leu-23), anti-TNF α FITC, anti-IFN γ Cy5, anti-IL-4 PE (4D9), and anti-IL10 APC.

Staining solution was prepared at a volume of 50 µl/test, for some of the samples half of cells were stained for the cytokines IFNγ and TNFα, and the others were stained for IL4 and IL10. This means that two types of staining solution were prepared in this study: CD4 PerCP (1:10 dilution), CD69 PE (1:10), IFNγ cy5 (1:400), TNFα FITC (1:800); andCD4 PerC P (1:10), CD69 FITC (1:10), IL4 PE (1:100), IL10 APC (1:400).

After permeabilization and washing, 50 µl staining solution was added into each tube and the pellet was resuspended by pipette and incubated for 30 min at room temperature in the dark. The cells were washed at 300g for 10 min at 4^0C and

resuspended with 300-500 ml 0.5% PBS/BSA. In some experiments, the samples can be stored at 4^0C in the dark for up to 24 hours prior to analysis.

Flow cytometric analysis was performed on a FACSCalibur™ flow cytometer. Data were acquired using CELLQuest™ software. After proper instrument setup, $CD4^+$ lymphocytes were gated. For this, typically 100,000 $CD4^+$ events were collected using FL3 (PerCP) as a fluorescent trigger.

Since all specific cytokine expression should occur within the $CD69^+$ (activated) cell subset, CD69 staining was included to exclude non-specific cytokine staining.. Forward scatter vs side scatter gating was employed in data analysis to exclude any $CD4^+$ monocytes.

Data were displayed as four-color dot plots in PAINT-A-GATE^{plus} software to measure the proportion of the double-positive(cytokine $^+$/$CD69^+$) cells. In this study, they were displayed as dot plots of TNFα FITC/IFNγ Cy5 (x-axis) and CD69 PE (y-axis) or IL4 PE/IL10 APC (x-axis) and CD69 FITC fluorescence (four decade log scales). Quadrant markers were positioned to include >99% of control Ig staining cells in the lower left quadrant.

Positive cells were subsequently quantified. Only cytokine-positive T cells which were also positive for the early activation antigen CD69 were counted. To analyse whether cytokines were produced by the same or different cells, $CD4^+$ T cells positive for 2 cytokines were also counted at the same time. An example of a FACS analysis is shown in Fig.3.

The FACS data were given in both, a qualitative and a quantitative manner. For the qualitative approach it was essential to determine a cutoff. This was done by 1. looking at the dot plots to judge whether a distinct population can be identified by eye (Fig.3); 2. seeing how many cells have to be eligible at least to allow for proper counting; 3. comparing the results between patients and controls; 4. adding two standard deviations to the mean percentage to be more certain that the differences measured are meaningful. With this approach I determined the minimally required cells to be at least 30 and set the cutoff to 0.02% of the cells gated.

3.11 Peptide-stimulation assay

Forty-six overlapping 18-mer G1 peptides, each overlapping by 12 AA, were put into 5 pools, with 8-10 in each pool. Pool 1 contained peptides 1-10, pool 2 peptides 11-19, pool 3 peptides 20-28, pool 4 peptides 29-38, pool 5 peptides 39-46.

For stimulation with peptides, mononuclear cell (MC) instead of whole blood was used. Immediately after the blood was drawn in heparinized syringes, MC of 13 AS patients were obtained by Ficoll-paque density centrifugation. The cells then were suspended in RPMI 1640 medium with 100 units/ml penicillin, 100µg/ml streptomycin 2mM L-glutamine, and 10% heat-incativated fetal calf serum. At least 1 x 10^6 cells/ml were stimulated with G1 pool of peptides (each peptide 5µg/ml) in the presence of anti-CD28, in the presence alone as negative control, or with SEB as positive control. Finally, staining with monoclonal antibodies directed against the surface markers CD4 and CD69, and against intracellular cytokines IFNγ and TNFα was performed (see cytokine staining and analysis by cytometry). For fine epitode mapping, fresh blood was taken again from patients who responded to pools of peptides and were stimulated with single peptides.

3.12 Isolation of G1-specific T cells using IFNγ secretion assay

The IFNγ secretion upon antigen stimulation was designed for the detection, isolation and analysis of live IFNγ-secreting leucocytes (113). It is especially useful for the isolation of antigen-specific Th1 cells after stimulation with specific antigen in vitro to induce secretion of IFNγ. The mechanism (Fig.1) of this technique is that an affinity matrix for IFNγ is generated by attaching IFNγ catch reagent to CD45 on the cell surface. The secreted IFNγ binds to the catch reagent on the surface of secreting cells and can subsequently be stained with a second IFNγ specific antibody. This IFNγ detection antibody is conjugated to Phycoerythrin (PE), allowing sensitive analysis by flow cytometry. Subsequently, cells can be magnetically labeled with anti-PE microbeads and enriched on a column which is placed in the magnetic field of a MACS separator. The magnetically labeled IFNγ secreting cells are retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained IFNγ secreting cells can be eluted as positively selected cell fraction, highly enriched with antigen-specific T cells. The cells can now be used for analysis or cell culture.

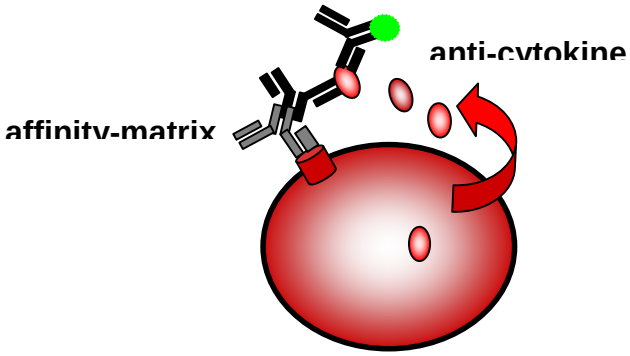


Figure 1. **Model of IFNγ secretion assay. Firstly,** IFNγ catch reagent attached on the surface of T cells by anti-CD45. Secondly, the secreted IFNγ bins to theCatch Reagent on secreting cells and can subsequently be stained with a second IFNγ specific antibody (IFNγ Detection antibody). Finally, MicroBeads were connected by anti-PE which make the cells magnetic and retained when they pass through collumn of separator.

In this study, IFNγ secreting cells were isolated from PB of AS patients. Two fractions (each contains 20 ml) of fresh heparin peripheral blood were incubated for 12 hours (at 37^0C, 5% CO_2) with G1 protein (20 µg/ml) in the presence of anti-CD28 (1 µg/ml) or without G1 protein (as control). At 12 hours, EDTA (2mM) was added, the cells were vortexed and incubated for 15 min at room temperature. Then, the blood was diluted by 1:1 with RPMI (without FCS, penicillin and streptomycin), each 10 ml diluted blood was mixed well with 40 ml RCLS and incubated for 15 min in ice water (was shaked every other 3-5 min) to lyse the red cells. The cells were spun down (1200 rpm, 10 min, 4^0C) and decanted, washed again by adding cold PBS/BSA buffer (without azid), and centrifuged at 1500 rpm for 10 min at 4^0C.

Subsequently, the cell pellet was resuspended in 160 µl of cold buffer (80 µl per 10^7 cells), 40 µl of IFNγ catch reagent (20 µl/10^7 total cells) was added (as 10^7 estimated cells in 10 ml blood), mixed well and incubated for 7 min on ice. After incubation, the cell suspension of 20 ml blood was directly aliquated into two 50 ml test tubes, 45 ml warm (37^0C) medium was added to dilute the cells, then incubated for 45 min (37^0C, 5% CO_2), and mixed well every other 5 min.

The samples were centrifuged at 1500 rpm for 10 min at 4^0C, supernatant was removed completely and washed again. The cell pellet was resuspended again in 160 µl of cold buffer, 40 µl of IFNγ detection antibody (PE) (20 µl for 10^7 cells, 1:5 dilution) was added and additional the staining reagents CD4 Cy5 and Beriglobin were put in, mixed well and incubated for 10 min on ice. Cells were washed with cold buffer, centrifuged at 1500 rpm for 10 min at 4^0C.

For magnetic labeling, cell pellet was resuspended in 160 µl cold buffer, 40 µl of anti-PE microbeads (1:5 dilution) was added, mixed well, and incubated for 15 min at 4^0C followed by washing of the cells (1500rpm, 10 min, 4^0C) and removing the supernatant completely. Finally, the cells were resuspened in 1 ml of cold buffer.

For magnetic separation, MS^+/RS^+ column was put in the magnetic field of the MACS separator and rinsed 3 times with 1 ml of cold buffer. Magnetically labeled cells were applied and pass through the column. The column was washed with 3 x 1 ml cold buffer, and the effluent was collected as negative fraction. Then, the column was removed from separator and placed in a suitable collection tube. Three ml of cold buffer was pipetted on top of the column, retained cells were flashed out by using the plunger supplied with the column. This step was repeated to increased the purity. Finally, the retained cells were eluted in buffer and used for analysis and further cell cultures.

3.13 Flow cytometry analysis of G1-specific T cells

Flow cytometric analysis was performed with the original fractions (before enrichment, A.) and with the enriched fractions (after enrichment, B.). Propidium iodide was added just prior to flow cytometric analysis at a final concentration of 0.5 µg/ml. A lymphocyte gate based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to improve the sensitivity of the analysis. Upon activation of the lymphocyte gate, dead cells were gated out according to PI fluorescence in a fluorescence 2 versus fluorescence 3 plot. Then antigen-specific T cells were analysed according to staining with CD4 Cy5 and anti-IFNγ PE (Fig.15).

3.14 Culture of G1-specific T cells

$IFN\gamma^+$ cells were put in a 96-well plate and cultured in the presence of recombinant IL2 (20 units/ml) in tissue culture medium, which contained RPMI 1640, 10% autologous serum, 2 mM L- Glutamine, Penicillin 100 units/ml, Streptomycin 100 µg/ml. IL2 was added every 3-4 day, the cells werefrequently observed under microscope and the expanded cells were transferred to other wells if necessary.

3.15 Restimulation of G1-specific T cells

For re-analysis of G1-specific T cells, the cells had to be left for at least 5 days without IL2. The cells were harvested, passed through 70 µm mesh filter to remove

clumps and washed with HBSS or RPMI (1500rpm, 10 min, 4^0C). Subsequently, autologous PBMC (as APCs) were labeled with CFDA-SE (procedure: cells were washed several times in PBS to remove protein-containing buffer, 5 µM CFDA-SE staining solution was prepared in PBS, the cells were resuspended at a concentration of 10^7/ml in staining solution and incubated for 4 min at room temperature, the reaction was stoped by washing the cells in medium containing 10% FCS or PBS/BSA at 1500rpm for 10 min at 4^0C, then washed again). Next, T cells were mixed with autologous PBMC as a 1:5 dilution and transferred into 15 ml test tubes, each tube contained at least 0.2 million T cells and adequate PBMC in 1 ml of tissue medium with RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 units penicillin and 100 µg streptomycin. Finally, G1 protein and other antigens of interest were added to different tubes, vortexed for 10 seconds and incubated for 6 hours in the presence of anti-CD28. Brefeldin A was added after 2 hours, the cells were harvested at 6 hours, fixed with 2% formaldinhyde and resuspended in PBS/BSA (see 2.8 of methods).

3.16 Staining and analysis of G1-specific T cells by flow cytometry

Stimulated cells were transferred into tubes for staining. After permeabilization, cells were washed once and staining was performed for 30 min at RT in the dark using a titrated mixture of fluorescent conjugated mAbs (anti-CD4 PerCP, anti-CD69 PE and anti-IFNγ Cy5).

For flow cytometric analysis, after activation of the lymphocyte gate, APCs were gated out according to CFDA-SE fluorescence in a fluorescence 2 versus fluorescence 1 plot. Then a gate for $CD4^+$ T cells was set, the percentage of IFNγ/CD69 double positive cells of the $CD4^+$ T cell subpopulation was indicated (see Fig.17 of results).

3.17 Clinical experiment about anti-TNFα treatment (infliximab)

twenty active AS patients were randomly selected into two groups as part of double-blind, placebo-controlled, multicenter trial of treatment of AS patients with infliximab. Patients of the first group underwent three infusions of 5 mg/kg infliximab at 0, 2, and 6 weeks, patients of the second group were treated with placebo at the same time points. After 6 weeks, patients from the placebo group were treated in a similar way with 5mg/KG infliximab at week 0, 2 and 6 weeks. Cytokine were

investigated before, 6 weeks and 12 weeks after treatment in the infliximab group, and before, 6 weeks during placebo treatment and 6 weeks after patients in the placebo group were switched to infliximab treatment. Peripheral blood from participatants was taken at basline, at 6 and 12 weeks, PBMC were isolated and stored at -80^0C until further analysis.

For analysis, cells were thawed (see 2.8 of methods) and put in the incubator overnight to come to life. Then 1 x 10^6 cells were cultured for 6 hours in the presence of 5 ng/ml PMA and 1 ng/ml ionomycin, or with a pool of 46 overlapping 18 mer peptides (5 µg/ml for each peptide) derived from the G1 domain of aggrecan in the presence of anti-CD28, and in the presence of anti-CD28 alone as negative control.

T cells were stained after in vitro stimulation as described before (106). Briefly, cells from PBMC were washed with PBS/BSA, centrifuged (300g, 10min, 4^0C), and cells were quadruple stained for CD3, CD8-surface markers and two intracellular cytokines, either IFNγ/ IL4 or TNFα/IL-10. All stainings were performed in FACS™ Permebealizing Solution. To avoid nonspecific binding of antibodies to Fc-receptors, all the staining was done in the presence of Beriglobin (3mg/ml).

Because stimulation with PMA/ionomycin induces a reduction of CD4-expression on the cell surface (36), CD4+ T cells were identified indirectly by gating on CD3-positive but CD8-negative lymphocytes. After gating either on CD3+/CD8+ or CD3+/CD8- (CD4) lymphocytes, data were analysed using CELLQuest software and displayed as dot plots of FITC (x-axis) and APC (y-axis) fluorescence. Quadrant markers were positioned to include > 99% of control Ig staining cells in the lower left quadrant.

3.18 Separation of cells by MACS and detection of ratio of CD4/CD8 and CD14/CD19 by flow cytometry

For cell separation (Fig.2), around 4 x 10^6 PBMC were taken and washed at 1500rpm for 10 min at 4^0C, cells were resuspended with 200 µl PBS/BSA buffer, 50 µl CD3 microbeads (1:5 dilution) were added and incubated for 15 min at 4^0C. The cells were put through column that was placed in the magnetic field of MACS separator. The magnetically labeled $CD3^+$ cells were retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained CD3+ cells were eluted with 3 x 1 ml cold PBS/BSA as positively selected cell fraction. The cells were washed at 1500rpm for 10 min at 4^0C.

Subsequently, the $CD3^+$ cells were stained with anti-CD4 Cy5 (1:400) and anti-CD8 PE (1:10), and the $CD3^-$ cells were stained with anti-CD14 PE (1:10) and anti-CD19 FITC (1:10) in the presence of Beriglobin (3mg/ml). After washing, cells were resuspended with 1 ml PBS/BSA, Propidium iodide (PI) was added (0.5 µg/ml). Then, CD4, CD8, CD14 and CD19 cells were checked by flow cytometry after the dead cells were gated out according to PI fluorescence in a fluorescence 2 versus fluorescence 3 plot. At last, calculated the ratio of CD4/CD8 and CD14/CD19.

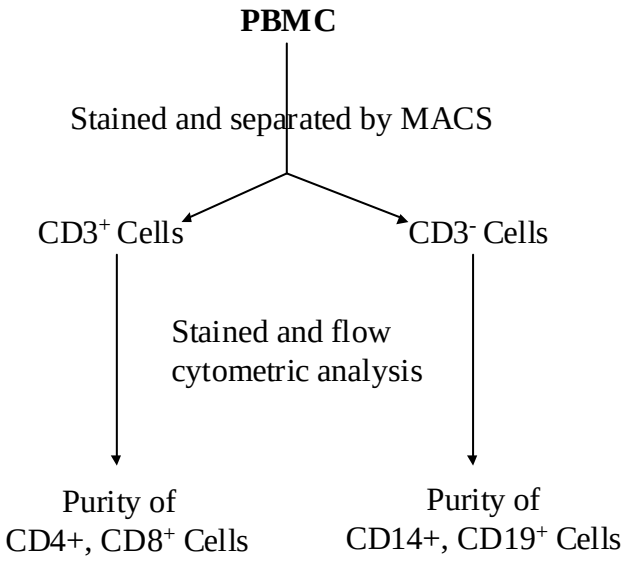


Figure 2. Model of separation and purity dectection for CD4+, CD8, CD14+ and CD19+ cells.

3.19 In vitro stimulation of macrophages With LPS and cytokine detection by ELISA

To test the effects of infliximab on cytokine production by macrophages, 1 x 10^6 PBMC from different time points were cultured with purified Lipopolysaccharide (LPS) at a concentration of 10 µg/ml for 18 hours, then cell-free supernatants were collected and frozen at -70^0C until the cytokine assay was performed.

Secretion of TNFα and IL10 was analysed in the supernatant using a sandwich ELISA with purified anticytokine Mab (capture) and biotinylated anticytokine antibodies (detecting), with a detection level of 20 pg/ml for both

cytokines using an ELISA reader from Dynatech. The ELISA protocol is briefly described as following:

The capture antibodies were coated in 96-well immunoassay plate with binding buffer (50 µl/well) at a concentration of 1-4 µg/ml at 37^0C for 1 hour. The plate was blocked with PBS supplemented with 10% heat-inactivated FCS at 37^0C for 1 hour. After washing with washing buffer, supernatants from samples and standards with known amounts of the appropriate cytokines were pipetted into the wells and incubated at 4^0C overnight. After washing, the detecting antibodies specific for the respective cytokines were added to wells and incubated at 37^0C for 1 hour. After washing, a horseradish peroxidase (HRP) conjugated streptavidin were added to the plates which were then incubated at 37^0C for 45 minutes. Finally, the substrate solution [subtrate buffer + o-phenylenediamine (OPD) Dihydrochloride] was added. After 10 minutes, the color development was stopped by 2N HCl and the absorbance was read at 490 nm using an ELISA reader (Dynatech MR5000). The amount of cytokines was determined by comparing the absorbance of the samples with standard proteins.

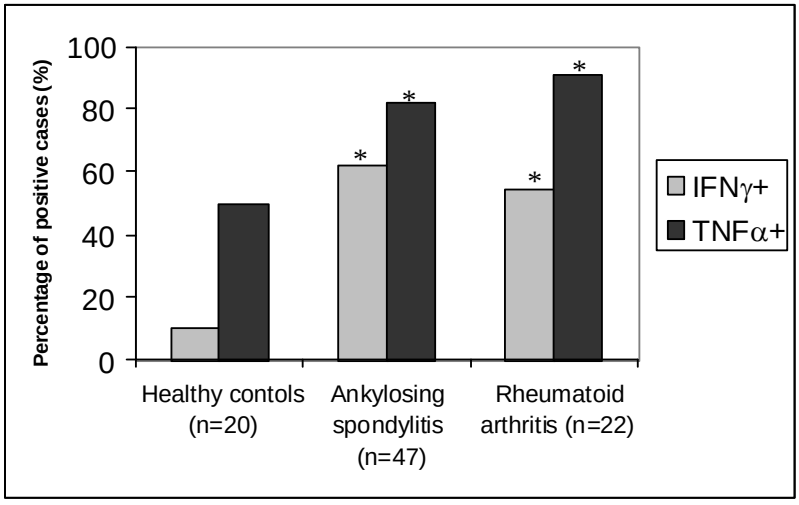
3.20 Statistical analysis

χ^2 test was used to compare frequency in different groups; Student's T test was used to analyse differences among medians of percentage of positive patients and to compare cytokine frequency induced by G1 between PB and SF; Wilcoxon-test was used to compare cytokine prodution between before and after placebo or anti-TNFα treatment. Differences were considered to be significant in case of a the two-tailed p-value of less than 0.05.

Results

1. Antigen (cartilage-derived) -specific cytokine secretion in ankylosing spondylitis and rheumatoid arthritis compared to healthy controls

As shown in Figure 3, there is an increased frequency of IFNγ positive T cells in PB specific for the G1-protein in AS and RA: 61.7% (29/47) of the AS patients and 54.5% (12/22) of the RA patients compared to only 10% (2/20) HC had increased percentages of IFNγ positive T cells in response to G1. TNFα positive CD4 T cells responding to G1-stimulation were even detected in a higher percentage in AS and RA patients but also in controls: 91.5% (43/47), 81.8% (18/22), 50% (10/20), respectively (Fig. 3). This difference between AS and RA compared to HC is significant for both cytokines ($p<0.05$). Most (26 out of 29 patients) of the IFNγ-positive CD4+ T cells were double positive for TNFα (not shown). There was no T cell response to gp39 and collagen II. An example for IFNγ secretion of PB $CD4^+$ T cells in response to stimulation with cartilage-derived antigens (G1, gp39, collagen II) is shown for one AS patient in Fig. 4.



*$p<0.05$, comparing with healthy controls.

Figure 3. T cell response to the G1-domainof aggrcan in PB. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the G1-domain of the proteoglycan aggrecan. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.

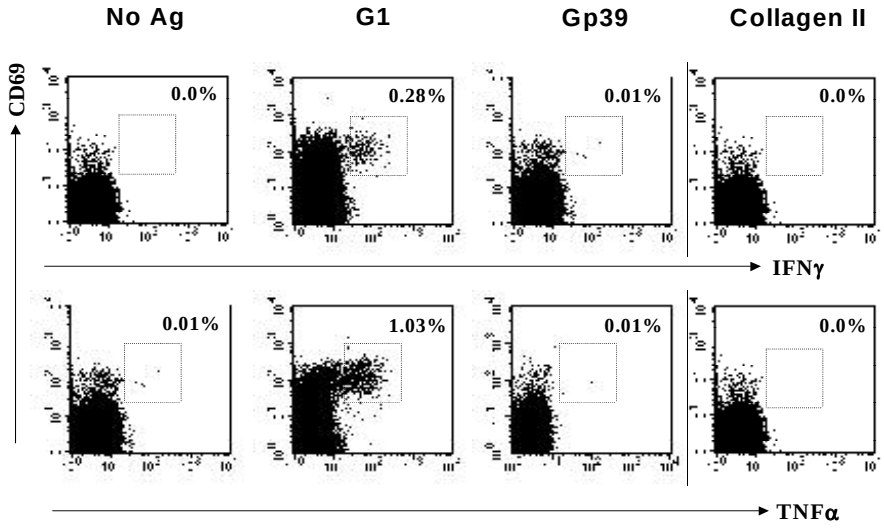
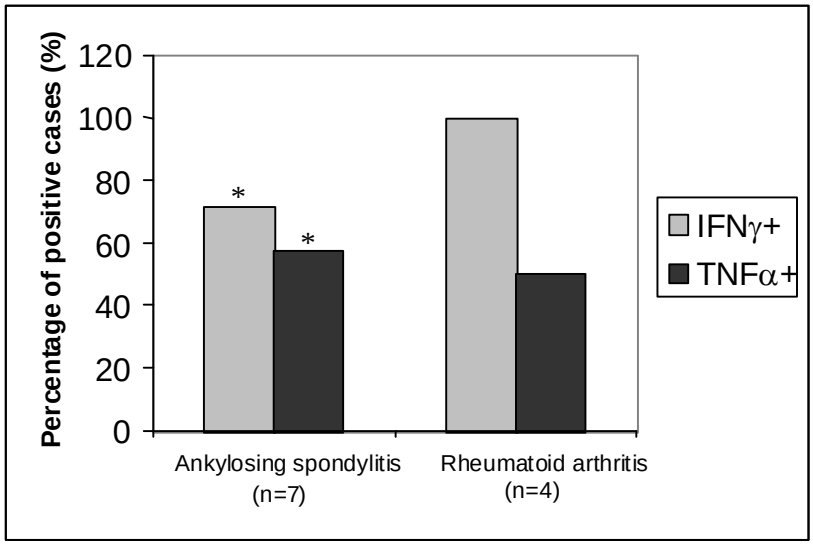


Figure 4. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) or with the human cartilage derived antigens glycoprotein (gp) 39 or collagen II in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.



*p>0.05, comparing with rhematoid arthritis.

Figure 5. T cell response to the G1-domain in SF. Percentage of patients with ankylosing spondylitis and with rheumatoid arthritis responding to the in vitro stimulation with the G1-domain of the proteoglycan aggrecan. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole synovial fluid**.

In SF, 71.5% (5 out of 7) of the AS patients responded to in vitro stimulation with G1 by IFNγ–secretion and 57.2% (4 out of 7) by TNFα (Fig. 5). In SF from RA patients, a response to G1 was detectable in all 4 patients (100%) as judged by IFNγ-production and in 50% by TNFα-production (Fig. 5). Interestingly, in SF the percentage of patients responding by IFNγ-secretion was higher than the percentage responding by TNFα-secretion while it was the other way around in PB (Fig. 3 and 5).

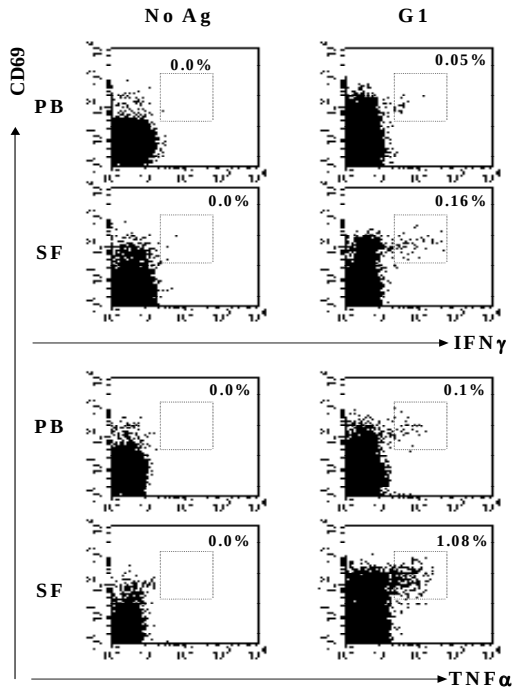


Figure 6. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) in a patient with **ankylosing spondylitis**. **The T cell response in synovial fluid (SF) is higher compared to peripheral blood (PB).** After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

An example for IFNγ secretion of SF CD4+ T cells in response to stimulation with these antigens is shown for one AS patient in Fig. 6. None of the AS patients showed a T cell response to gp39 or to collagen II (an example is shown in Fig. 4) and none of the RA patients showed a T cell response to collagen II (an example is shown in Fig.7; gp39 was not tested in RA). The G1 specific T cell response in SF (mean ± SD: 0.26 ± 0.23% for IFNγ; 0.43 ± 0.39 % for TNFα) was significantly ($p=0.01$ for IFNγ, $p=0.007$ for TNFα) stronger than that in PB (mean ± SD: 0.06 ± 0.05% for IFNγ; 0.07 ± 0.03% for TNFα). An example is shown in Fig. 6 for one AS patient.

No increased percentages of IL4- or IL10-positive CD4+ T cells were observed after stimulation with G1, gp-39 or collagen II in any of the three groups (data not shown).

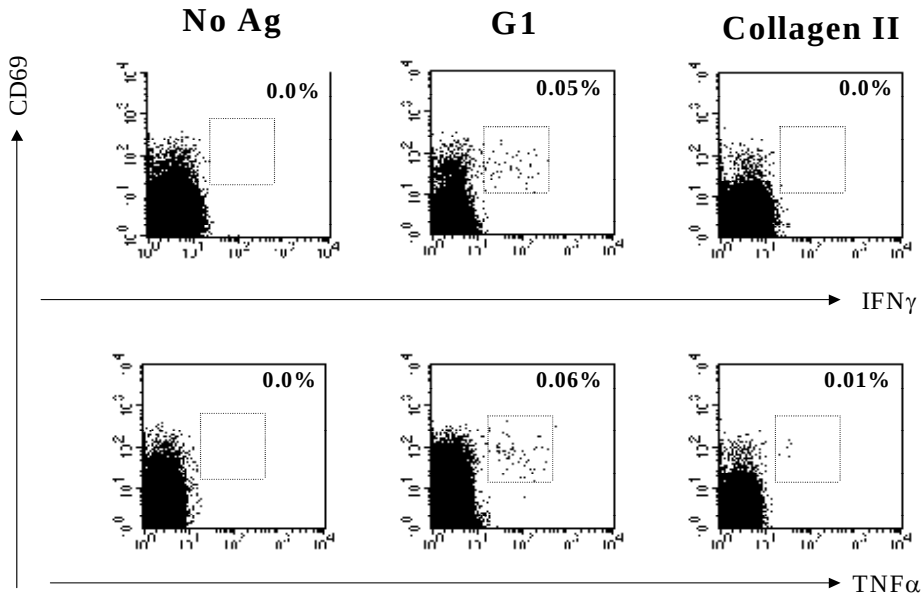


Figure 7. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) or with the human cartilage derived antigen collagen II in a patient with **rheumatoid arthritis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

2. Characterization of immunodominant G1- epitopes

When the response of CD4+ T cells derived from peripheral blood after stimulation with G1-pools of peptides was investigated in 13 of the AS patients, in 6 out of 13 (46.2%) patients a response to pool 4, containing peptides 29-38, was observed, but not to any of the other 4 pools (Table 4, Fig 8). No pool-specific response was detectable in the other 7 AS patients. Restimulation of peripheral blood T cells from the 5 responding patients with single peptides (all out of pool 4) indicated that peptide 35 (in 4 patients) and peptide 30 (in one patient) (Table 4, Fig. 8) were stimulatory. Peptide 35 is located within residues 292 to 309 of the G1-domain (AGMDMCSAGWLADRSVRY) while peptide 30 is located within residues 252 to 269 (EGEVFYATSPEKFTFQEA).

Table 4. T cell response to G1 pools of peptides and single peptide in ankylosing spondylitis patients.

Patient	Positive pool[#]	Positive peptide*
Pat.1	Pool 4 (peptides 29-38)	Peptide 35
Pat.2	Pool 4 (peptides 29-38)	Peptide 35
Pat.3	Pool 4 (peptides 29-38)	Peptide 30
Pat.4	Pool 4 (peptides 29-38)	Peptide 35
Pat.5	Pool 4 (peptides 29-38)	Peptide 35
Pat.6	Pool 4 (peptides 29-38)	not tested

[#] None of the other 4 pools of peptides was stimulatory.

* None of the other 9 peptides out of pool 4 was stimulatory.

Peptide 35 (AA-sequence): AGMDM CSAGW LADRS VRY

Peptide 30 (AA-sequence): EGEVF YATSP EKFTF QEA

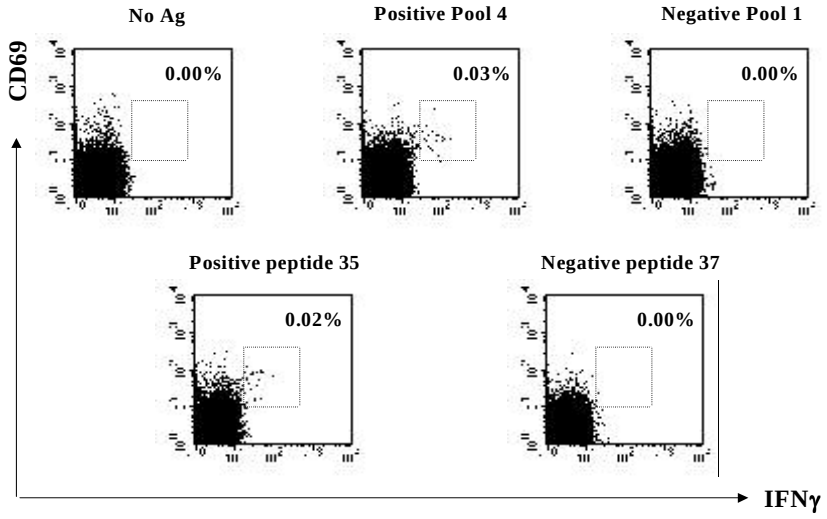
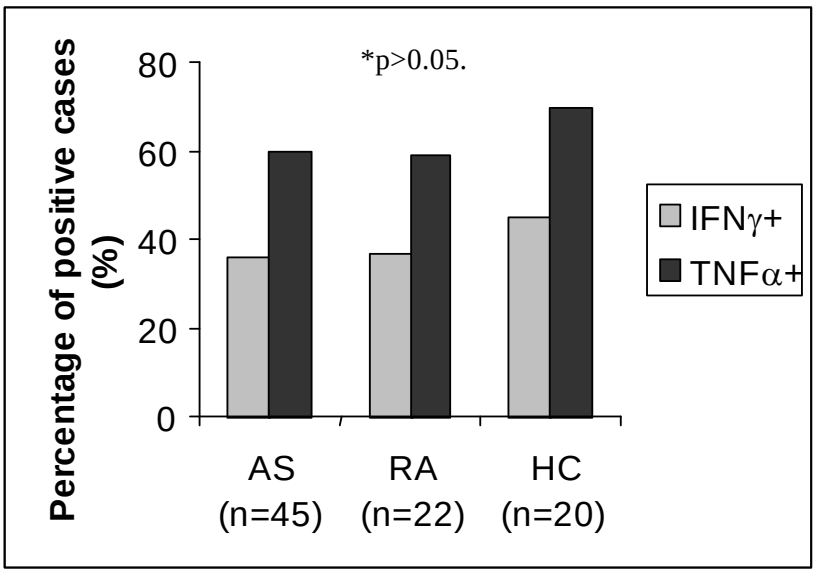


Figure 8. Example of an **ankylosing spondylitis patient** with an antigen-specific response to one of the **pools of peptides** (Pool 4), but not to Pool 1, derived from G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag). Out of Pool 4, containing peptides 29-38, only the single peptide 35 but not peptide 37 was recognized by this patient's CD4+ T cells. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

3. T cells response to human hsp60 in patients and healthy controls

IFNγ^+ CD4 T cells responsive to h-hsp60 were detected in the AS, RA patients and HC, with an incidence of 36% (16/45), 36.5% (8/22), and 45% (9/20), respectively; The corresponding percentage of TNFα^+ CD4 T cells was 60% (27/45), 59% (13/22) and 70% (14/20). There was no significant difference between AS or RA and HC ($p>0.05$) (Fig.9). Fig.10 shows an example for an h-hsp60-specific IFNγ^+ or TNFα^+ response of PB CD4$^+$ T cells in AS, RA and HC.

For SF, 71.4% (5 out of 7 patients) AS- and 75% (3 out of 4 patients) of RA-cases showed a T cell response to h-hsp60 by IFNγ-secretion. 85% (6/7) of AS- and 75% (3/4) of RA-cases showed a TNFα-secretion in response to h-hsp60. In comparison with PB, hsp60-specific IFNγ/TNFα-response were stronger (mean ± SD: 0.18 ± 0.3 in SF vs 0.09 ± 0.08 in PB for IFNγ; 0.35 ± 0.64 vs 0.15 ± 0.05 in PB for TNFα), but the difference was not significant ($p>0.05$ for both cytokines).



*There is no significant difference among all groups for both cytokines.

Figure 9. T cell response to h-hsp60 in PB. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the h-hsp60. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.

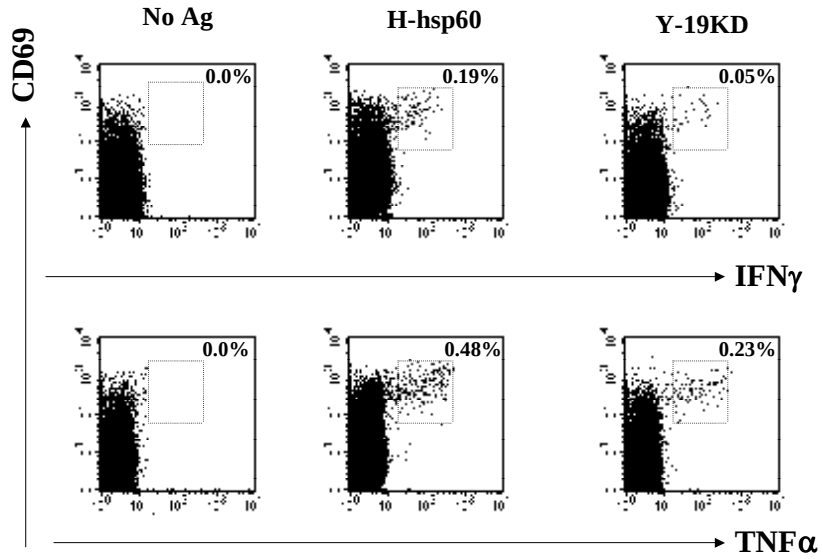
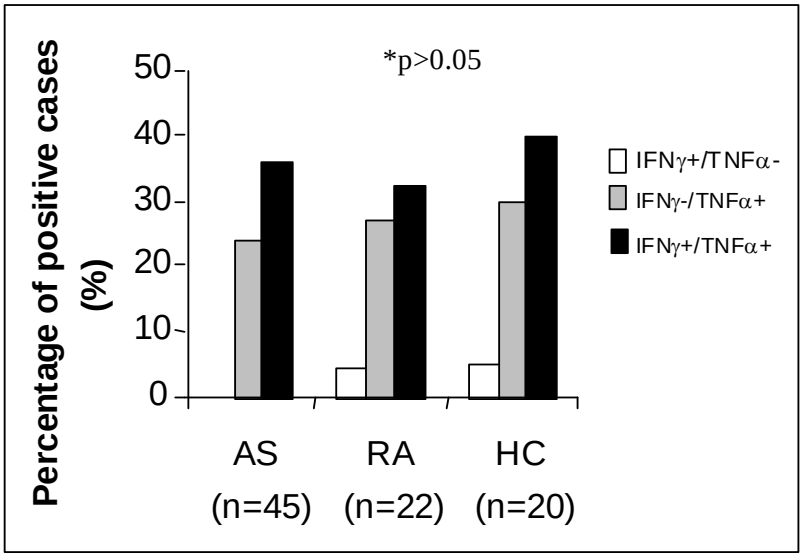


Figure 10. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.



*There is no big difference among all groups for combination analysis.

Figure 11. Combining analysis of h-hsp60-specific IFNγ and TNFα in PB. Percentage of cases with IFNγ/TNFα single or double positive responding to the in vitro stimulation with h-hsp60 in patients with ankylosing spondylitis, with rheumatoid arthritis and of controls. Analysis was done with **whole peripheral blood**.

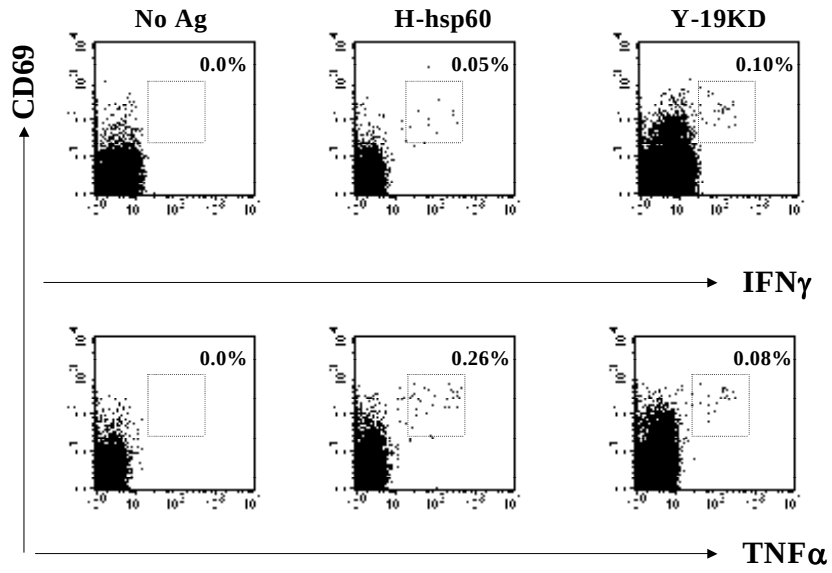


Figure 12. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a patient with **rheumatoid arthritis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

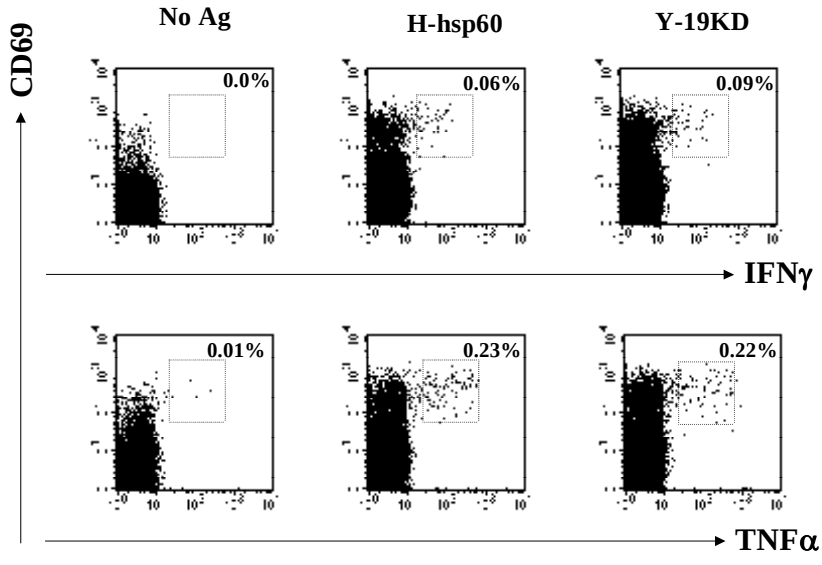


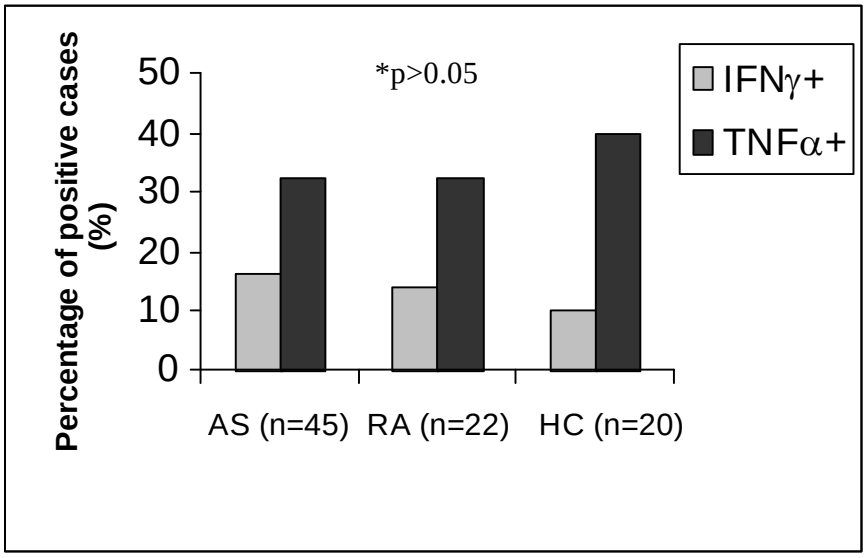
Figure 13. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a healthy control. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

A combined analysis for IFNγ and TNFα secretion indicates that 0% (0/45) of $IFN\gamma^+/TNF\alpha^-$ (IFNγ single positive), 24% (11/45) of $IFN\gamma^-/TNF\alpha^+$ (TNFα single positive) and 36% (16/45) of $IFN\gamma^+/TNF\alpha^+$ (double positive) were detected in AS patients after stimulation with h-hsp60 (Fig. 11); The percentages of $IFN\gamma^+/TNF\alpha^-$, $IFN\gamma^-/TNF\alpha^+$ and $IFN\gamma^-/TNF\alpha^+$, in RA, were observed with an incidence of 4.5% (1/22), 27% (6/22) and 32% (7/22) (Fig. 11), respectively (an example of T cell response to h-hsp60, in one patient with RA, was shown in figure 12); The corresponding frequency in HC is 5% (1/20), 30% (6/20) and 40% (8/20) (Fig.11). No significant difference was shown among different groups ($p>0.05$). An example of antigen-specific response to h-hsp60, in a healthy control, was shown in figure 13.

4. T cells response to yersinia-19KD in patients and healthy controls

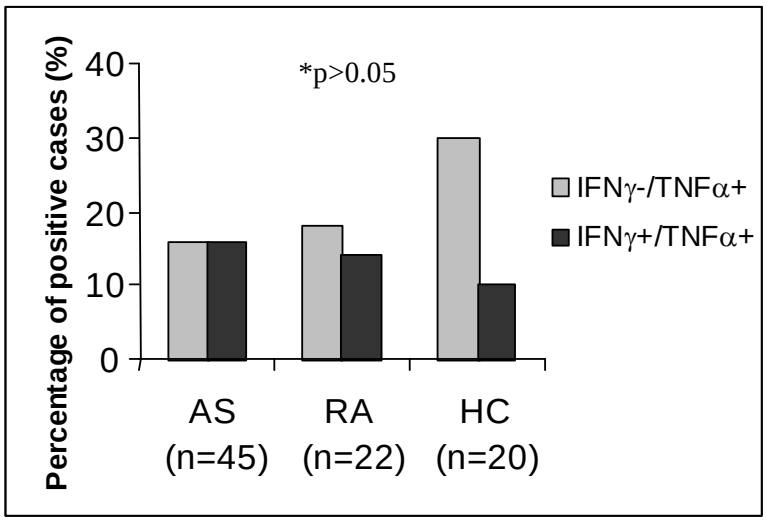
A Y-19KD-specific T cell response could be detected in all 3 groups (Fig. 10, 12, 13). 16% (7/45), 14% (3/22), 10% (2/20) of samples with $IFN\gamma^+$ $CD4^+$ T cells induced by yersinia-19KD were observed in AS, RA patients and controls, respectively (Fig. 14). For y-19KD-specific $TNF\alpha^+$ $CD4^+$ T cells, the percentage was 32% (14/45), 32% (7/22), 40% (8/20), respectively, in the corresponding groups (Fig. 14). There was no significant difference among different groups. The incidence of $IFN\gamma^-/TNF\alpha^+$ was detected with a percentage of 16% (7/45), 18% (4/45), 30% (6/20) in AS, RA and HC, respectively; The $IFN\gamma^+/TNF\alpha^+$ frequency showed an incidence of 16% (7/45), 14% (3/22) and 10% (2/20), respectively, in the corresponding groups. No single $IFN\gamma^+$ ($IFN\gamma^+/TNF\alpha^-$) positive cells were found in any of the 3 groups (Fig.15). There was no difference between any of groups ($p>0.05$).

Although a higher percentage of AS patients (42.8%, 3 out of 7 patients for IFNγ; 71.4%, 5 out of 7 patients for TNFα) compared to RA (25%, 1 out of 4 patients for IFNγ; 50%, 2 out of 4 patients for TNFα) showed a synovial T cell response to the 19KD protein, this difference was non-significant, possibly due to the small number of patients.



*There is no big difference among different groups for both cytokines.

Figure 14. T cell response to y-19KD in peripharal blood. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the y-19KD. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.



*There is no big difference among different groups for combination analysis.

Figure 15. Combining analysis of y-19KD-specific IFNγ– and TNFα–secretion in peripharal blood. Percentage of cases with single TNFα or IFNγ and TNFα double positive responding to the in vitro stimulation with y-19KD in patients with ankylosing spondylitis, with rheumatoid arthritis and of controls. No single IFNγ+ case, responding to the in vitro y-19KD stimulation, was observed in any of the groups. Analysis was done with **whole peripheral blood**.

5. Relationship of cytokine producion by h-hsp60 and Y-19KD

In this study, PB of 67 patients (AS 45, RA 22) underwent cytokine secretion analysis. Almost all Y-19KD specific IFNγ^+ samples were accompanied by h-hsp60 specific IFNγ^+ production (just 1 exception). As table 5 shows, IFNγ^+ section was observed in 24 samples including 9 responses to both h-hsp60 and Y-19KD, 14 single responses to h-hsp60, 1 only response to Y-19KD. There is a significant relationship of IFNγ producing between by h-hsp60 and by 19KD ($p<0.05$).

Furthermore, all Y-19KD specific TNFα^+ samples were accompanied by h-hsp60 specific TNFα^+ secretion. There were 40 samples out of 67 samples responding by TNFα-secretion, of which the cases with a TNFα response to both h-hsp60 and 19KD were 21, 19 patients only responded to h-hsp60 alone; none of them responded only to Y-19KD (Table 6). A significant correlation could be observed in TNFα response between h-hsp60 and 19KD ($p<0.05$).

6. Non-specific cytokine secretion in AS and RA

By intracellular cytokine staining and flow cytometric analysis, I investigated whether a difference in cytokine secretion, induced by SEB, could be detected at single cell level in different groups. Of which, only IFNγ showed a significant difference among different groups when measured. Levels of IFNγ were lower in AS patients (mean ± SD 2.28 ± 1.87%) than those both in HC (4.09 ± 3.57%, $p=0.005$) and in RA (3.43 ± 3.24%, $p=0.049$); TNFα secretion was a little bit lower in AS patients (8.30 ± 5.51%) than those both in RA patients (9.41 ± 5.58%) and in HC (10.88 ± 6.35%), but the difference was not significant. IL10 secretion was similar in AS and RA patients (0.06 ± 0.05% versus 0.05 ± 0.04%). Also no marked difference of IL4 producing CD4+ T cells was observed in any of the patients' groups (AS: 0.82 ± 0.70% versus RA: 0.96 ± 0.65%) (not detected in HC).

7. Enrichment of G1-specific T cell

For further epitope analysis, G1-specific T cells were enriched by the IFNγ secretion assay, as described in Methods (see 3.12 of materials and methods). As Fig. 16 shows, a high frequency of G1-specific IFNγ^+ CD4 T cell (61.28%, cellular

Table 5. Relationship between h-hsp60 and Y-19KD for IFNγ producing cells*.

	$IFN\gamma^+$ CD4+ T cells after stimulation with h-hsp60	$IFN\gamma^-$ CD4+ T cells after stimulation with h-hsp60
$IFN\gamma^+$ CD4+ T cells after stimulation with Y-19KD	11	1
$IFN\gamma^-$ CD4+ T cells after stimulation with Y-19KD	22	53

*$p<0.05$, χ^2 test

Table 6. Relationship between h-hsp60 and Y-19KD for TNFα producing cells.

	$TNF\alpha^+$ CD4+ T cells after stimulation with h-hsp60	$TNF\alpha^-$ CD4+ T cells after stimulation with h-hsp60
$TNF\alpha^+$ CD4+ T cells after stimulation with Y-19KD	29	0
$TNF\alpha^-$ CD4+ T cells after stimulation with Y-19KD	25	33

$p<0.05$, χ^2 test

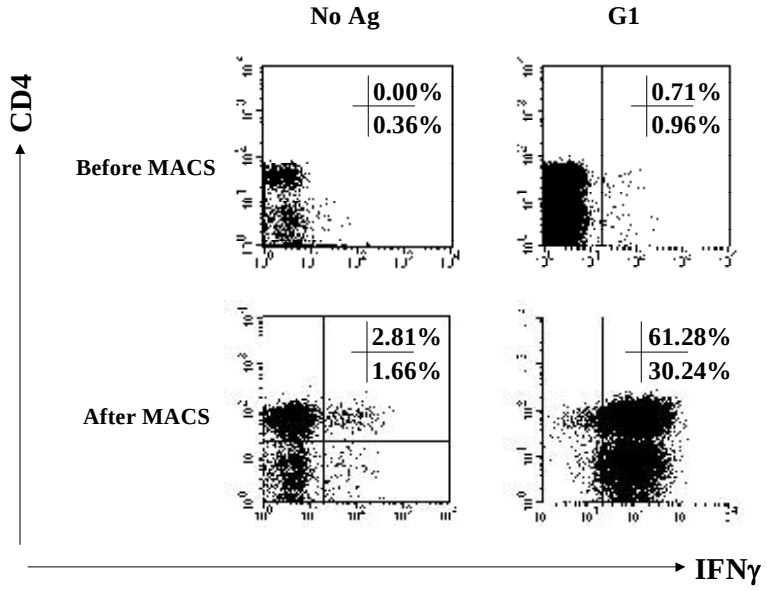


Figure 16. Example of G1-specific $CD4^+$ T cell enrichment compared to stimulation without antigen (Ag), in a patient with **ankylosing spondylitis**. After staining for T cell surface marker and cytokine IFNγ bound on the cellular surface, dead cells were gated out according to PI fluorescence in fluorescence 2 versus fluorescence 3 plot and a gate for CD4+ T cells was set. The percentage of IFNγ positive cells of the CD4+ T cell subpopulation is indicated. For more details see Methods section.

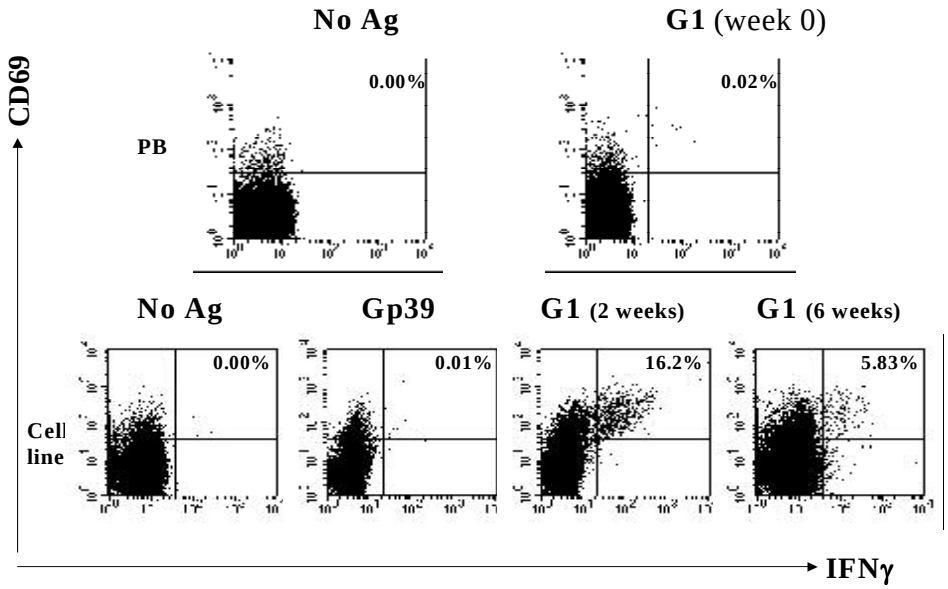


Figure 17. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan at different time points (2 or 6 weeks in culture) from the same **ankylosing spondylitis**. For the cell line, after staining for T cell surface markers and intracellular cytokines and after gating out antigen presenting cells according to CFDA fluorescence in a fluorescence 2 versus fluorescence 1 plot, a gate for CD4+ T cells was set. The percentage of IFNγ/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

surface staining) was present after second MACS in the G1 stimulated fraction, but in the CD4 T cell fraction without stimulation just few of IFNγ$^+$ CD4 T cells were obtained (2.81%). After several days of culture of G1-specific T cells isolated by MACS, a good antigen specificity was shown upon restimulation of this cell line with the G1 protein. An example is shown in Fig.17 indicating that 16.2% of G1-triggerred IFNγ$^+$ CD4 T cell could be observed in the G1-specific cell line at 2 weeks of culture. This frequency of IFNγ-positive T cells was much higher than that (0.02%) obtained by direct stimulation of PB of the same patient at baseline. What is more, another antigen (gp39) is not stimulatory to this cell line (Fig.17). However, it seems that the antigen specificity of the cell line became weaker after a 6 weeks in culture. Figure 17 shows that just 5.83% of G1-triggerred IFNγ$^+$ T cells was detected in the same cell line after 6 weeks of culture.

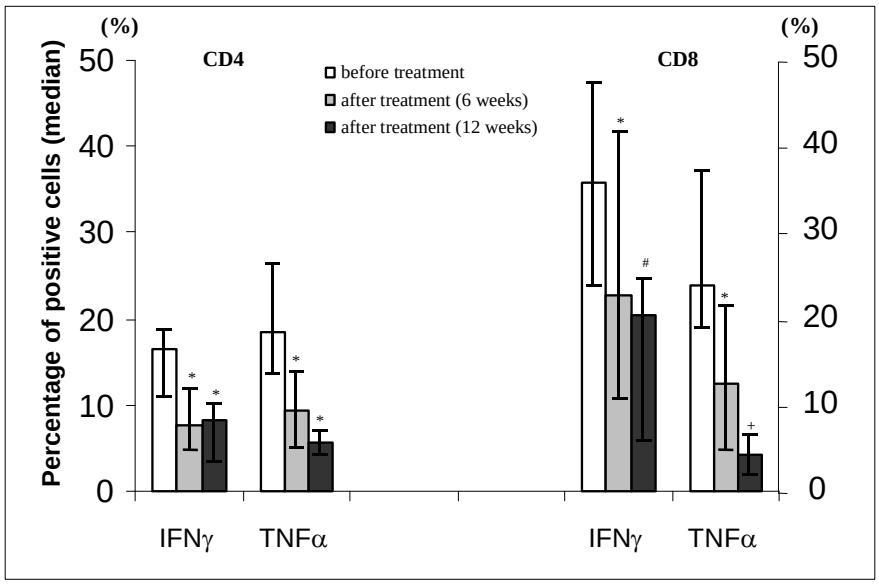
8. Monitoring of cytokine productions of CD4$^+$ and CD8$^+$ T cells after infliximab treatment

8.1 Infliximab treatment induces a decrease in the number of IFNγ and TNFα positive T cells after non-specific in vitro-stimulation.

For the infliximab group, treatment with 2 infusions of infliximab resulted in a significant decrease in the number of IFNγ- and of TNFα- positive T cells upon PMA/ionomycin stimulation after 6 weeks compared to before treatment both in the CD4+ subpopulation [median (25^{th} – 75^{th} percentile) IFNγ: 16.5% (11.1 – 18.8%) vs 7.8% (4.9 – 12%), p<0.005; TNFα: 18.4% (13.5 – 26.4%) vs 9.3% (5.2 – 14%), p<0.005.] and in CD8$^+$ subpopulation [IFNγ: 35.7% (23.8 – 47.4%) vs 22.8% (10.7 – 41.9%), p<0.005; TNFα: 23.8% (18.9 – 37.2%) vs 12.4% (4.7 –21.6%), p<0.005] (Fig.18). An example for the downregulation of IFNγ and TNFα production by infliximab is shown in Fig.19.

After patients had received the third infliximab infusion at week 6, no further decrease in the production of IFNγ [8.12% (3.4 – 10.2%)], but a non-significant decrease in the production of TNFα [5.62% (4.1 – 7.2%)] by CD4+ T cells could be observed at week 12 after non-specific stimulation in vitro. The difference was significant when the numbers before and after treatment at 12 weeks were compared

(p<0.005 for both cytokines) (Fig.18). For the cytokine production of $CD8^+$ T cells, a further significant decrease in the frequency of IFNγ and TNFα secreting cells was shown after 12 weeks [IFNγ: 20.36% (5.6 – 24.6%) , p<0.005 compared to the value before treatment and p=0.037 compared to that after 6 weeks; TNFα: 4.34% (2.12 – 6.6%), p<0.005 compared both to the value before treatment and to that after 6 weeks] (Fig.18) .



*p<0.005, comparing with that before treatment
#p<0.005, comparing with those both before treatment and at weeks
+p<0.005, comparing with that before treatment and p=0.037, comparing with that at weeks

Figure 18. Comparison of non-specific cytokine production between before and after infliximab treatment (at 6 weeks and 12 weeks)**.** A significant decrease of production of IFNγ and of TNFα by $CD4^+$ and $CD8^+$ T cells, upon PMA/ionomycin stimulation, was observed after infliximab treatment. The medians and range of non-specific cytokine production are indicated in the figure.

In contrast, no change of IFNγ or TNFα-secretion was observed during treatment with placebo upon PMA/IONO stimulation (Fig.20) [(before treatment vs placebo treatment at 6 weeks) IFNγ: 12.99% (8.55 – 14.04%**)** vs 11.25% (7.51 – 17.82%) and TNFα: 9.03% (6.18 – 10.14%**)** vs 9.93% (7.11 – 12.97%) by CD4+ T cells, p>0.05 for both cytokines]; The correspondent number of cytokine-positive CD8+ T cells was: IFNγ: 23.67% (14.99 – 36.74%**)** vs 29.38% (13.38 – 38.78%); TNFα: 8.59 % (5.71 – 18.18%) vs 9.27% (7.24 – 20.41%), p>0.05 for both cytokines.

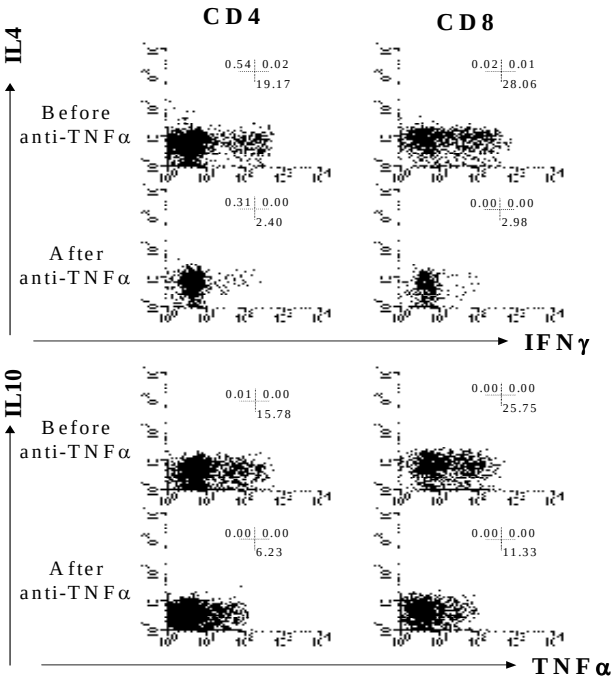
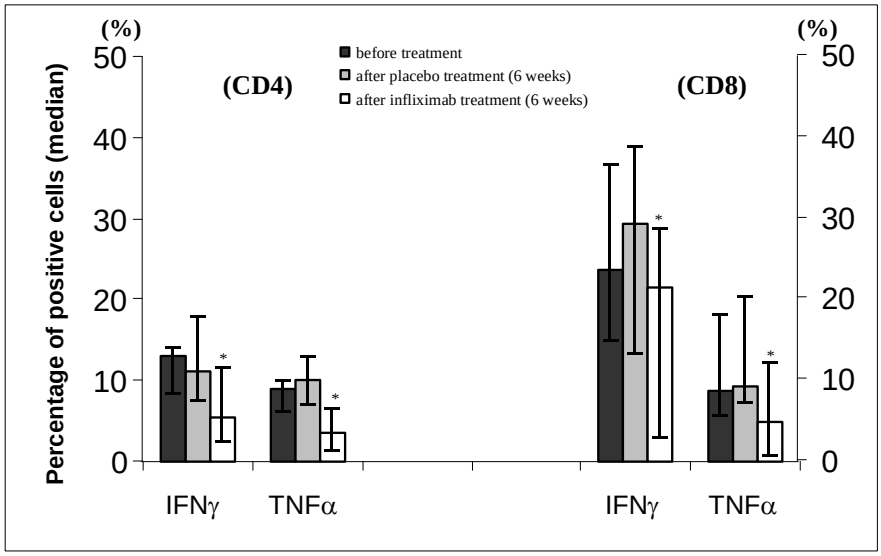


Figure 19. Example of the decrease of non-specific cytokine production by infliximab upon PMA/ionomycin stimulation in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cell and indirect gate for $CD4^+$ T cell were set. The percentage of IFNγ, IL4, TNFα, IL10, IFNγ/IL4- or TNFα/IL10-double-positive cells of the $CD4^+$ or $CD8^+$ T cell subpopulation is indicated.



*p<0.02, comparing with those before treatment and after placebo treatment.

Figure 20. Comparison of non-specific cytokine production between before and after placebo/infliximab treatment. A similar non-specific cytokine production was detected in time points of without and with placebo treatment, but a significant decrease of production of IFNγ and of TNFα by $CD4^+$ and $CD8^+$ T cells, upon PMA/ionomycin stimulation, was observed again after the patients accepted infliximab treatment. The medians and range of non-specific cytokine production are indicated in the figure.

After patients from placebo group were treated with infliximab, again a significant downregulation of antigen non-specific cytokine production by CD4 and CD8 was observed after 6 weeks [IFNγ: 5.36% (2.52 – 11.78%) by CD4 and 21.4% (2.9 – 28.9%) by CD8; TNFα: 3.53% (1.44 – 6.65%) by CD4 and 4.95% (0.81 – 12.24%) by CD8, $p<0.05$ in comparison with those before treatment for both cytokines] (Fig.20)

8.2 Infliximab treatment induces a decrease of IFNγ and TNFα by T cells after antigen-specific in vitro-stimulation.

A higher number of IFNγ- and TNFα- positive CD8+ T cells was detected than those of CD4+ T cells upon G1 peptides stimulation in all 20 AS patients before treatment (IFNγ: mean ± SD, 0.62% ± 0.46% vs 0.12% ± 0.11%, $p<0.005$; TNFα: 0.52% ± 0.4% vs 0.18% ± 0.2%, $p<0.05$). An example is shown in Fig.21.

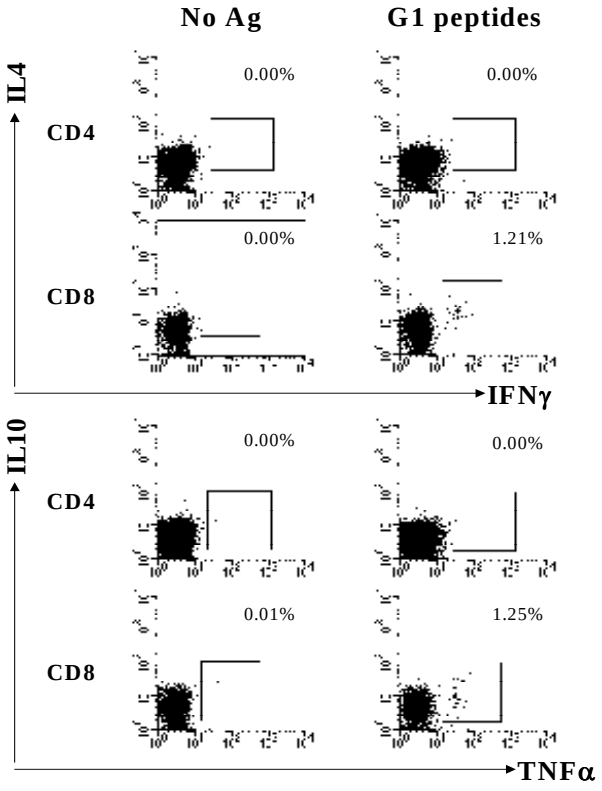
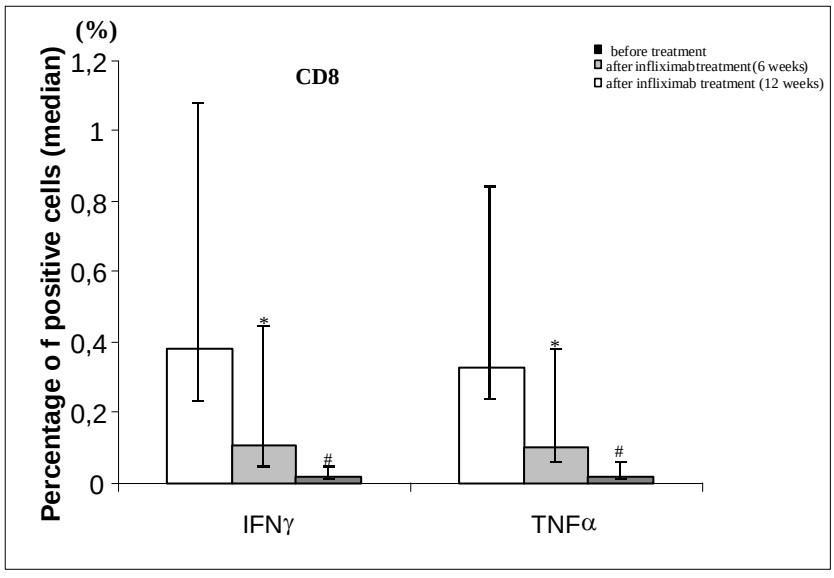


Figure 21. Example of an antigen-specific response to the G1 peptides compared to stimulation without antigen (Ag) in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cell and the indirect gate for $CD4^+$ T cell were set. The percentage of IFNγ and TNFα positive cells of the CD4+ or CD8+ T cell subpopulation is indicated.

Upon specific stimulation with G1-peptides, there was a significant reduction after 6 weeks of treatment for IFNγ positive CD8+ T cells (before treatment vs after treatment) [0.38% (0.23 – 1.08%) vs 0.11% (0.05 – 0.45%), p<0.02) and for TNFα positive CD8+ T cells [0.33% (0.24 – 0.84%) vs 0.1% (0.06 – 0.38%), p<0.02) (Fig.22). An example for the decrease in IFNγ- and TNFα-positive CD8+ T cells after G1-specific stimulation is shown in Fig.23. In comparison to $CD8^+$ T cells, however, the decrease in the numbers of IFNγ- or TNFα-positive $CD4^+$ T cells [IFNγ: 0.08% (0.03 – 0.17%) vs 0.05% (0.017 – 0.1%), p>0.05; TNFα: 0.08% (0.06 – 0.3%) vs 0.04% (0.017 – 0.1%), p>0.05] during treatment was not significant.

I also found a further decrease in the G1-specific IFNγ- and TNFα-positive $CD8^+$ T cells after 12 weeks of treatment [IFNγ: 0.015% (0.01 –0.05%); TNFα: 0.02% (0.01 – 0.06%), p<0.002 in comparision with that before treatment and p<0.05 in comparision with that at 6 weeks] (Fig.22). However, again no significant change in the number of cytokine-producing CD4+ T cells was detected.



*p<0.02, comparing with that before tretment .
#p<0.002, comparing with that before treatment and p<0.005, comparing with that at 6 weeks.

Figure 22. Comparison of the antigen-specific cytokine production between before and after infliximab treatment (at 6 weeks and 12 weeks). A significant decrease of the production of IFNγ and of TNFα by $CD8^+$ T cells, upon G1 peptides stimulation, was observed after infliximab treatment. The medians and range of antigen-specific cytokine production are indicated in the figure.

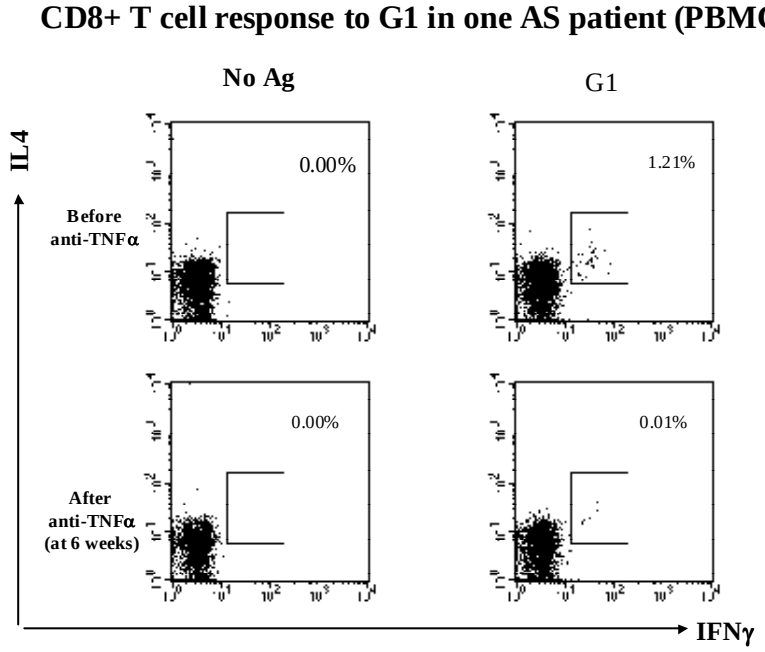
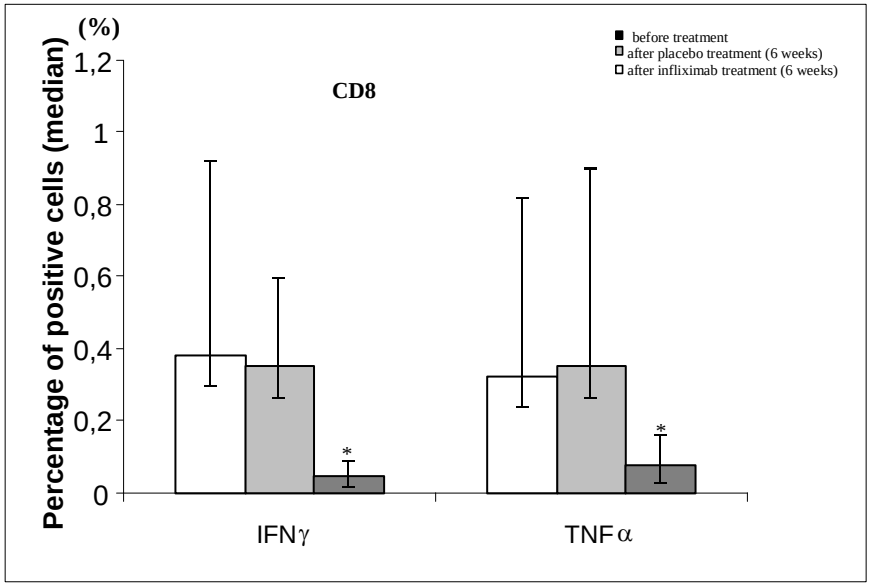


Figure 23. Example of decrease of antigen-specific cytokine production during infliximab treatment upon stimulation with G1 peptides in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cells and indirect gate for $CD4^+$ T cells were set. The percentage of IFNγ and TNFα positive cells of the $CD8^+$ T cell subpopulation is indicated.



*p<0.02, comparing with those before treatment and after placebo treatment .

Figure 24. Comparison of antigen-specific cytokine production between before and after 6 weeks of placebo/infliximab**.** A similar antigen-specific cytokine production was detected before and after 6 weeks of placebo treatment, but a significant decrease of the production of IFNγ and of TNFα by $CD8^+$ T cells, upon G1 peptides stimulation, was observed again after the treatment was switched to infliximab (at 6 weeks of infliximab treatment). The medians and range of non-specific cytokine production are indicated in the figure.

In patients treated with placebo, no significant difference in the antigen-specific cytokine production between before and after placebo treatment was observed. For $CD4^+$ T cells, IFNγ: 0.07% (0.02 –0.14%) vs 0.06% (0.015 – 0.14%); TNFα: 0.08% (0.03 – 0.2% vs 0.09% (0.03 – 0.23%), p>0.05; for $CD8^+$ T cells, IFNγ: 0.38% (0.30 – 0.92%) vs 0.35% (0.26 – 1.0%); TNFα: 0.32% (0.24 – 0.82%) vs 0.35% (0.26 – 0.9%), p>0.05 for both cytokiens (Fig.24). After the patiens were switched to infliximab treatment, an significant decrease of antigen specific cytokine production by CD8 upon G1-peptides stimulation was observed [IFNγ: 0.05% (0.02 – 0.09%) and TNFα: 0.08% (0.03 – 0.16%), p<0.02 comparing with those at baseline for both cytokines (Fig.24)].

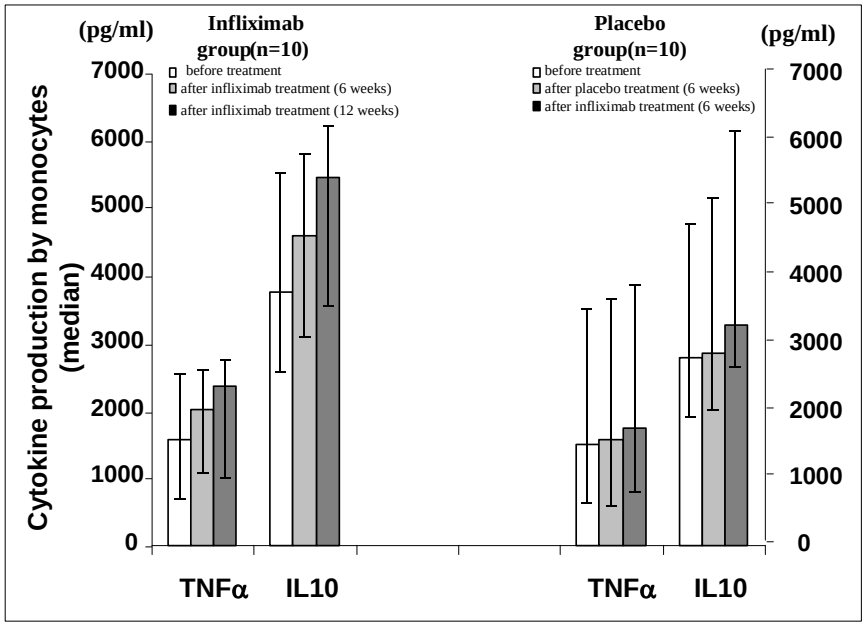
8.3 No change in IL-4- or IL-10-positive T cells during treatment

In both the infliximab and the placebo group, no significant change of antigen non-specific IL4 and IL10-secretion was observed during treatment (data not shown, p>0.05). G1-peptides-specific stimulation of T cells did not induce IL-4 or IL-10-secretion before or after treatment.

9. Effect of infliximab treatment on cytokine production by monocytes

No significant change in the numbers of cytokine-production by monocytes was observed in the infliximab group after 6 weeks upon in vitro stimulation with LPS (Fig.25) [median (25^{th} – 75^{th} percentile), before vs after treatment, TNFα: 1581.28 pg/ml (694.14 – 2531.85 pg/ml) vs 2024.42 pg/ml (1086.02 – 2610.05 pg/ml); IL10: 3765 pg/ml (2570 – 5525 pg/ml) vs 4605 pg/ml (3082.5 – 5815pg/ml) (p>0.05 for both cytokines)]. Also after 12 weeks of treatment, no significant change of cytokine production by monocytes was observed: TNFα: 2368 pg/ml (1011.5 – 2765.52 pg/ml); IL10: 5460.2 pg/ml (3542.1 – 6230.8 pg/ml), p>0.05 comparing with that before treatment for both cytokines. For the placebo group, a similar amount of cytokine production upon LPS stimulation was detected before and after placebo treatment [median (25^{th} – 75^{th} percentile), before vs after placebo treatment, TNFα: 1483.96 pg/ml (610.72 – 3500.67 pg/ml) vs 1575.18 pg/ml (608.77 – 3666.32 pg/ml); IL10: 2785 pg/ml (1917.5 – 4782.5 pg/ml) vs 2869.32 pg/ml (2032.5 – 5167.5pg/ml) (p>0.05

for both cytokines) (Fig.25)]. After the patients of the placebo group were switched to infliximab, again, there was still no marked change in the cytokine secretion (Fig.25) [median (25^{th} – 75^{th} percentile), TNFα: 1751.58 pg/ml (757 – 3866.35 pg/ml); IL10: 3280 pg/ml (2632.5 – 6167.5pg/ml) (p>0.05, in comparison with those before and after 6 weeks of placebo treatment for both cytokines)].



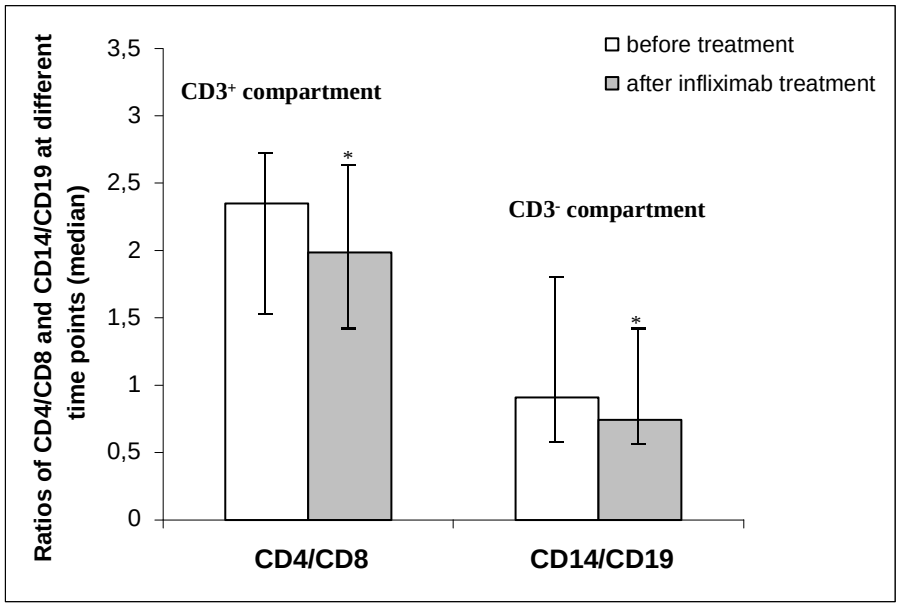
*p>0.05, there is no significant difference between before and after treatment for both cytokines in two groups.

Figure 25. Comparison of non-specific cytokine production by ,monocytes between before and after placebo/infliximab treatment. A similar non-specific cytokine production upon LPS stimulation was detected between before and after infliximab or between before and after placebo. The medians and range of non-specific cytokine production are indicated in the figure.

10. Change of ratio of CD4/CD8 in $CD3^+$ cells and of CD14/CD19 in $CD3^-$ cells after infliximab treatment

The cells at week 0 and week 6 after infliximab treatment from all 20 patients were investigated for the percentage of lymphocytes and macrophages. After the cells were separated into $CD3^+$ and $CD3^-$ fractions by MACS, the relative numbers of CD4 and CD8 cells were detected inside the $CD3^+$ fraction, and of CD14 and CD19 cells inside the $CD3^-$ fraction by flow cytometry. Subsequently, the ratio of CD4/CD8 cells was calculated in the $CD3^+$ population and CD14/CD19 cells in the $CD3^-$ population. The results indicated that 10 out of 20 patients had a decrease of CD4/CD8 ratio and the others had an increase after infliximab treatment. The general

ratio [median (25^{th} – 75^{th} percentile), before vs after treatment with infliximab]: 2.35 (1.53 – 2.72) (mean 2.23) vs 1.98 (1.42 – 2.63) (mean 2.13) (Fig.26), thus there was no clear difference (p>0.05). For $CD3^-$ cells, a decrease of CD14/CD19 ratio was observed in 13 out of 20 patients, an increase in 7 out 20 patients; again the general ratio did not show a significant difference [0.91 (0.57 – 1.81) vs 0.74 (0.56 – 1.4), p>0.05, Fig.26].



*p>0.05, comparing with that before infliximab treatment.

Figure 26. Comparison for ratios of CD4/CD8 and CD14/CD19 between before and after infliximab treatment. After $CD3^+$ and $CD3^-$ T cells were separated by MACS, a similar ratios of CD4/CD8 in the $CD3^+$ compartment and of CD14/CD19 in the $CD3^-$ compartment was detected by flow cytometric analysis between before and after infliximab, The medians and range of CD4/CD8 and CD14/CD19 ratios are indicated in the figure. For more details see methods.

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).

Summary

The pathogenesis of two important inflammatory rheumatic diseases, RA and AS, is not clear. In both diseases an immune response against an unknown putative antigen could play a crucial role. T cell responses to antigens derived from bacteria such as klebsiella or to autoantigens derived from the cartilage such as proteoglycan have been tested, but no convincing evidence for their involvement in the pathogenesis has been obtained so far.

In this study, I applied the more sensitive and more specific technique of antigen-specific cytometry to investigate the T cell response to h-hsp60 and y-19kd as well as various cartilage-derived autoantigens. Taking IFNγ-secretion of CD4+ T cell as primary outcome parameter, I set out to quantify the antigen specific T cell response in peripheral blood and synovial fluid of patients with AS or RA, and controls to answer the questions which antigen-specific T cells are detectable in AS patients and compare this to RA patients and controls.

The results indicate that a similar T cell response to h-hsp60 and y-19kd is present in patients with AS and RA compared to healthy controls, which suggests that these two antigens are not primarily involved in the pathogenesis of AS and RA. For cartilage-derived autoantigens, on the basis of the relative frequency of IFNγ^+ cells among CD4+ T cells, the results showed that the G1 domain of the proteoglycan 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%). No T cell responses to HC gp39 and collagen II was observed in AS-patients in the present study. Importantly, the response of synovial fluid CD4+ T cells to the G1-domain was shown to be significantly higher compared to PB. Further analysis indicated that two T cell epitopes of G1 domain were identified to be immunodominant in AS: AA residues 292 to 309 and 252 to 269. These data suggest that the G1 domain of aggrecan could be a target of the immune response in AS.

A further aim of this study was to assess the possible effect of anti-TNFα treatment on the capacity of T cells and macrophages to produce cytokines. Treatment of active ankylosing spondylitis with the monclonal anti-TNFα antibody infliximab is clinically highly effective. The precise mechanism of action, however, is not known. I investigated the cytokine response in 20 active AS patients during

infliximab therapy. The results indicated that infliximab could downregulate the T cell (but not macrophage) production of the proinflammatory cytokines IFNγ and TNFα at week 6 and 12, but no effect on IL4- and IL10-production was seen. This downregulation of the proinflammatory cytokines is probably a relevant mechanism for the clinical efficacy.

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Abbreviations used in this thesis

AA	amino acid
AS	ankylosing spondylitis
AIDS	acquired immune deficiency syndrome
APC	allophycocyanin
APCs	antigen present cells
BSA	bovine serum albumin
CD	cluster of differentiation
CFDA-SE	(6)-Carboxyfluorescein diacetate succinimidyl ester
CIA	collagen II induced arthritis
Cy5	phycoerythrin-cyanine5
DMSO	dimethyl sulfoxide
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony stimulating factors
HBSS	Hanks' balanced salt solution
HC	healthy control
HC gp39	human cartilage glycoprotein 39
HLA	human leucocyte antigen
HSP	heat shock protein
IL	interleukin
IFN	interferon
MACS	magnetic-activated cell sorting
M-CSF	monocyte/macrophage colony-stimulating factors
MHC	major histocompatability complex
MNC	mononuclear cells
MAb	monoclonal antibody

MRI	magnetic resonance imaging
PB	peripheral blood
PBS	phosphate buffered saline
PerCP	peridinin-chlorophyll
PE	phycoerythrin
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
RCLS	red cell lysing solution
RT	room temparature
SEB	staphylococcus enterotoxin B
SF	synovial fluid
SpA	spondyloarthropathies
SSC	side scatter
uSpA	undifferentiated spondyloarthropathies
TCR	T cell receptor
TNF	tumor necrosis factor
Y-19KD	yersinia 19KD

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