Regulation of proteoglycan-specific immune responses in arthritis

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Regulation of proteoglycan-specific immune responses in arthritis

Regulatie van proteoglycaan-specifieke immuunreacties in artritis

(met een samenvatting in het Nederlands)

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Abbreviations

Ag	Antigen
APC	Antigen presenting cell
AS	Ankylosing spondylitis
CFA	Complete Freund's adjuvant
CII	Collagen type II
ConA	Concanavalin A
CS	Chondroitin sulfate
DDA	Dimethyldioctadecylammonium bromide
DMARD	Disease-modifying anti-rheumatic drug
EBV	Epstein-Barr virus
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
Grp	Glucose-regulated protein
HLA	Human leukocyte antigen
hPG	Human proteoglycan
HSP	Heat shock protein
ID	Intradermally
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
IP	Intraperitoneally
JIA	Juvenile idiopathic arthritis
KS	Keratan sulfate
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mPG	Mouse proteoglycan
Mt	*Mycobacterium tuberculosis*
NSAID	Non-steroid anti-inflammatory drug
OA	Osteoarthritis
OVA	Ovalbumin
PB	Peripheral blood
PBMC	Peripheral blood monocular cell
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PG	Proteoglycan
PGIA	Proteoglycan-induced arthritis
PLN	Popliteal lymph nodes
RA	Rheumatoid arthritis
S.I.	Stimulation index
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of mean
SF	Synovial fluid
TCR	T cell receptor
Tg	Transgenic
Th1/2	T helper 1/2
TNF	Tumor necrosis factor
Tr1	Regulatory T cell type 1
Tregs	Regulatory T cells

Chapter 1

General Introduction

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1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases characterized by chronic inflammation of the synovium of diarthrodial joints (1). It can lead to long-term joint damage, resulting in chronic pain, loss of function and disability. Primarily the small joints of the extremities are affected, but as the disease progresses more of the large joints become involved too. The chronic inflammatory process induces changes in the cellular composition (cellular infiltration) and the gene expressing profile of the synovial membrane, resulting in hyperplasia of the synovial membrane, which causes structural damage of cartilage, bone and ligaments (2). Extra-articular disease affecting a variety of organs occurs in the majority of patients and is a significant factor in morbidity and mortality of people with RA (3). The severity of RA encompasses a wide spectrum, ranging from self-limiting disease to chronic progressive disease, causing varying degrees of joint destruction and clinically evident extra-articular organ involvement.

RA occurs in 0.5-1.0% of the population worldwide (4,5). The prevalence is about two to three times more common in women than in men. Although the cause or causes of RA remain elusive, the general consensus is that factors contributing to its occurrence and course (clinical heterogeneity) are probably both genetic and environmental. The main risk factors for the disease include genetic susceptibility, sex and age, smoking and infectious agents. In addition, hormonal, dietary, socioeconomic, and ethnic factors seem to contribute (4-6).

The major goals of treatment of the arthritis are to reduce pain and discomfort, prevent deformities and loss of joint function, and to maintain a productive and active life. Inflammation must be suppressed and mechanical and structural abnormalities corrected or compensated by assistive devices. The introduction of new therapies such as tumor necrosis factor-alpha (TNFα)-blocking agents and new treatment strategies, especially early and aggressive therapy, including combinations of several disease-modifying anti-rheumatic drugs (DMARDs) have improved the outcome for RA patients (7). Unfortunately, these therapies form not a cure, as continuous systemic immunosuppression is required to maintain clinical benefits. Consequently, the long-term side effects are unsure while the costs are high (8,9).

2. Immune responses in RA

RA is a chronic inflammatory condition that involves many elements of the immune response (10). The synovium (or synovial membrane) is normally a relatively acellular delicate structure consisting of one or two layers of synoviocytes. In RA, the synovium becomes hypertrophic and edematous. Angioneogenesis, recruitment of inflammatory cells due to production of chemokines, local retention and cell proliferation do all contribute to the accumulation of cells in the inflamed synovium. Locally expressed degradative enzymes digest the extracellular matrix and destroy the articular structures (11). The synovial membrane that extends to the cartilage and bone is known as pannus. It actively invades and destroys the periarticular bone and cartilage at the margin between synovium and bone. Multiple cell types participate in the pathogenesis of RA and the following section will introduce the major contributing cell populations.

T cells

Consideration of the possibility that T cells might be actively involved in the pathogenesis of RA first became prominent in 1980s (12). The innate immune system might prepare the synovium for infiltration by T cells and the subsequent immune events in the joint (13). Activated T cells that are abundantly present in the inflamed joints of RA patients can stimulate other cells (e.g. B cells, macrophages and fibroblast-like synoviocytes) (11,13-15). These T cells are found to participate in the complex network of cell- and mediator-driven events leading to inflammation and joint destruction (13,16,17). However, several parts of the T cell pathway are still hypothetical and further research is needed to provide conclusive evidence. The role of T cells in RA will be described in more detail in the next paragraph.

B cells

B cells play several critical roles in the pathogenesis of RA (18). They are the source of autoantibodies being produced in RA and contribute to immune complex formation and complement activation in the joints (8,19). Patients with RA frequently have rheumatoid factors (RF); antibodies of the IgM or IgG subclasses mostly reactive with the constant region of their autologous IgG molecules. Multiple other autoantibodies have been found in RA, with recent interest focused on those directed at cyclic citrullinated peptides (20). Antibodies that are directed to citrullinated protein (21) detect cyclic citrullinated peptides in many different proteins and are present in about 80% of the RA patients. Several lines of evidence suggest that citrullinated antigens have direct involvement in the rheumatoid disease process. Anti-cyclic citrullinated peptide antibodies precede the clinical development of synovitis by many years (22,23). B cells are also very efficient antigen-presenting cells, and can contribute to T cell activation (24). B cells both respond to and produce chemokines and cytokines that promote leukocyte infiltration into the joints, formation of ectopic lymphoid structures, angiogenesis, and synovial hyperplasia. The important role of B cells in the disease etiology is supported by the recent success of B cell depletion therapy using rituximab (25).

Macrophages

The major effector cells in the pathogenesis of arthritis are synovial macrophages and fibroblasts (2). Activated macrophages are critical in RA, not only due to macrophage-derived cytokines (in particular TNFα and interleukin-1 (IL-1)) in the synovial compartments (26), but also because of their localization in strategic sites within the destructive pannus tissue (27,28). In addition beside tissue macrophages, also circulating monocytes and other cells of the myelomonocytic lineage contribute to disease (26,29-31). The variety and extent of macrophage-derived cytokines in RA and their widespread effect indicate that macrophages are local and systemic amplifiers of disease severity and perpetuation (32).

Fibroblast-like synovial cell

Among the many cell types present in the rheumatoid joint, the fibroblast-like synovial cell (FLS) is one of the most prominent. It is now accepted that the FLS is not only space-filling, but directly responsible for cartilage destruction,

and also drives both inflammation and autoimmunity (33). There is evidence for proliferation and expression of inflammatory cytokines and chemokines by FLS in inflamed synovia (34,35).

In summary, the cell-cell interactions that occur in RA synovium are multiple, complex and fundamentally important to the pathogenesis and outcome of this disease (36). In addition to the cell populations considered in detail above, osteoclasts, chondrocytes, mast cells, dendritic cells and other cell types are important in the events that occur within RA pannus and in adjacent cartilage and bone. Besides the cognate cell-cell interactions that require direct contact between two different types of cells, interactions between cell populations in RA synovium can also be mediated by secreted molecules, such as cytokines (36). Cytokines play a central role in the initiation and perpetuation of synovial inflammation (37). They are believed to activate resident synovial cells to produce protolytic enzymes that mediate destruction of the cartilage, ligaments and tendons of the joints. Many of the cytokines thought to play a role in initiating joint destruction are probably produced as a result of local T cell and macrophage activation. The cytokine system is a highly complex network with cytokines cross-regulating their expressions and function (2). Numerous cytokines, including IL-1, IL-8, TNFα and interferon-gamma (IFNγ), have been detected in synovial fluid (SF). TNFα and IL-1 have been identified as key players in synovial inflammation and are direct targets of successful anti-cytokine treatment of RA (2).

There is substantial evidence that RA has an autoimmune component, besides the fact that no infectious cause is proven. First, the fact that the disease affects multiple joints is consistent with the process being systemic (38). Second, the disease is associated with autoantibodies and the formation of immune complexes (39). Third, autoreactive T cells are present in the inflamed joints (40). Fourth, RA (like other systemic autoimmune diseases) is significantly more common in women than in men (39). Finally, there is a marked association between disease and the expression of specific HLA subtypes, which is consistent with the expansion of specific T cell subsets that are presumably activated by specific epitopes (41).

Over the last few years, susceptibility to autoimmunity has been regarded not so much as a predisposition to generating autoaggressive effector cells, but rather as a failure to regulate the immune response (42). Considering the current concepts of immunity, a defect in immunoregulation could be acquired at multiple points during both early and late phases of an autoimmune response. For $CD4^+$ T cells, the initial ligand-receptor interaction between MHC-peptide and the T cell receptor (TCR) is crucial, since this determines qualitative and quantitative aspects of the signals transduced through the TCR. Useful understanding of the role of T cells in RA will need to place T cell function in context of the incompletely understood cell-cell interactions that are likely to be keys to the pathogenesis of this disease (43).

3. Role of T cells in arthritis

The extension of the contribution of T cells to the pathogenesis of RA remains a matter of intense debate. It is apparent that taking RA as simply a T cell-mediated or T cell-independent disease represents a too narrow view (13), as in complex diseases such as RA it is unlikely that there is a single ‘guilty’ effector cell. The association between

specific alleles encoded within the MHC class II region and the development of RA has provided the first strong evidence that CD4+ T cells play a role in the pathogenesis of this chronic inflammatory disease (42).

Much attention has been focused on the role of T cells in autoimmunity for two main reasons. First, T helper cells are the key regulators of all immune responses to proteins. Second, several autoimmune diseases are genetically linked to the MHC, and the function of MHC is to present peptide antigens to T cells. T helper cell abnormalities may also lead to autoantibody production because T helper cells are necessary for the production of high affinity antibodies against protein antigens. An autoantigen has not been unequivocally identified in RA, but the synovium clearly is the site of antigen-specific T cell responses. Using *in vitro* proliferation assays with T cells from RA patients, many putative autoantigens have been identified as candidate antigens. Type II collagen (CII), proteoglycan (PG) aggrecan, cartilage link protein and many relatively joint-specific antigens (e.g. matrix metalloproteinases) have been implicated (44-46).

Tissue infiltrating T cells, in particular $CD4^+$ T cells, are a consistent feature of rheumatoid synovitis (43,47). This antigen driven infiltration may predominate during the early phase of inflammatory response. T cells could be recruited through bystander activation, or by stimulation of self antigens released from inflamed tissue. Initial stimulation of T cells by complexes of antigenic peptide and disease-associated MHC class II molecules could profoundly influence the expression of pro-inflammatory cytokines. Data suggest that during the chronic phase of the disease it may be the cytokine milieu that sustains and maintains pathogenic T cells (Table 1) (42).

Table 1. Characteristics of chronically activated T cells in the synovium of patients with RA.

T cells are found in follicular lymphoid aggregates
The cell-surface phenotype (e.g. CD45RO, CD69, CD44 expression) suggests chronic immune activation
T cells are terminally differentiated, with significant telomere loss
Synovial T cells are hyporesponsive to TCR ligation
Synovial T cells exist in an environment favoring cell survival
There is an imbalance of pro- and anti-inflammatory cytokines, with a predominance of macrophage products in inflamed joints
There is a bias towards the development of T helper 1 cells

Adapted from Cope AP (42).

T cell hyporesponsiveness arises through subsequent sustained expression of inflammatory cytokines. The hyporesponsive state of synovial T cells in RA directly correlates with impaired TCR-mediated signaling transduction (48). Impaired T cell activation could promote and perpetuate the chronic inflammatory response through the loss of mechanisms of tolerance that are dependent upon intact TCR signal transduction pathways (49). Hyporesponsive T cells might function as effector cells and sustain the chronic inflammatory process through predominantly antigen independent mechanisms. It is proposed that by reversing T cell hyporesponsiveness, antigen-independent responses serve to regulate the inflammatory process.

In addition, to direct autoimmune attack by effector T cells, arthritis might result from defective homeostatic control of immunity by regulatory T cells (Tregs) (17). Synovial T cells can be divided into several subsets that either promote or inhibit inflammation. Both pathological responses to antigen and a failure of regulatory function need to be considered as ways in which T cells could perform a critical role in human arthritis.

The regulation of immune responses to self-antigens is a complex process that involves maintaining self-tolerance while retaining the capacity to mount robust immune responses against invading microorganisms (50). Peripheral tolerance mechanisms are needed to control autoreactive T cells. One control pathway is the absence of costimulatory signals that renders T cell activation incomplete and commits T cells to anergy or cell death (51). A second major control mechanism involves suppressive (immunomodulatory) signals that are delivered by Tregs (51). Two major subsets of Tregs can be distinguished: natural and adaptive (acquired) Tregs (50). Certain natural Tregs ($CD4^+CD25^{+\ bright}$ T cells) actively down regulate the activation and proliferation of autoreactive T cells (52). Such cells are capable of dampening an immune response by conventional $CD4^+$ T cells specific for the autoantigen, as shown in transplantation. The production of Tregs is a key function of the thymus in self-tolerance. The development of Tregs in the thymus stresses the fact that there are many potentially autoreactive T cells that must be permitted to enter the periphery in order to assure protection from pathogens. Adaptive Tregs can develop either from $CD4^+CD25^+$ natural Tregs or by altering the activity of T helper cells and include type 1 Tr cells (Tr1) that secrete high levels of IL-10 (53), and Th3 cells which primarily secrete high levels of transforming growth factor-beta (54).

Autoantigens in RA

In the classical model of autoimmunity, tissue specificity is determined by the tissue-specific recognition of autoantigens that elicit adaptive immune responses. Both T cell and antibody responses have been found to a variety of putative autoantigens in RA patients (55). RA associated antigens fall into two major groups: first, those that are locally expressed in the joint. Prominent candidate antigens are CII (56,57), human cartilage glycoprotein 39 (gp39) (44), and PG (58,59). These antigens can induce autoimmunity and arthritis in rodent models, but whether the reactivity participates in the primary pathogenesis of RA or reflects tissue degradation remains unknown. Second group of antigens are those proteins not associated with the joint, and consist of:

1) highly conserved foreign antigens with human homologues, in which the initiating antigenic stimulus may occur through infection. The Epstein-Barr virus (EBV) encoded glycoprotein, like other bacterial proteins, contains the QKRAA shared epitope motif (MHC class II determinant) and has been a strong candidate for over 25 years as environmental infectious agent involved in RA pathogenesis (60). Other conserved candidates include heat shock proteins (HSPs), which are major bacterial antigens. Antibodies and T cells reactive with HSP65 and DnaJ class of HSPs are abundant in the SF of RA patients (61).

2) post-translationally altered proteins, such as citrullinated filaggrin, to which autoantibodies show high specificity but low sensitivity for RA (21) and the Fc portion of IgG, which is recognized by rheumatoid factor.

3) ubiquitous proteins, such as glucose-6-phosphate isomerase (G6PI) (62), p205 (63), and HSPs secreted during stress, such as endoplasmic reticulum immunoglobulin binding protein BiP (64,65). Despite the presence of many

putative critical antigens there is for most of them no solid evidence yet that they are implicated in the pathogenesis of RA (40).

Taken together, numerous research studies have scrutinized the role for T cells and their mechanisms of action in RA. A crucial point remains unresolved: do T cells activated by one or more autoantigens initiate the disease process, or is the influence of T cells secondary to a synoviocyte-driven inflammatory response originating within the joints(17)? Probably the cause and the driving forces are polygenic and multifactorial, and understanding the disease will require a detailed basic analysis of disease mechanisms. Animal models are excellent tools for such analysis.

4. Experimental models for RA

To understand the complexity of the pathogenesis of RA and for preclinical testing of new therapeutic agents, animal models are a necessity. To be able to evaluate and select suitable animal models for RA it is crucial to reproduce some of the basic features of RA in such models (66). Hallmarks are first, tissue-specificity; RA is characterized by a tissue-specific, inflammatory attack affecting diarthrodial joints. Although systemic manifestation can be prominent, the predominant inflammatory attack is directed towards peripheral joints. Second, chronicity; in RA chronicity is an essential characteristic. The disease course may proceed with identifiable relapses, but there is usually steady progression of joint destruction. Third, MHC class II association; the genetic influence is significant though not prominent and points towards an important role of class II genes in the MHC. In particular, certain structures near the peptide-binding pocket of HLA-DR4 molecules are highly associated with RA (67,68).

Many models have been described and each represents different aspects of the disease. The models described for RA so far can be divided into three principal groups: 1) cartilage protein-induced, 2) adjuvant induced, and 3) spontaneous. As arthritis is mediated by a specific immune attack on cartilage in peripheral joints, it is not surprising that several cartilage proteins, such as PG (69), CII (70), gp39 (44) and cartilage oligomeric matrix protein (COMP) (71) have been shown to be arthritogenic in different animal strains.

In our studies we used the proteoglycan (aggrecan)-induced arthritis (PGIA) model because this model reproduces several features of RA in which we are especially interested. PGIA is a chronic arthritis model which makes it especially useful to test immunomodulating agents over a longer period. Furthermore, PGIA is an antigen-induced arthritis model fundamentally controlled by T cells making it possible to generate a T cell receptor (TCR) transgenic (Tg) mouse and study the role of antigen-specific T cell responses in RA. In addition, PGIA is induced by a cartilage matrix component and there is growing evidence that, at least in a subset of patients with RA, antigen-specific T cell responses to cartilage matrix proteins do develop (45,59,72-76). Among the candidate autoantigens (58,77), cartilage PG aggrecan is one of the target autoantigens in RA joints (45,59,72-76).

5. Proteoglycan (aggrecan)-induced arthritis model

Aggrecan (Fig. 1) is a complex macromolecule consisting of a large core protein (~2,400 amino acids) to which glycosaminoglycan and oligosaccharide side chains are covalently attached (78,79). These side chains include approximately 100 chondroitin sulfate (CS) chains, 30 keratan sulfate (KS) chains and shorter N- and O-linked oligosaccharides. Link protein, a small glycoprotein, stabilizes the noncovalent linkage between aggrecan and hyaluronan (HA) to form the PG aggregate that may contain as many as 100 aggrecan monomers.

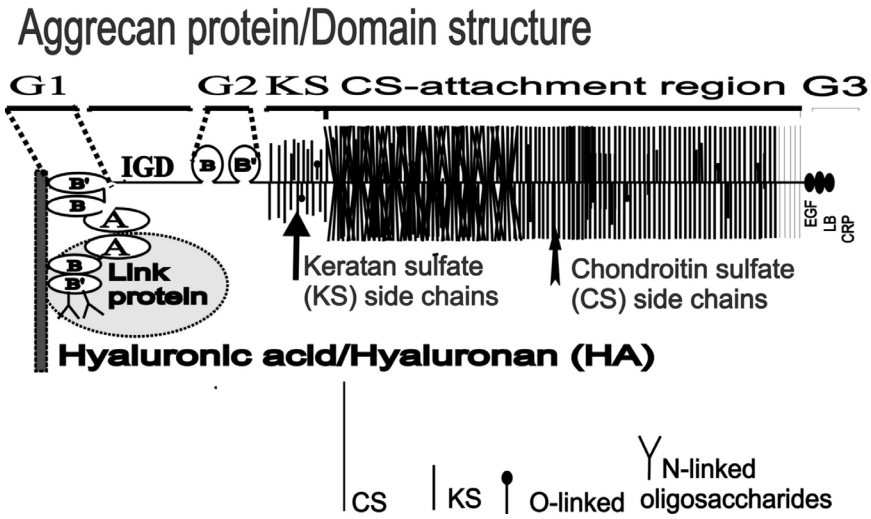

Figure 1. *Schematic presentation of the structure of cartilage proteoglycan (PG) aggrecan. Cartilage PG consists of a central protein core to which glycosaminoglycan (GAG), chondroitin sulfate (CS), and keratan sulfate (KS) side chains are attached together with O-linked and N-linked oligosaccharides. (Hundreds of PG aggrecan molecules bind to a single hyaluronan chain, stabilized by link protein, forming large, multimillion Dalton size aggregates.) The various domains/subdomains of the central protein core are the G1 domain with A, B, and B' loops; IGD (interglobular domain between G1 and G2 domains); G2 domain with B and B' loops; KS (a KS-rich region between the G2 domain and the CS attachment region); CS attachment regions; and a G3 domain containing EGF (epidermal growth factorlike), LB (lectin-binding), and CRP (complement regulatory protein-like) subdomains. Approximately 100-120 CS side chains are attached to a long, but restricted, region of the core protein (CS attachment region). Although most of the KS chains are localized in a narrow region (KS), KS side chains are also present along the entire core protein and frequently mask T cell epitopes. CS and KS side chains mask the T cell epitopes of the core protein, and many of the T cell epitopes can be processed by the MHC only if the GAG side chains are removed. Adapted from Glant TT and Mikecz K (138) with the approval of Humana Press (Totowa, NJ).*

The G1 and G2 N-terminal globular domains of aggrecan and its G3 C-terminal domain have distinct properties that function as integral parts of the aggrecan core protein. Approximately half of the aggrecan molecules in adult cartilage lack the G3 domain due to proteolytic cleavage during matrix turnover. The core protein of aggrecan is heavily degraded during inflammatory processes, which results in the loss of function of articular cartilage (58,77,80). Immunization of BALB/c mice with partially deglycosylated human PG (hPG) induces chronic progressive polyarthritis and spondylitis (Fig. 2) (81). This PGIA model has many similarities with human RA, as indicated by clinical assessments (Fig. 3), radiographic analyses, scintigraphic bone scans, laboratory tests, and histopathology of peripheral joints (69,81-83). The development of the disease is based upon T and B cell responses cross-reactive between the immunizing human and mouse (self) cartilage PG (69,83,84). Although the production of mouse PG-specific antibodies precedes inflammation and shows a high correlation with the incidence of arthritis, neither these antibodies nor PG-specific B cells alone can transfer the disease to naïve syngeneic mice (82,85,86).

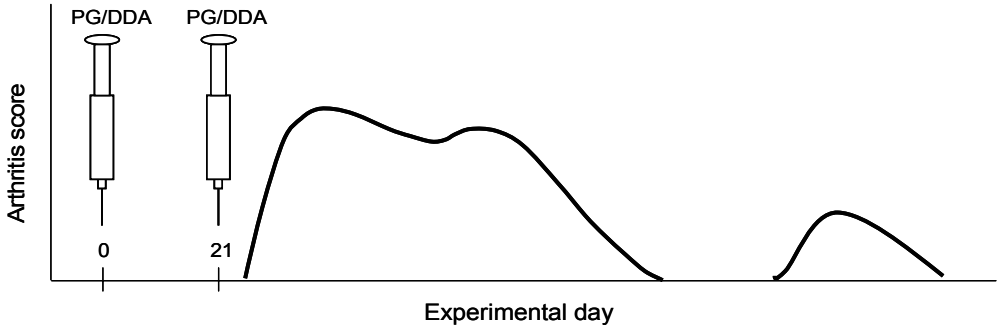

Figure 2. *Schematic presentation of the immunization scheme and arthritis course. To induce proteoglycan (PG)-induced arthritis young retired breeder female BALB/c mice are immunized with PG (100 μg human PG protein in 100 μl PBS) mixed with dimethyldioctadecylammonium bromide (DDA; 2 mg in 100 μl) on days 0 and 21. It is typical that, if an animal once develops any small symptom of inflammation e.g., in the interphalangeal joint of one paw, this mouse will develop severe arthritis sooner or later without additional injections. Usually, the small joints are first involved. Along the progression of the disease, more and more joints are involved until severe deformities and ankylosis develops. Disease may flare up in one or in a few joints, when the inflammation in other joints is regressed (95,138).*

Adoptive transfer of PGIA requires the presence of both T and B cells from arthritic animals (82,87,88), and a rapid accumulation of mouse PG-specific Th1 cells in the synovium appears to be the most critical component of the development of arthritis (69,89). It is very likely that an autoantigen-driven mechanism of joint inflammation becomes local and self-sustaining by PG (cartilage) degradation in the mouse joint. Autoreactive T cells subsequently migrate to and proliferate in the synovium and joint draining lymph nodes (85). Here self-peptides are present in relatively high concentrations as a result of the normal turnover of the cartilage matrix, which may be higher when an increased PG degradation occurs in the inflamed joint. Once an animal develops arthritis, more and more joints become involved and repeated "spontaneous" episodes of inflammation result in complete deterioration of articular cartilage and lead to deformities of the peripheral joints (69).

PGIA is postulated to be a fundamentally T cell-dependent, but PG-specific B cell and antibody mediated autoimmune model (69). Several lines of evidence indicate that $CD4^+$ T cells play an important role in PGIA (69,84,90). First, susceptibility to PGIA is associated with MHC class-II ($H\text{-}2^d$ haplotype in BALB/c and $H\text{-}2^k$ in C3H mice) (69,84,91-93), and the disease is prevented when $CD4^+$ T cells are depleted either *in vivo* (90) or *in vitro* prior to adoptive transfer (82). Moreover, immunization of BALB/c mice with hPG induces a dominant Th1 T cell response (89,94,95) and treatment of arthritic mice with IL-4 can prevent disease development by inducing a switch from a Th1-type to a Th2-type response. The importance of $CD4^+$ Th1-cells was further underlined by the observation that IL-4-deficient BALB/c mice developed a significantly more severe form of the disease than wild-type BALB/c mice (96). Finally, the transfer of disease requires *in vivo* or *in vitro*-activated $CD4^+$ T cells from arthritic animals (82,87), or an arthritogenic PG epitope-specific $CD4^+$ T cell hybridoma (5/4E8) (97).

Although $CD4^+$ T cells are necessary for the development of PGIA (89,90,97-99), both autoreactive T cells and B cells, and possibly autoantibodies are required for the development of a severe chronic relapsing arthritis in this model (69,83,84,99,100). PG-specific B cells regulate the initiation of autoimmune murine arthritis by functioning as antigen presenting cells and by producing autoantibodies (99). While arthritic serum alone did not induce disease (82), autoantibodies are important for initiating inflammation in the joint by binding to the cartilage surface (89), and initiating chemokine and cytokine responses in the joint (101).

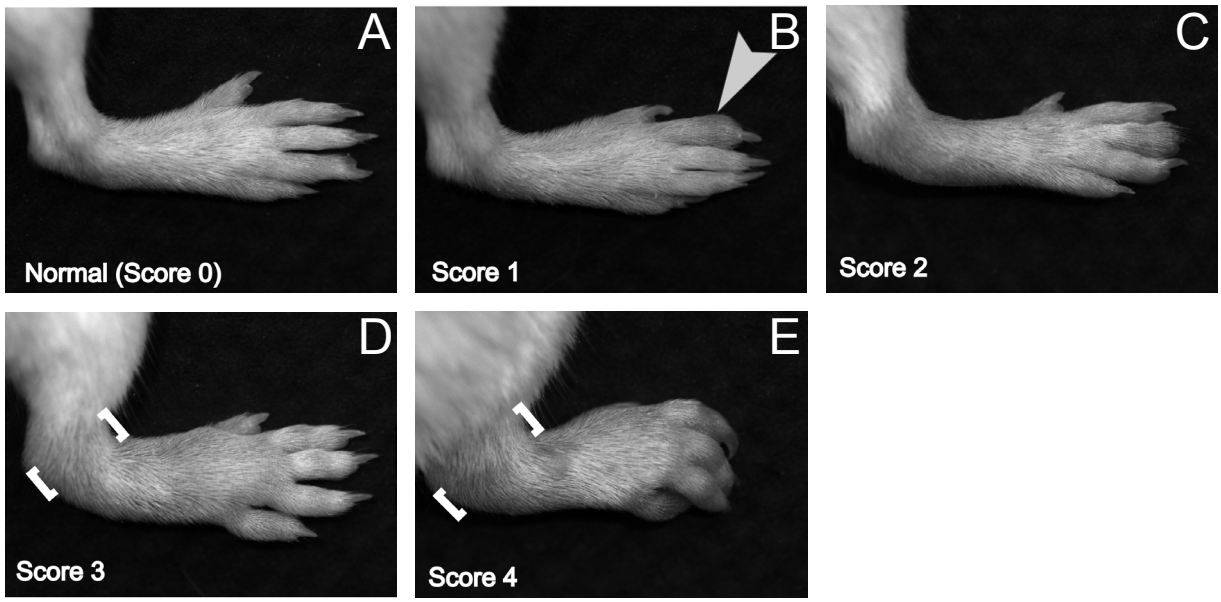

Figure 3. *Representative macroscopic images of a right hind paw before the onset of arthritis (**A**, normal) and subsequently as the disease progressed. Corresponding and representative clinical (arthritis) scores from 1 to 4 are shown in B-E. Arrowhead in **B** shows the inflamed second digit, whereas swollen digits 2-5 of the paw can be seen a few days later (**C**). White brackets in **D** and **E** indicate massive swelling of the ankle joints as well, and swollen and ankylotized fingers can be seen in E. Adapted from Glant TT and Mikecz K (138) with the approval of Humana Press (Totowa, NJ).*

6. Immune responses to proteoglycan in RA

Cartilage is one of the few immunologically privileged tissues in the body in that it is essentially avascular and therefore not subjected to close immunological surveillance. Only when degraded can its often uniquely antigenic cell fragments and matrix molecules become exposed, released and subsequently "recognized" by the immune system. Some structural changes, such as partial deglycosylation (102) or cleavage of the core protein of PG by various metalloproteinases (103,104), occur *in vivo* during the normal turnover of cartilage PG, but these processes are more extensive in inflammatory conditions. Fragments released during PG degradation may trigger and/or maintain local immune reactions in the synovial joints in arthritis-susceptible animals, and perhaps in humans as well. Indeed, immune responses to human cartilage PG have occasionally been detected in patients with RA, juvenile idiopathic arthritis (JIA), and ankylosing spondylitis (AS) (45,58,72,75) supporting the hypothesis that, among other candidate autoantigens, cartilage PG might be a target of the autoimmune inflammatory attack in arthritic joint lesions. Li *et al.* demonstrated the presence of G1 autoreactive T cells in peripheral blood (PB) and synovium of RA patients (45). Studies of Zou *et al.* showed that after antigen-specific stimulation with the G1 protein, $CD4^+$ T cells of the majority of AS and half of the RA patients secreted significant amounts of IFNγ and TNFα, which clearly suggested recognition of the G1 domain of aggrecan (59). Recently they also showed that a G1 peptide-specific $CD8^+$ T cell response is present in patients with AS and RA, but not in healthy controls (105).

The presence of PG-specific immunity/autoimmunity in RA has raised many questions as to whether the immunity is causal, a consequence of cartilage destruction or a result of a molecular mimicry. It may be a secondary event after cartilage destruction caused by other mechanisms. Nonetheless, the demonstration of such immune response in both PB and SF in RA patients is very interesting, particularly since immunity to G1 causes the induction of an inflammatory erosive polyarthritis in BALB/c mice (PGIA model) (69,84).

7. Heat shock proteins

Heat shock proteins (HSPs) constitute a group of proteins that are highly immunogenic, present in all organisms, with the potential to trigger immunoregulatory pathways (61). More specifically, immunity to HSPs can suppress immune responses that occur in various inflammatory conditions, such as in RA, JIA and related disorders (61,106-108). The synovial tissue of patients with arthritis is characterized by a chronic inflammatory process leading to an alteration of cellular homeostasis and finally to severe tissue damage. A variety of different stressors (e.g. proinflammatory cytokines) is present in the inflamed synovial tissue of RA patients, each of which has the potential to induce upregulation and unleash an HSP-specific immune response (109-111). It has been shown that HSPs are expressed in the synovial membrane in patients with RA and JIA but not in non-inflamed synovial tissue (111-113).

HSPs, also called stress-proteins, become markedly over-expressed by all cells under conditions of stress: such as increased temperature (fever); viral infection; exposure to pro-inflammatory mediators, such as TNFα and IFNγ; oxidative stress (114-117). HSPs carry out crucial housekeeping functions that are important for the survival of prokaryotic and eukaryotic cells. As molecular chaperones they interact with unfolded or partially folded protein subunits and facilitate correct folding, assembly and translocation of proteins and they protect cells from the effects of various stresses. HSPs are classified into families on the basis of their molecular weight (including the HSP10, HSP40, HSP60, HSP70, HSP90 and HSP100 families). HSPs are highly evolutionary conserved (115,118,119), with significant interspecies homologies e.g. some mammalian family members have highly conserved microbial homologues (120-122). HSPs are strongly immunogenic molecules; immune responses to bacteria are dominated by responses to HSP including the recognition of conserved bacterial epitopes giving rise to cross-recognition of self-HSP. The physiological significance of this aspect of immune behavior is possibly connected to the regulatory control of the inflammatory process, as shown in several systems described below.

Although HSPs have well described roles as chaperones for intracellular proteins, the significance of the immunoregulatory capacity of HSPs in arthritis is only now becoming clear (122-124). The first evidence for a role of HSPs as antigens in inflammatory responses was obtained in 1988 in the rat model of heat-killed-mycobacteria-induced adjuvant arthritis (125). From then on many studies have shown the potential role for immune responses to HSP in the immune regulation of arthritis, in experimental models with HSP60 (121,126-128) and first clinical trials in patients with HSP peptides (129,130). Less is known about HSP70 (antigenically unrelated to HSP60), which is one of the most conserved HSPs (118).

The HSP70 family is very large with most organisms having multiple members. Most eukaryotes have at least a dozen different HSP70 found in a variety of cellular compartments. Some of the better known mammalian members are the constitutive cytosolic member (HSC70 or HSP73), the stress-induced cytosolic form (HSP70 or HSP72), the ER form immunoglobulin binding protein (BiP or glucose-regulated protein (Grp) 78), and the mitochondrial form mHSP70 (or mito-HSP70, or Grp75) (115). Some of the cytosol-resident HSPs of the HSP70 family belong to the group of HSPs most highly induced by stress. HSP70 is highly overexpressed in the synovium of patients with arthritis (65,111,120). Interestingly, non-steroid anti-inflammatory drugs (NSAIDs) (116) and gold (131) can also induce the expression of HSP70 which might promote HSP-directed immunoregulation. Moreover, animal studies

showed that treatment with the anti-rheumatic drug OM-89, containing HSP60 and HSP70, leads to HSP60- and HSP70-specific immunity, indicating that this immunity might be crucial for its clinical efficacy (132,133).

Kingston *et al.* demonstrated that HSP70 from *Mycobacterium tuberculosis* has modulatory effects on experimental rat arthritis (134). Later Tanaka *et al.* showed that activation of T cells recognizing an epitope of Mt HSP70 can protect against adjuvant arthritis (135) and Wendling *et al.* showed that a conserved Mt HSP70 sequence prevented adjuvant arthritis upon nasal administration and induced IL-10-producing T cells that cross-reacted with the mammalian self-HSP70 homologue (136). Prakken *et al.* demonstrated that the induction of IL-10 and inhibition of experimental arthritis are specific features of HSPs that are absent for other evolutionarily conserved immunodominant proteins (137). Recently, a mammalian HSP70 protein, BiP (Grp78), reduced the severity of adjuvant arthritis and disease suppression was found to be related to the induction of regulatory T cells cross-reactive with self-HSP70 that triggered the production of IL-10 (136,137).

Recent studies showed that HSPs are critical antigens in the immune regulation of certain chronic inflammatory diseases, and are important in protection from disease (61,108). As HSPs are present at the site of inflammation and have been described as relevant targets of T cell responses, they provide promising immunomodulatory candidates for site-specific intervention of arthritis. Initial clinical trials with a DnaJP1 peptide (*E. coli* HSP40) in RA (129,130) have indicated the potential of HSPs as a source of immunomodulatory peptides. For further development of HSPs for therapy, better understanding of the immunomodulation of chronic inflammatory arthritis and the immunomodulation mechanism of HSPs in this disease is essential.

8. Outline of the thesis

The aim of this thesis is to extend our knowledge about the pathogenesis and immunomodulation of arthritis. In chapter 2 the application of dimethyldioctadecylammonium bromide (DDA), a powerful adjuvant that does not have the side effects of the conventionally used Freund's adjuvants, in the PGIA model is described. A significantly reduced onset period and a more severe arthritis was achieved in BALB/c mice immunized with cartilage PG in DDA, which makes the PGIA model much more convenient and interesting for studying the immune pathogenesis and immunoregulation. In the third chapter the anti-inflammatory role of HSP70 is further analyzed in the PGIA model. HSP70 treatment could dramatically suppress the development of inflammation and subsequent tissue damage in PGIA. Moreover, we demonstrated that HSP70 preimmunization resulted in an altered immune response as shown by proliferation and a regulatory cytokine profile to both HSP70 and PG resulting in a regulatory response. Together these data underline the therapeutic potential of HSP70 in arthritis via the induction of IL-10. In chapter 4 the generation is described of a new Tg mouse which has a PG-specific TCR. This Tg mouse will allow us to study the role of antigen-specific T cells in the development of autoimmune arthritis. The immunological features of these TCR-Tg mice are further characterized and explored in chapter 5. A single PG injection could provoke a severe form of PGIA, and splenocytes from naïve TCR-5/4E8-Tg mice, after a mild *in vivo* activation, or cells from hPG-immunized arthritic TCR-5/4E8-Tg mice could adoptively transfer arthritis into syngeneic BALB/c.SCID recipient mice. Given the relative paucity of useful models of chronic arthritis, these TCR-5/4E8-Tg mice may constitute a valuable and novel tool for studying mechanisms of autoimmune regulation of arthritis, and for developing T cell-directed immune modulating strategies. In chapter 6 the immune response to PG epitopes in RA patients is further investigated. The results indicate that some PG-specific peptides, mostly those located in the G1 domain of human PG, can induce T cell proliferation and cytokine production *in vitro*. The findings described in this thesis and their possible contribution to the understanding of the pathogenesis of RA and their implications for the design of HSP-derived therapeutic interventions are discussed in chapter 7.

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Chapter 2

Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype

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Abstract

Objective. To apply and analyze the mechanisms of action of dimethyldioctadecylammonium bromide (DDA), a powerful adjuvant that does not have the side effects of conventionally used complete Freund's adjuvants in proteoglycan-induced arthritis (PGIA) and collagen-induced arthritis (CIA).

Methods. PGIA and CIA were generated using standard immunization protocols with cartilage proteoglycan (PG) aggrecan or human type II collagen (CII) emulsified with CFA, and compared with PGIA and CIA generated using immunization protocols in which the same antigens were used in combination with the adjuvant DDA. Immune responses to immunizing and self PGs and CII, and the incidence, severity, and onset of arthritis were monitored throughout the experiments. In addition, a new, inexpensive, and powerful method of inducing arthritis using crude cartilage extracts is described.

Results. A significantly reduced onset period and a more severe arthritis were achieved in BALB/c mice immunized with cartilage PGs in DDA. PGs from bovine, ovine, and porcine cartilage, which otherwise have no effect or have only a subarthritogenic effect, and crude extracts of human osteoarthritic cartilage induced a 100% incidence with a very high arthritis score in BALB/c mice. The overall immune responses to either CII or PG were similar in antigen/CFA-immunized and antigen/DDA-immunized animals, but the Th1/Th2 balance shifted significantly toward a Th1 bias in DDA injected animals with either PGIA or CIA.

Conclusion. DDA, which was first used in autoimmune models, is a potent nonirritant adjuvant, which eliminates all undesired side effects of the Freund's adjuvants. DDA exerts a strong stimulatory effect via the activation of nonspecific (innate) immunity and forces the immune regulation toward Th1 dominance. These lines of evidence also suggest the possibility that seemingly innocuous compounds may exert an adjuvant effect in humans and may create the pathophysiologic basis of autoimmunity in susceptible individuals via the activation/stimulation of innate immunity.

Introduction

Several lines of evidence indicate that the effector mechanism that initially attacks the small joints in rheumatoid arthritis (RA) is T cell driven. As a result, an aggressive synovial pannus develops that destroys articular cartilage and bone, leading to massive ankylosis and deformities of the peripheral joints. The disease has a progressive character, with involvement of more and more joints over time. While the primary target organ is the synovial joint, there is no clear evidence that any macromolecule of cartilaginous tissues, bone, or synovium would be a preferential autoantigen. Nevertheless, the most relevant animal models of RA appear to be those induced by cartilage matrix components, such as type II collagen (CII) or proteoglycan aggrecan (PG).

Systemic immunization of genetically susceptible rodents with cartilage-specific CII (1,2) or of BALB/c or C3H mice with human cartilage PG (3-7) leads to the development of progressive polyarthritis (8). Both models use 100-150 μg of antigenic protein (either CII or cartilage PG) in Freund's complete adjuvant (CFA). Mycobacterial heat-shock proteins (HSPs) present in CFA are known to be very potent nonspecific immunostimulators, and these proteins significantly contribute to, and potentiate, the systemic T cell response (9,10). In subsequent injections, the antigen can be administered in emulsion with mineral oils (Freund's incomplete adjuvant; IFA) that do not contain mycobacterial components. Mineral oils or oil derivatives, such as pristane, can also provoke inflammatory reactions in the synovial joints of certain (susceptible) rodent strains (11-13). Due to an unknown mechanism, peripheral joints are the target organs in RA, and a number of animal models can simulate the human disease, but all require antigen challenge with adjuvant (14,15).

In an attempt to avoid the effect of bacterial HSPs in autoimmune models of arthritis, as well as CFA-induced irritation and granuloma formation with subsequent adhesions in the peritoneal cavity (15), we used a positively charged lipophilic quaternary amine (dimethyldioctadecylammonium bromide; DDA) instead of CFA. With the use of DDA as adjuvant, the time of onset of arthritis was significantly earlier and the severity was significantly greater in PG-induced arthritis (PGIA). In collagen-induced arthritis (CIA), a significantly higher incidence was achieved.

The combination of the positively charged lipophilic adjuvant DDA ($C_{38}H_{80}NBr$) with the negatively charged cartilage PG seems to be powerful and simultaneously avoids the detrimental and destructive effects of CFA. We also provide herein a simple and convenient protocol for the preparation of crude cartilage extract that can be used to induce PGIA in BALB/c mice with maximum incidence (100%) and severity. The use of crude cartilage extract with DDA does not require the purification of PG by cesium chloride gradient ultracentrifugation, and PGs from human (osteoarthritic), canine, porcine, and bovine cartilage all proved equally arthritogenic in genetically susceptible BALB/c mice.

Materials and Methods

The use of human materials was approved by the Institutional Review Board, and the use of animals for immunization and arthritis induction was approved by the Institutional Animal Care and Use Committee, of Rush University.

Isolation of antigenic components from cartilage

Crude (total) cartilage extracts. Total cartilage extracts were obtained by 4M guanidinium chloride extraction (6,16) from newborn and adult human and bovine articular cartilage, bovine nasal cartilage, ewe and swine articular cartilage, chicken sternal cartilage, cartilaginous skeletal tissue from newborn mice, and rat chondrosarcoma. These cartilage extracts were further purified by gradient centrifugation to obtain highly purified cartilage PGs or were dialyzed against water and lyophilized (hereafter called crude cartilage extract).

High-density cartilage PG. High-density cartilage PG was purified by repeated CsCl gradient ultracentrifugation under dissociative conditions (in 4M guanidinium chloride), as described in detail elsewhere (6,16). Purified cartilage PGs were deglycosylated for immunization or were left untreated. None of the purified PGs ("native PG") or the crude cartilage extracts without deglycosylation induced arthritis, and the results with deglycosylated cartilage samples are therefore presented.

Purified PGs or crude extracts of normal or osteoarthritic human cartilage were treated with chondroitinase ABC (Seikagaku America/Associates of Cape Cod, Falmouth, MA), 5 units/100 mg of PG or 5 units/1 g of crude cartilage extract in 0.1M Tris–acetate buffer, pH 7.6, for 24 h at 37°C, to deplete chondroitin sulfate side chains (3,6,16). Alternatively, we achieved the same level of chondroitin sulfate depletion by digesting samples with testicular hyaluronidase (Worthington Biochemical, Lakewood, NJ), using 5,000 units/100 mg of purified PG or 5,000 units/g of crude cartilage extract in sodium acetate buffer (pH 5.0) containing 50 mM NaCl and 50 mM Mg_2SO_4 (24 h at 37°C) (5).

PG samples or crude extracts of cartilage isolated from skeletally mature adults of different species (Table 1) were subsequently digested with endo-β-galactosidase (Seikagaku America), 0.1 unit/100 mg of PG or 0.1 unit/1 g of crude cartilage extract in sodium acetate buffer, pH 5.8, to remove residual keratan sulfate side chains present in adult (aging) cartilage PGs (5,6,16). Since the keratan sulfate and chondroitin sulfate side chains may mask a number of dominant/arthritogenic epitopes, the depletion of these glycosaminoglycan side chains is critical in order to "retrieve" dominant arthritogenic T cell epitopes of the core proteins of PG (4,5,8).

A PG/glycosaminoglycan-free crude cartilage extract was obtained by diethylaminoethyl (DEAE; Whatman, Clifton, NJ) ion-exchange chromatography, as described previously (17). The unbound fraction was retrieved at 0.15M NaCl and was further absorbed with hyaluronan-Sepharose (18) to remove glycosaminoglycan-free G1-domain fragments of PG and cartilage link protein (5). Samples were dialyzed against water and lyophilized. The absence of glycosaminoglycans in the PG-free fractions of crude extracts of human cartilage (Table 1) was confirmed by monoclonal antibodies (mAb) to chondroitin 4-sulfate (clone BE123), chondroitin 6-sulfate (clone MK302), and keratan sulfate (clone EFG11) (Chemicon, Temecula, CA). The absence of the G1 domain was confirmed by

mAb G18 (5) and the absence of the link protein by a rabbit polyclonal antibody LP1-R18 raised against a link protein-specific synthetic peptide (19). The DEAE-bound fraction was eluted with 2.0M NaCl, deglycosylated with chondroitinase ABC and endo-β-galactosidase (human adult articular cartilage PG + ABC/endo-β-galactosidase) as described above, and used as a positive control for immunization (Table 1).

Human cartilage residues remaining after guanidinium chloride extraction were washed exhaustively with cold water, equilibrated in 0.5M acetic acid, and then digested with pepsin (1 mg of enzyme/g wet weight of cartilage residue; Worthington) at 4°C as described previously (6,20,21). Type II collagen was purified by sequential and repeated precipitation with NaCl in 0.5M acetic acid at 4°C.

Animals and immunization protocols

More than 1,200 mice of different strains, ages, and both sexes were purchased from the National Cancer Institute (NCI; Frederick, MD), Charles River Laboratories (Kingston, NY, Raleigh, NY, and Portage, MI colonies), Harlan (Madison, WI), Taconic Farm (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME). Female SCID mice of BALB/c background (NCI/NCrC.B-17-scid/scid) were obtained from the NCI and maintained under germ-free conditions; these mice were used for transfer experiments.

Female and male mice (12-16 weeks of age) of different inbred strains as well as their F_1 hybrids were used to compare selected immunization protocols. In experiments in which the effects of different cartilage PGs and/or crude cartilage extracts, with or without treatment of different glycosidases, were compared (Table 1 and Fig. 1), we immunized female retired breeder BALB/c mice that had been purchased from the NCI. All mice were housed in the same room of the Comparative Research Center at Rush University. When a new experimental group was immunized, 10-15 female retired breeder BALB/c mice were also immunized with deglycosylated human cartilage PGs in DDA or CFA; these groups were used as positive/reference control groups.

Mice were injected intraperitoneally with 100 μg of deglycosylated cartilage PGs or crude extracts (both measured and normalized to PG core protein) (6) or with human cartilage CII, except where indicated otherwise. A fine cationic liposome form of DDA (micelle) was obtained by heating a 10-mg/ml DDA suspension (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS; pH 7.4) to 56-63°C for 15-20 minutes and then cooling on ice (22). Alternatively, the same effect of micelle formation can be achieved by heating the DDA in a microwave oven. This DDA micelle form was mixed with an equal volume of antigen (1 mg of CII/ml or 1 mg of PG core protein/ml of PBS), and the mixture was either shaken for 20-30 minutes at room temperature or vortexed for 15-20 seconds. DDA and antigen/DDA (CII/DDA or PG/DDA) emulsions were freshly prepared on the day of immunization and stored on ice until the time of injection. The antigen/DDA micelle emulsion (200 μl total) was injected intraperitoneally (IP) or, where indicated, subcutaneously (SC) or intradermally (ID).

Four major groups of animals were treated according to 1 of the following "standard" immunization protocols. In protocol 1 (PG/CFA), an IP injection of 100 μg of PG protein in 100 μl of PBS (pH 7.4) emulsified with 100 μl of CFA (Difco, Detroit, MI) was given on day 0. On days 21 and 42, the same dose of PG in IFA (Difco) was injected IP. In protocol 2, the same antigen dose, injection time points, and IP approach were used, but the 100 μg of PG in 100 μl

of PBS was mixed with 1 mg of DDA micelle in 100 μl of PBS. In protocol 3, 100 μg of CII was dissolved in 0.1M acetic acid, and the volume was adjusted to 1 mg of CII/ml of PBS to prepare an emulsion with an equal volume of CFA. The emulsion containing 100 μg of CII was then injected either IP or ID into the proximal tail on days 0 and 21. In protocol 4, the same dose of CII (100 μg) in 100 μl of DDA micelle (1 mg) was also used for either IP or ID immunization.

In addition to these 4 "standard" immunization protocols, we used 5 other approaches. In protocols 5 and 6, different doses (protocol 5) of PGs or crude cartilage extracts (protocol 6) were mixed with different amounts of DDA and injected IP, SC, or ID. A minimum of 0.5 mg of DDA per injection was required to achieve the maximum effect (incidence and severity) in BALB/c mice, but the onset of arthritis was faster when 1 mg of DDA micelle was used. Higher doses did not change any immune or clinical parameters of arthritis induction, and the IP immunization was the most effective for inducing arthritis. Concentrations higher than 20-25 mg/ml of DDA micelle were insoluble, and 3.5-4.0 mg of DDA in 100 μl per mouse per injection in 100 μl resulted in 15-20% mortality within 4-5 days after IP injection.

In protocol 7, we also used PG/DDA in micelle form (heated and cooled as described above), or the water-insoluble DDA was simply suspended in PBS at room temperature and mixed with PG. Although the PG/DDA suspension produced a slightly higher Th1 response and the arthritis onset was 1-3 days earlier than when PG/DDA micelle was used (data not shown), the PG/DDA suspension required constant shaking, which made the immunization more complicated and less reproducible. In protocol 8, we also used purified cartilage PG (100 μg of core protein) in emulsion with DDA micelle (1 mg) for immunization as a standard method, and we compared the results with those obtained when the PG and DDA were injected separately into different areas (e.g., DDA injected IP and PG injected SC or vice versa). IP administration of DDA appeared to be critical to achieving a maximum adjuvant effect in PGIA, and ID administration appeared to be critical in CIA. In contrast, IP injection of DDA was not an irritant, and no granuloma formation or adhesion occurred. Therefore, unless indicated otherwise, we routinely used 100 μg of PG core protein in 100 μl of PBS with 1 mg of DDA micelle in 100 μl of PBS for IP immunization of BALB/c mice purchased from NCI.

Along with these preliminary/supplemental experiments, we also immunized BALB/c mice with keyhole limpet hemocyanin, ovalbumin, bovine serum albumin (BSA), or methylated BSA (mBSA) (100 μg of antigen/injection; all from Sigma-Aldrich) in DDA (protocol 9). Arthritis was scored, and T and B cell responses and cytokine levels were measured as described below for PG/DDA-immunized animals.

Assessment of arthritis

The paws of all immunized mice were examined twice weekly until day 21, and then daily thereafter. Abnormalities due to arthritic changes of the joints were recorded. The appearance of joint swelling was recorded as the time of onset of arthritis. A standard scoring system based upon swelling and redness (range 0-4 for each paw; maximum possible score 16 per animal) was used for the assessment of disease severity (3,8,21,23). The limbs and spine of

arthritic and nonarthritic mice were dissected, fixed, decalcified, sectioned, and the tissue sections were stained with hematoxylin and eosin for histopathologic examination.

Measurement of antigen-specific T cell responses, antibodies, and cytokines

Sera were collected from immunized mice during the immunization period (once or twice each week from the retroorbital venous plexus) and at the end of the experiments. Spleen and lymph node cells were collected at the end of the experiments. PG-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (7,23,24). Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with human or mouse cartilage PGs (0.1 μg of protein/well), and the free binding sites were blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and levels of both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG (Accurate, Westbury, NY) or rat mAb to mouse IgG1 or IgG2a (Zymed, South San Francisco, CA) as secondary antibodies (23). Serum antibody levels were calculated relative to the corresponding mouse IgG isotype standards (all from Zymed) or mouse serum immunoglobulin fractions (Sigma-Aldrich) (23,24). The total serum IgG fraction was determined by a capture ELISA method (23) using mouse κ-chain-specific peroxidase-labeled rat mAb for detection (BioSource, Camarillo, CA).

Antigen-specific T cell responses were measured in quadruplicate samples of spleen cells or PG/adjuvant-primed lymph node cells (3 x 10^5 cells/well) cultured in the presence of 25 μg of PG protein/ml or 25-50 μg of CII. Antigen-specific interleukin-2 (IL-2) production was measured in 2-day-old supernatant by CTLL-2 bioassay, and T cell proliferation was assessed on day 5 by 3H-thymidine incorporation (5-7,23,24). The antigen-specific T cell response was expressed as a stimulation index (S.I.), which was calculated as the ratio of the counts per minute of 3H-thymidine incorporated in antigen-stimulated cultures relative to that in nonstimulated cultures. Production of antigen-specific interferon-γ (IFNγ), IL-4, IL-12, and tumor necrosis factor α (TNFα) was measured in cell culture supernatants (3 x 10^6 cell/ml) on day 4 using a capture ELISA method (BD Biosciences, San Diego, CA, or R&D Systems, Minneapolis, MN) as described previously (7,24). Cytokines (IFNγ, IL-1α, IL-6, IL-10, IL-12, and TNFα) in the sera of immunized animals were measured by ELISA at the end of the experiments.

Characterization of cell surface markers

The expression of cell surface markers was assayed by flow cytometry. Briefly, either 1 x 10^6 unseparated spleen cells obtained at different time points of immunization, lymph node cells from antigen-primed mice (100 μg of antigen with 100 μl of CFA or DDA micelle were injected into the footpad and cells harvested 9 days later), or cells harvested from peritoneal lavage fluid were incubated with fluorescein isothiocyanate- or phycoerythrin-labeled or biotinylated mAb to CD45/B220 (B cell marker), CD3, CD4, CD8, CD25, CD28, CD44, and CD69 (T cell and T cell activation markers), mAb to CD68 or F4/80 (monocyte/macrophage marker), mAb to Gr-1 (myeloid cell lineage marker, e.g., neutrophil leukocytes), and mAb to CD11c (activated dendritic cell marker). Antibodies were purchased from BD Biosciences, R&D Systems, or BioSource.

Cells were washed twice in PBS containing 1% BSA and 0.02% sodium azide. The cells labeled with biotinylated primary antibodies were incubated with streptavidin-R-phycoerythrin (BD Biosciences) or streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR) for an additional 30 minutes at 4°C. After 2 more washes, the cells were fixed in 2% buffered formalin, and samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson).

Cell isolation and transfer of arthritis

Single-cell suspensions were prepared from the spleens of arthritic BALB/c mice. Donor arthritic mice were immunized with cartilage PG in CFA or in DDA as described above for protocols 1 and 2. Mononuclear cells were isolated on Lympholyte M (Zymed) and used for adoptive transfer of arthritis as described previously (23). In all transfer experiments, 1.5 x 10^7 unseparated spleen cells were injected IP on day 0 with 100 μg of cartilage PG (measured as protein), and 1 x 10^7 spleen cells from arthritic donors were injected on day 7 without cartilage PG into SCID mice. There were absolutely no differences in the incidence, onset, severity, or histopathologic features of adoptively transferred arthritis in SCID mice that received donor cells from PG/CFA-immunized (23) or PG/DDA-immunized (data not shown) mice.

Statistical analysis

Statistical analysis was performed using SPSS version 7.5 software (SPSS, Chicago, IL). The Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. Both the 5% significance level and the 1% significance level were used.

Results

Incidence and severity of PGIA in arthritis-susceptible BALB/c and C3H colonies

DDA was first used in our laboratory when we studied how HSPs affect the onset and severity of PGIA (25), since we wanted to avoid the undesired effects of the bacterial HSPs present in CFA. A group that received PG with DDA (PG/DDA) IP developed more severe PGIA significantly earlier than did BALB/c mice immunized with the same dose of PG with CFA and IFA (Fig. 1).

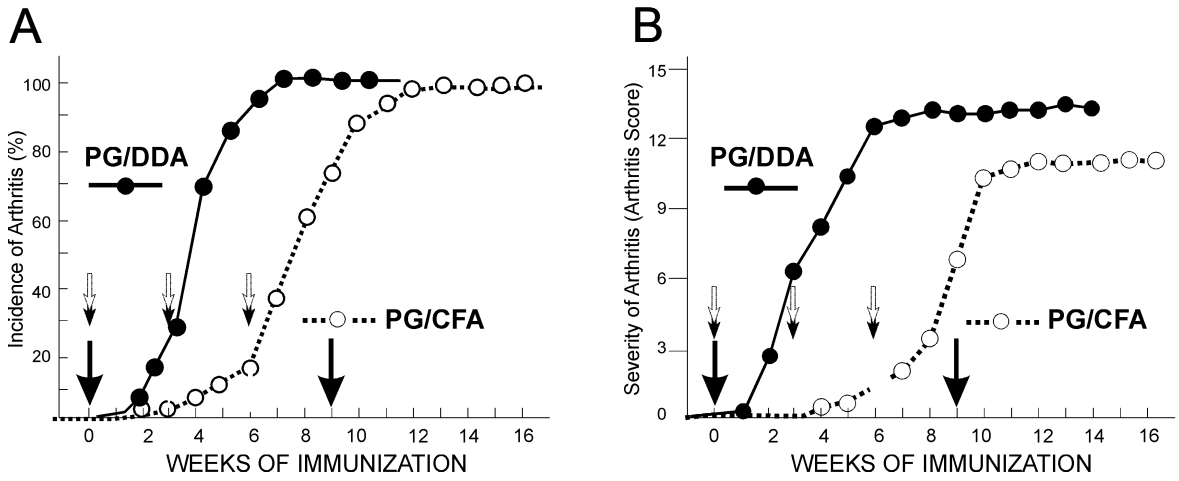

Figure 1. *Incidence and severity of proteoglycan-induced arthritis (PGIA) in BALB/c mice immunized with human cartilage proteoglycan aggrecan (PG) in Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (IFA), or dimethyldioctadecylammonium bromide (DDA). Human PG was deglycosylated of both chondroitin sulfate and keratan sulfate side chains (see Materials and Methods).* ***A****, The incidence of arthritis was assessed twice a week.* ***B****, Each paw of each animal was scored for inflammation (0-4 scale) resulting in an arthritis score for each immunized animal (range of possible scores 0-16). Arrows mark the injection times for PG/CFA (solid arrows), PG/IFA (shaded arrows), and PG/DDA (open arrows with solid arrowheads). The cumulative results of 4 independent experiments (n = 10-15 animals per experiment) are shown.*

Since PGIA can be induced only in genetically susceptible BALB/c and C3H strains, but the severity and susceptibility are different in different colonies of the same murine strain, we compared the major clinical parameters (arthritis onset, severity, and incidence) in commercially available BALB/c (6) and C3H colonies (7). While the intergroup (substrain) differences (50-100% incidence with arthritis scores of 5.1-12.4) occurred in BALB/c mice, the individual differences among BALB/c colonies disappeared by week 9 in PG/DDA-immunized mice (data not shown). The incidence was 100%, and the mean ± SD arthritis score in 9 different BALB/c colonies (n = 462) was 10.6 ± 3.6 at week 9 (Fig. 2B); this was not observed in PG/CFAimmunized BALB/c mice at this time point (Fig. 1).

In contrast to the BALB/c strain, the extreme differences among C3H colonies, for example, between C3H/HeJ and C3H/HeJCr, 2 colonies which are otherwise derived from the same founder (7), remained significant in PG/DDA-immunized mice (compare columns 4 and 5 in Fig. 2B). Only 28% of the C3H/HeJ mice (from The Jackson Laboratory) developed arthritis, and with very low arthritis scores (1.9 ± 1.2), after up to 4 antigen injections (column 5, Fig. 2B, panel 2), whereas the incidence was 100% in C3H/HeJCr mice (from the NCI), with an arthritis score of 5.7 ± 2.3 (column 4, Fig. 2B).

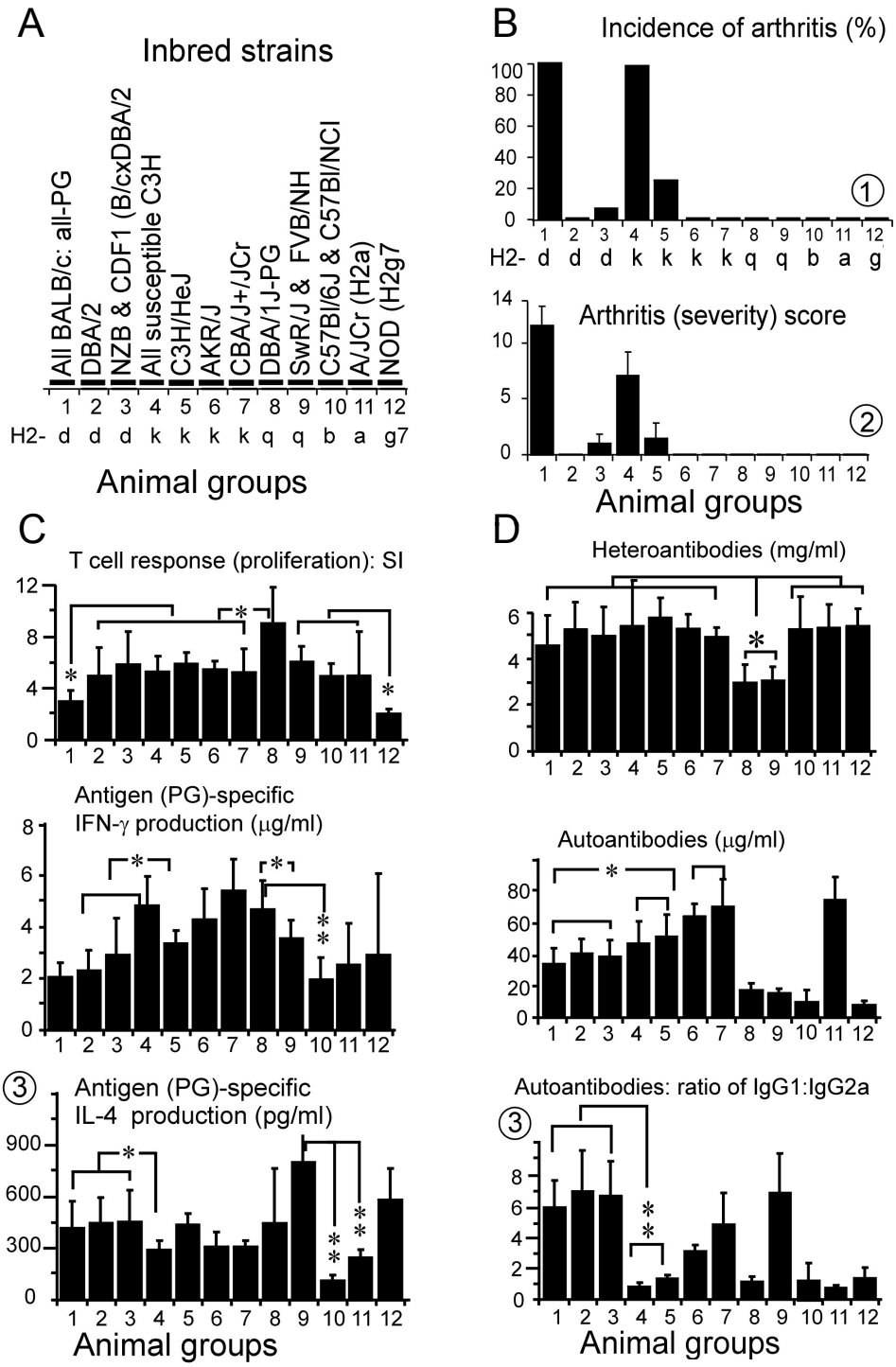

Figure 2. *Comparative analysis of 9 BALB/c substrains, 3 C3H substrains, F1 hybrids of BALB/c x DBA/2 (CDF_1) (see refs. 6, 7, and 8, respectively), and an additional 10 inbred strains with different H-2 haplotypes. Female mice were immunized intraperitoneally with deglycosylated human cartilage PG in DDA as shown in Fig. 1, except that all animals, regardless of their stage of arthritis, received 4 PG/DDA injections. All animals were killed on days 91-92 of immunization (4 weeks after the fourth antigen injection). Data from the end of the experiments in all animals, whether arthritic or nonarthritic, are shown (n = 6-16 animals per group).* ***A****, Inbred mouse strains that were studied.* ***B****, Incidence of arthritis, and arthritis scores.* ***C****, Antigenspecific T cell responses: proliferation (expressed as a stimulation index; S.I.), production of interferon-γ (IFNγ), and production of interleukin-4 (IL-4).* ***D****, Serum level of heteroantibodies, serum level of autoantibodies, and the IgG1:IgG2a ratios in PG/DDA-immunized mice. Significant differences between groups or pooled groups are indicated: * = P<0.05; ** = P<0.01. The corresponding major histocompatibility complex haplotype of each strain is shown across the bottom of A and B (panel 1). In each panel, the groups are shown in the same order as in A. Values are the mean and SD. See Fig. 1 for other definitions.*

Although all BALB/c mice and the highly susceptible C3H colonies uniformly reached 100% incidence of arthritis by weeks 9-10 in response to PG/DDA immunization (Fig. 2B, panel 1), there were significant differences in the final arthritis scores in BALB/c mice (arthritis score 11.6 ± 1.6) compared with the highly susceptible C3H colonies (arthritis score 7.2 ± 2.1) (Fig. 2B).

PGIA susceptibility in inbred murine strains

Susceptibility to PGIA is determined by both major histocompatibility complex (MHC) and non-MHC genes (8,24), and the different colonies of susceptible BALB/c (H-2^d) and C3H (H-2^k) strains exhibit variabilities in both the severity and incidence of PGIA (Fig. 2B). Therefore, the next evident question was whether other inbred murine strains with the same (H-2^d or H-2^k) or different haplotypes can develop PGIA in response to PG/DDA immunization.

While all murine strains, regardless of their H-2 haplotype or genetic background, responded well to PG/DDA immunization (Figs. 2C-D), we could not find any strain other than BALB/c and C3H that was susceptible to PGIA (Fig. 2B, panel 1). This was especially interesting because a number of murine strains carried the same class II alleles (either H-2^d or H-2^k haplotype) and exhibited similar, or occasionally even higher, T cell and B cell responses to PG immunization compared with the susceptible BALB/c or C3H colonies (Figs. 2C-D).

Arthritogenic effect of immunization with DDA and PGs isolated from various species

In previous studies, we tested cartilage PGs from various species for their ability to induce arthritis in BALB/c mice when used with Freund's adjuvants (4,8). PGs from fetal human and pig cartilage and from adult human and dog cartilage were the only ones that could induce arthritis in BALB/c mice, but only if the glycosaminoglycan (chondroitin sulfate or both chondroitin sulfate and keratin sulfate) side chains were removed (5,6). Cartilage PGs from other species either did not induce arthritis or induced only a weak and transient inflammation at a very low incidence (<10%) in BALB/c mice immunized with PG in Freund's adjuvants.

Using DDA as adjuvant, we retested a few, relatively easily accessible cartilage PGs of various species. Quite unexpectedly, PGs from fetal and adult bovine cartilage and from adult pig and sheep cartilage proved as arthritogenic as PGs isolated from newborn or adult human cartilage when they were injected with DDA. Moreover, crude cartilage extract from osteoarthritic cartilage proved to be excellent arthritogenic material if both the chondroitin sulfate and keratin sulfate side chains of PG were removed (6); it was as effective as the highly purified human cartilage PG (Table 1).

Thus, when using lipophilic adjuvant DDA for immunization, PGs with relatively poor arthritogenicity in Freund's adjuvants can induce arthritis with maximum incidence and high severity in mice of the BALB/c strain (Table 1). While cartilage macromolecules other than PG (Table 1) in crude extracts have also provoked immune responses, these molecules of PG- and link protein-free cartilage extracts were not arthritogenic in BALB/c mice (see PG-free extract of human articular cartilage, Table 1).

Table 1. Arthritogenic effect of different species of cartilage PGs on BALB/c mice and their immune responses to mouse cartilage PG*

Cartilage PG source (deglycosylation)	Day of earliest onset†	Arthritis incidence‡	Day of maximum incidence§	Cumulative severity score, mean ± SD	Proliferation, S.I., mean ± SD¶	Serum autoantibody to mouse PG, mean ± SD mg/ml	
						IgG1	IgG2a
Human newborn articular cartilage PG (ABC)	26	15/15 (100)	38	11.8 ± 3.8	3.8 ± 1.3	0.5 ± 0.1	0.1 ± 0.0
Human adult articular cartilage PG (ABC/endo-β-gal)	26	15/15 (100)	46	12.4 ± 1.5	2.7 ± 0.3	0.4 ± 0.2	0.1 ± 0.0
OA-24; human OA cartilage extract (ABC/endo-β-gal)	26	44/44 (100)	48	9.9 ± 1.7	2.4 ± 1.0	0.3 ± 0.1	0.1 ± 0.1
Human adult articular cartilage PG (ABC)	42	9/9 (100)	58	8.8 ± 3.4	2.9 ± 2.1	0.3 ± 0.2	0.1 ± 0.0
Canine articular cartilage PG (ABC)	27	15/15 (100)	47	12.8 ± 1.6	4.7 ± 3.0	0.5 ± 0.2	0.1 ± 0.0
Bovine nasal/articular cartilage PG (ABC)	47	20/20 (100)	66	8.8 ± 3.2	2.7 ± 0.1	0.1 ± 0.0	ND
Fetal bovine articular cartilage PG (ABC)	48	9/9 (100)	68	13.5 ± 2.4	2.4 ± 0.2	0.1 ± 0.0	ND
Porcine articular cartilage PG (ABC/endo-β-gal)	32	9/9 (100)	51	12.2 ± 1.8	2.8 ± 0.5	0.1 ± 0.0	0.1 ± 0.0
Porcine articular cartilage PG (ABC)	32	4/5 (80)	68	12.8 ± 2.9	2.0 ± 0.2	0.2 ± 0.0	ND
Sheep articular cartilage PG (ABC/endo-β-gal)	45	5/5 (100)	70	7.8 ± 3.0	2.1 ± 0.3	0.1 ± 0.0	ND
Chicken sternal cartilage PG (ABC)	NA	0/14 (0)	NA	NA	5.6 ± 2.2	0.1 ± 0.0	ND
Newborn mouse PG	NA	0/8 (0)	NA	NA	1.1 ± 0.1	0.2 ± 0.1	ND
Rat Swarm chondrosarcoma PG (ABC)	NA	0/12 (0)	NA	NA	1.0 ± 0.2	0.7 ± 0.2	0.1 ± 0.0
PG-free human adult articular cartilage extract	NA	0/10 (0)	NA	NA	6.4 ± 1.4	ND	ND
PBS + DDA	NA	0/22 (0)	NA	NA	0.9 ± 0.2	ND	ND
Type II collagen from human adult articular cartilage	NA	0/15 (0)	NA	NA	3.6 ± 1.4	ND	ND

** Mice were immunized with purified cartilage proteoglycan (PG) aggrecan (100 μg of PG core protein in 100 μl of phosphate buffered saline; PBS) and dimethyldioctadecylammonium bromide (DDA; 1 mg/100 μl). The OA-24 group received crude cartilage extract from osteoarthritic human cartilage (~100 μg of PG core protein). Native (nonglycosylated) cartilage PGs did not induce arthritis when used in emulsion with either of the Freund's adjuvants (4,8) or with DDA (results not shown). The type of deglycosylation required to retrieve the arthritogenic potential of a given cartilage PG sample is shown. Chondroitin sulfate side chains were removed by chondroitinase ABC (ABC) treatment; both chondroitin sulfate and keratan sulfate side chains were depleted by chondroitinase ABC and endo-β-galactosidase (ABC/endo-β-gal) treatment. Each animal received 4 injections, regardless of whether arthritis developed at any immunization time point. Mice were killed at the same time on days 91-92. Experiments were repeated 2 or 3 times (except those groups with 5 mice), and the results are summarized. Results for the bovine nasal and bovine articular cartilage are combined. NA = not applicable; ND = not detected.*

† The day when the earliest onset of arthritis (first arthritic animal) was diagnosed in the group.

‡ Number of arthritic animals/all immunized animals (%).

§ The day when all mice in the immunized group developed arthritis, except in negative (NA) groups or in the group labeled with the double asterisk.

¶ Antigen-specific T cell proliferation in the presence of the same antigen as used for immunization (50 μg protein/ml), expressed as the stimulation index (S.I.).

Histopathologic features of the peripheral joints and spine of PGIA-susceptible mice

In the mice immunized with PG in DDA, the clinical scores for the paws corresponded well to the histopathologic abnormalities in the small peripheral joints, as described for the "classic" PG/CFA-induced form of PGIA (3,4,6-8). Joint inflammation started with mononuclear (mostly lymphocyte) and polymorphonuclear infiltration, which was soon accompanied by massive cartilage degradation, followed by bone erosion (Figs. 3B-D).

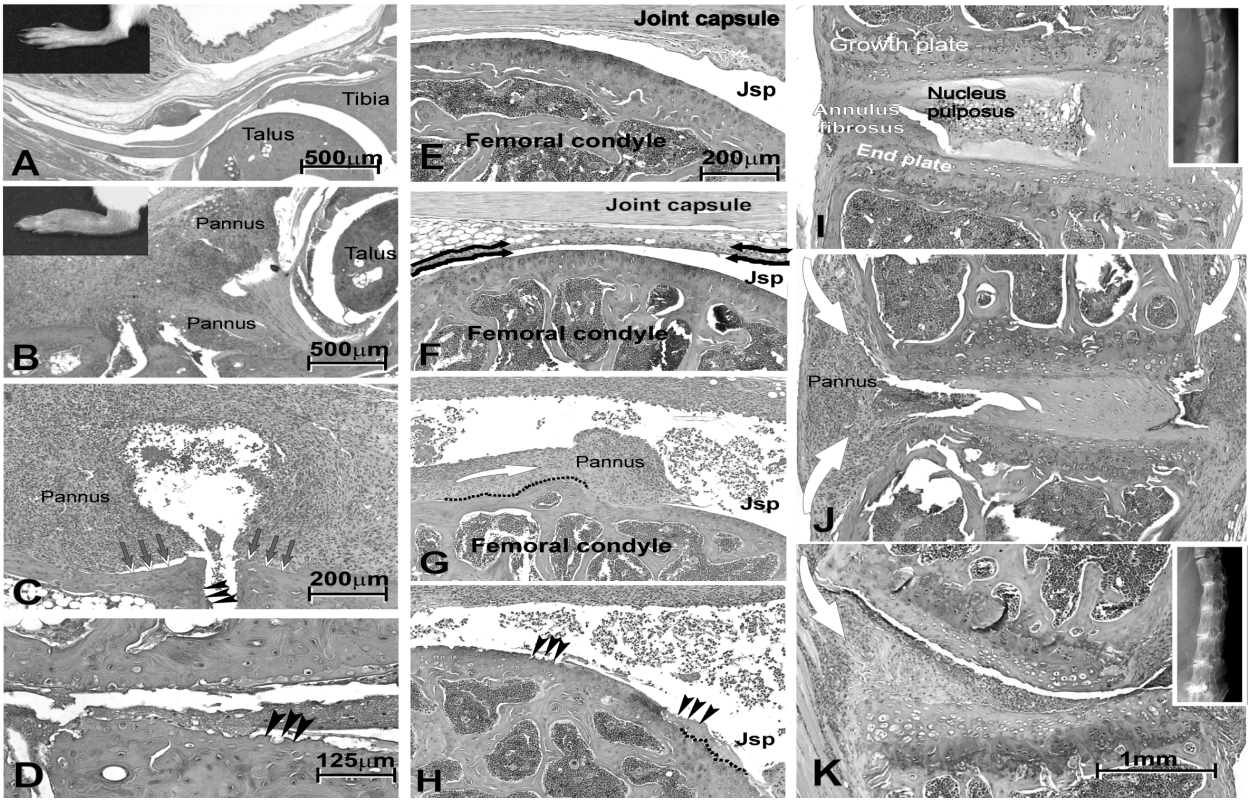

Figure 3. *Clinical appearance and histopathologic and radiographic features of PGIA in BALB/c mice immunized with PG/DDA.* ***A****, Normal ankle joint.* ***B****, Heavily inflamed ankle joint 2-3 weeks after the onset of arthritis. Insets, Photographs of the hind paws from which the respective tissues were obtained.* ***C*** *and* ***D****, Higher-magnification views of tarsometatarsal joints at 2 weeks and 4 weeks, respectively, after arthritis onset. In the acute/subacute phase of the disease, the same joint may have massive lymphocyte and neutrophilic granulocyte infiltration, as well as extensive cartilage and bone erosions (B-D). The erosions are the consequences of invasion by the aggressive pannus-like synovial tissue (pannus). Shaded arrows with white arrowheads in C indicate the borders between bone and bone-resorbing soft tissue (pannus). Arrowheads in C, D, and H indicate areas of cartilage erosion.* ***E****, Normal knee joint. Jsp = joint space.* ***F****, Knee joint obtained on the day of arthritis onset, showing very early inflammation, as indicated by thickening of the synovial lining cell layer (parallel arrows).* ***G*** *and* ***H****, Knee joints obtained 4 days and 12 days, respectively, after arthritis onset. Arthritis onset was recorded as paw inflammation. Dotted lines in G and H and arrowheads indicate cartilage erosions. Curved white arrow in G indicates proliferation of a pannus-like granulomatous tissue at the bone–cartilage junction.* ***I-K****, Representative micrographs of intervertebral discs from a normal, nonimmunized animal (I), and from mice with PGIA (J and K).* ***J****, Histologic features of a relatively acute (early) onset of spondylitis (~2-3 weeks after the onset of inflammation in the peripheral joints) with hyperproliferative pannus-like granulation tissue (curved white arrows). This aggressive, pannus-like tissue destroys the annulus fibrous and resorbs the nucleus pulposus.* ***K****, Histologic features of a completely resorbed intervertebral disk, with segments of vertebrae ankylotized by fibrotic tissue and chondrophytes, ~8-10 weeks after the onset of peripheral joint inflammation. Insets, Radiographs of the spines from which the respective tissues were obtained. All tissue sections were stained with hematoxylin and eosin. See Fig. 1 for other definitions.*

While these histopathologic abnormalities were very similar to those previously described in either BALB/c or C3H mice immunized with PG in Freund's adjuvants (3,6,7), comparison of the 2 immunization protocols revealed remarkable differences in the knee joint (Figs. 3E-H) and spine (Figs. 3I-K). For example, inflammation usually can be seen in the ipsilateral knee joint 2-3 weeks after the onset of paw inflammation (small joints) in PG/CFA-immunized BALB/c mice. In contrast, both small joints and ipsilateral large joints (Figs. 3F-G) were simultaneously inflamed in PG/DDA-immunized mice, but the periarticular inflammation (edema and cellular infiltration) was less pronounced in the large joints. A second remarkable difference was that, simultaneously with the synovial joint inflammation, massive spondylitis was detected in all arthritic BALB/c mice that had been immunized with PG/DDA

(Figs. 3J-K). This was especially unusual, because spondylitis typically only appeared 2-4 months after peripheral (synovial) joint inflammation in BALB/c mice that had been immunized with PG/Freund's adjuvants (3,6-8).

Mechanisms of action of DDA as adjuvant

As briefly summarized in Materials and Methods, a large number of preliminary and supplemental experiments (protocols 5-9) were performed to determine the optimum conditions (dose, combination, and route) of PG/DDA immunization for induction of PGIA. The overall results of T cell- and B cell-mediated immune responses to human PGs in PG/CFA- or PG/DDA-immunized BALB/c mice, however, were highly comparable, and serum levels of proinflammatory cytokines were even more pronounced in PG/CFA-immunized animals than in those injected with PG/DDA (Table 2).

Table 2. Immune responses and antigen-specific cytokine production by spleen cells after immunization of BALB/c mice with cartilage PG in CFA or in DDA*

	After first injection		After second injection		After third injection	
	PG in CFA	**PG in DDA**	**PG in CFA**	**PG in DDA**	**PG in CFA**	**PG in DDA**
T cell proliferation, S.I.	2.1 ± 0.25	4.25 ± 0.63†	3.42 ± 1.06	3.21 ± 0.95	3.49 ± 1.13	3.46 ± 1.10
Serum antibody level, mg/ml						
IgG1 to human PG	<0.02	ND	6.62 ± 3.90	3.36 ± 1.42†	10.6 ± 0.42	9.6 ± 1.52†
IgG2a to human PG	<0.02	<0.02	0.09 ± 0.04	0.06 ± 0.05	0.24 ± 0.06	0.38 ± 0.12
IgG1 to mouse PG	<0.02	0.10 ± 0.01	0.28 ± 0.18	0.06 ± 0.04†	0.65 ± 0.13	0.45 ± 0.09†
IgG2a to mouse PG	ND	ND	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.05 ± 0.00
Serum cytokine level, pg/ml						
IL-1β	28.5 ± 12.3	10.8 ± 0.11‡	20.7 ± 0.22	15.0 ± 2.60†	23.7 ± 5.60	19.7 ± 0.16†
TNFα	77.3 ± 28.4	47.2 ± 12.3‡	405 ± 55.4	239 ± 23.4‡	587 ± 44.2	441 ± 55.7†
IL-6	98.6 ± 11.3	72.9 ± 18.3‡	101 ± 34.6	86.6 ± 19.6‡	158 ± 28.2	118 ± 18.3‡
IL-10	ND	10.9 ± 0.09	10.1 ± 12.4	3.5 ± 0.11	4.2 ± 1.8	5.4 ± 0.08
Antigen-specific cytokine production by spleen cells, pg/10^8 cells/ml						
IFNγ	ND	55.0 ± 9.6	12.1 ± 4.3	55.0 ± 9.40	46.8 ± 12.1	107 ± 32.4
IL-4	ND	ND	41.2 ± 5.8	48.2 ± 13.2	25.8 ± 9.70	34.8 ± 8.60
IFNγ:IL-4 ratio	NA	NA	0.29	1.14‡	1.81	3.07‡
IL-12	ND	ND	ND	11.2 ± 2.60	ND	23.0 ± 11.3
TNFα	ND	ND	9.28 ± 0.9	1.20 ± 0.02†	10.0 ± 4.72	6.40 ± 0.91†
Ratio of lymphocytes in spleen						
$CD3^+$:$B220^+$	NE	NE	NE	NE	0.83 ± 0.12	0.81 ± 0.06
$CD4^+$:$CD8^+$	NE	NE	NE	NE	0.95 ± 0.02	1.13 ± 0.04†

** Assays were performed 9-11 days after the intraperitoneal injection. Values are the mean ± SD. PG = proteoglycan aggrecan; CFA = Freund's complete adjuvant; DDA = dimethyldioctadecylammonium bromide; S.I. = stimulation index; ND = not detected or the same amount was measured in nonstimulated and PG-stimulated spleen cell cultures; IL-1β = interleukin-1β; TNFα = tumor necrosis factor-α; IFNγ = interferon-γ; NA= not applicable; NE = not evaluated.*

† P = 0.05 versus PG in CFA-immunized group.

‡ P = 0.01 versus PG in CFA-immunized group.

Although some studies indicate that the use of DDA for vaccination provokes a more dominant delayed-type hypersensitivity reaction, accompanied by an antibody response that is higher than that obtained with the use of many other adjuvants, including CFA (26), we could not confirm those observations. The serum antibody levels to either PG or CII, or against a number of arthritis-irrelevant antigens (such as keyhole limpet hemocyanin, BSA, or ovalbumin; data not shown) were similar or even lower when injected with DDA than with CFA. In contrast, we found extensive differences in antigen-specific IgG1:IgG2a and IFNγ:IL-4 ratios when the 2 adjuvants were compared throughout the immunizations (Tables 2 and 3). Again, while the overall effects of DDA and CFA on the T cell response (measured as PG-specific T cell proliferation and IL-2 production) were highly comparable (Table 2), antigen (PG)-specific cytokine production by either PG-primed spleen cells (Table 2) or peripheral lymph node cells (data not shown) was significantly different and was clearly shifted toward Th1.

Table 3. Incidence, severity, and immune responses in DBA/1J mice immunized with human CII in CFA or DDA*

	ID injection		ID injection		IP injection	
	CII in CFA, males	CII in DDA, males	CII in CFA, females	CII in DDA, females	CII in CFA, males	CII in DDA, females
In vitro T cell proliferation in the presence of 25 μg/ml of human CII						
Stimulation index	3.4 ± 1.0	2.9 ± 0.8	2.7 ± 1.0	2.4 ± 1.6	3.1 ± 1.6	2.3 ± 1.6
Serum anti-mouse CII antibodies, μg/ml						
IgG1	407 ± 28	490 ± 133	398 ± 54	452 ± 78	426 ± 114	341 ± 222
IgG2a	48 ± 17	23 ± 14	66 ± 21	53 ± 22	19 ± 11	20 ± 9.1
Clinical symptoms						
Day of earliest onset	30	30	30	30	40	32
Maximum arthritis score	5.8 ± 3.4	8.2 ± 2.2†	5.2 ± 2.4	8.3 ± 3.2†	2.4 ± 0.6	2.4 ± 1.2
Maximum incidence, %	85	100†	90	100	25‡	25‡

** DBA/1J mice were immunized with 100 μg of human type II collagen (CII) emulsified with 100 μl of Freund's complete adjuvant (CFA) or with 100 μl of dimethyldioctadecylammonium bromide (DDA; micelle form) and injected intradermally (ID) into the proximal tail or intraperitoneally (IP). Mouse CII was used for measuring autoantibodies; human CII was used for measuring T cell proliferation. Values are the mean ± SD of 2 independent experiments (n = 20 mice per group).*
† P = 0.05 versus group injected with CII in CFA.
‡ P = 0.01 versus each of the groups injected intradermally.

To gain insight into the local mechanisms of DDA action and to understand why DDA supported PGIA more powerfully, BALB/c mice were injected IP with PBS, PBS/IFA, PBS/CFA, or PBS/DDA, with or without cartilage PG antigen, and cells obtained from a peritoneal lavage and from the spleen were harvested at 6 h, 12 h, every 24 h thereafter until day 9, on days 14 and 21 after the first injection, and 9 days after the second or third injection. As expected, the cell number was significantly higher, and reached a peak after 24-48 h, in the peritoneal lavage fluid from all adjuvant-injected groups compared with the PBS- or PG/PBS-injected groups, and these levels never returned to normal (data not shown). The cell influx contained predominantly neutrophilic granulocytes (up to 70-85%), and the ratio of the neutrophils was consistently highest in the CFA-injected groups at every time point evaluated. The cell number in peritoneal lavage fluid from DDA-injected groups was approximately two-thirds to one-half the cell number in fluid from CFA-injected animals by days 9-14 (data not shown). The cells consisted almost

exclusively of F4/80$^+$ macrophages (mean ± SD, 79 ± 11%) and CD3$^+$ T cells (7.0 ± 2.6%), and more than 60% of the CD4$^+$ cells were activated (CD4$^+$/CD69$^+$). Moreover, the ratio and the total number of CD11c$^+$ cells (activated dendritic cells) in the peritoneal lavage fluid were highest in the PG/DDA-injected group (data not shown).

Application of DDA in the induction of CIA

Induction of PGIA required IP immunization of genetically susceptible strains of mice with deglycosylated cartilage PGs, and the synergistic adjuvant effect of DDA was significantly higher than the effect of even the most powerful batch of CFA. Therefore, the question of whether DDA could be used to replace CFA in other frequently used arthritis models was evident. We obtained the same results using mBSA/DDA immunization for antigen-induced arthritis (data not shown), as described previously for mBSA/CFA immunization (18).

The most important data for CIA are summarized in Table 3. The onset of CIA was highly comparable in various groups of DBA/1 mice, but 100% incidence was reached only in CII/DDA-immunized mice after 2 antigen injections, with a significantly higher arthritis score in CII/DDA-immunized mice than in CII/CFA-immunized mice (Table 3). The route of immunization (ID versus IP), however, was a critical component of arthritis induction (Table 3), and the incidence reached only 60% even after the third IP injection of CII given either in CFA or DDA (data not shown). It is important to note here that although both murine strains (BALB/c and DBA/1) responded well to immunization with either human PG or CII, DBA/1 mice did not develop arthritis in response to PG/DDA immunization (data not shown), and BALB/c mice did not show signs of arthritis after CII/DDA (Table 1) or CII/CFA immunization (6).

Discussion

We present herein a summary of the results of our studies using a powerful adjuvant DDA for immunization and arthritis induction. DDA belongs to the group of lipophilic quaternary amines that were described more than 35 years ago as a potential adjuvant (27). DDA as an adjuvant has been used successfully and without side effects in vaccines administered to children and pregnant women (28,29) It is also widely used as a detergent in cosmetic compounds and fabric softeners (for review, see ref. 30). It is a positively charged compound (MW 631 daltons) with a monovalent counter-ion (Br), and 2 long alkyl chains that are hydrophobic (27,30). Because of the lipophilic chains, DDA is poorly soluble in water but forms a semicolloidal polycationic liposomal micelle structure at a temperature of 40°C or higher (22,30). DDA is a highly potent immunostimulator, especially with negatively charged antigens, provoking a strong delayed-type hypersensitivity (30-33). It is a powerful, nonirritant adjuvant and, via T cell stimulation, significantly enhances antigenspecific B cell activation and immunoglobulin production (26,34). A special benefit of the use of DDA in rodent models of autoimmunity is that this adjuvant forces the immunoregulation toward Th1 (35-37). DDA can enhance the adjuvant effect of other adjuvants (14) and even potentiate the arthritogenic effect of IFA (14) or CFA (38) in oil adjuvant-susceptible strains of rats. However, DDA as an adjuvant has never previously been tested in any autoimmune model.

We compared the effects of DDA with those of CFA and IFA in arthritis-susceptible murine strains to gain insight as to how the DDA could achieve an even more superior effect than the Freund's adjuvants. While the overall immune responses (antibody production and antigen-specific T cell responses) were highly comparable when human PGs in CFA or in DDA were used, suboptimal doses of cartilage PGs or PGs having only a suboptimal arthritogenic effect when injected with CFA were as effective as the human cartilage PGs in provoking PGIA. Moreover, crude extracts obtained from osteoarthritic cartilage, when appropriately deglycosylated, induced arthritis in a manner similar to that of the highly purified human cartilage PG, whereas other cartilage matrix macromolecules in the crude extract did not induce arthritis in BALB/c mice.

There are a number of inducible animal models of human autoimmune diseases, all of which require immunization of genetically susceptible strains of rodents and non-human primates with a target organ-specific antigen in adjuvant (15,39,40). Depending on the model and species, 1-4 injections of antigen are required, and at least 1 of these injections should be given with CFA as the adjuvant (15). Therefore, CFA seems to be a critical component in the induction and achievement of a high incidence and severity of autoimmune diseases in rodent models. The use of the same (auto)antigen in IFA, Alhydrogel (aluminum hydroxide gel), or synthetic adjuvants is either insufficient to induce the disease, or the incidence and severity are far below those of the antigen/CFA-induced disease. Thus, the nonspecific activation of innate immunity (macro-phages, dendritic cells) and T cells by mycobacterial components such as muramyldipeptide (peptidoglycan), HSP, and trehalosedimycolate (a glycolipid equivalent with lipopolysaccharide of Escherichia coli), in mineral oil (14,15) is a critical component in the provocation of immune reactions to self antigens in autoimmune models.

While a large number of adjuvants can be used for immunization of animals or for vaccination of humans, until now CFA has remained the most potent and powerful adjuvant in all experimental models of autoimmunity. The use of CFA, however, induces several side effects, including immune reactions to mycobacterial components, and, as a

highly irritating compound, CFA induces sterile inflammation, followed by local granulomatous tissue formation and severe adhesions, especially in the peritoneal cavity (15).

In our comparisons of the 2 adjuvants (CFA and DDA), only a few notable differences between the PG/CFA-immunized mice and the PG/DDA-immunized mice were noted. These were as follows: 1) significantly higher levels of serum proinflammatory cytokine and PG-specific IgG1, but not IgG2a, in PG/CFA-injected mice (Table 2); 2) significantly higher ratios of antigenspecific IFNγ to IL-4 and significantly lower ratios of IgG1 to IgG2a in PG/DDA-immunized mice than in PG/CFA-immunized mice; 3) the highest ratio of activated $CD3^+$,$CD44^{High}$ cells in PG/DDA-primed lymph nodes; 4) an increased ratio of $CD4^+$ to $CD8^+$ cells in the spleens of arthritic mice (Table 2) but not in PG/DDA-primed lymph nodes; and 5) a significantly increased number of $CD4^+$/$CD69^+$ cells in peritoneal lavage fluid from PG/DDA-primed than in that from PG/CFA-primed BALB/c mice. Moreover, at least a 2-4-fold increase in macrophage influx into the peritoneal cavity, accompanied by more $CD11c^+$ dendritic cells but significantly fewer polymorphonuclear cells, was characteristically observed in DDA-injected mice.

Among the adjuvants tested to date, only CFA and DDA proved to express sufficient power to provoke arthritis in PG- or CII-immunized genetically susceptible strains of mice. In terms of humoral and cellular immune responses to PG or other antigens and production of related cytokines, however, CFA is at least an equivalent, and frequently, an even more effective, adjuvant than DDA (Tables 2 and 3), and both CFA and DDA induced significantly higher immune responses and cytokine production than did PG/IFA. However, the production of antigen-specific Th1 and Th2 cytokines and the shift of the Th1/Th2 balance toward Th1 were significantly more pronounced in animals immunized with PG/DDA than in those immunized with PG/Freund's adjuvants (Table 2). As a result, the use of DDA accelerated the development of a more severe arthritis, and suboptimum doses of PG or CII antigens were able to induce inflammation via a more potent activation of the innate immunity.

A potential or critical role of innate immunity in arthritis induction is consistent with the findings of studies that used only adjuvants (nonspecific stimulators) in genetically susceptible strains of rodents (14,15). Observations from these studies of oil-, pristane-, and squalene-induced arthritis (13-15) together with our observations raise the question of whether the "adjuvants" can also play a role in, and/or contribute to, joint inflammation in genetically susceptible humans. Can immunostimulatory molecules from microbes, environmental compounds (cosmetics or laundry detergents), or endogenous "self" adjuvants (such as lipid squalene) in fact cause or contribute to joint inflammation? Since a number of potential autoantigens (type II collagen, proteoglycan aggrecan, link protein, gp-39, or glucosephosphate isomerase) can be identified in various subsets of RA patients, it may be an attractive hypothesis that a nonspecific stimulation of innate immunity might be an initial component in the disease mechanism. A defect in immune regulation, the production of cytokines/chemokines, and the possible involvement of joint-derived or joint-independent autoantigens (such as rheumatoid factor) may be only subsequent, although clearly detectable, events in an initial nonspecific activation of innate immunity. While the relevance of this hypothesis requires extensive studies, our observations using an innocuous component as adjuvant seem to support this possibility.

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Chapter 3

HSP70-specific immune responses are anti-inflammatory and inhibit proteoglycan-induced arthritis

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Abstract

Objective. Immune responses to heat shock proteins (HSPs) can be involved in the suppressive regulation of various inflammatory autoimmune diseases. The aim of this study was to explore the capacity of immune responses to HSP70 to prevent or arrest inflammatory damage in an antigen-induced arthritis model and to search for the mechanisms involved.

Methods. The anti-inflammatory effect of a single dose of microbial (mycobacterial) HSP70 in adjuvant (DDA) was investigated in proteoglycan (PG)-induced arthritis (PGIA), a progressive autoimmune murine model of rheumatoid arthritis (RA).

Results. HSP70 pretreatment significantly delayed arthritis onset and dramatically reduced severity. Joint sections of HSP70-pretreated arthritic mice showed very mild leukocyte infiltration, less reactive synovial cell proliferation, and consequently almost no cartilage damage compared to the joints of control animals. The protective effect of HSP70 was accompanied with increased HSP70-specific and PG-specific T cell proliferation, IFNγ and IL-10 production. Interestingly, not only after restimulation *in vitro* with HSP70, but also with PG enhanced IL-10 production was observed in animals that were protected by the administration of HSP70.

Conclusion. HSP70 immunization can suppress the development of inflammation and subsequent tissue damage in PGIA. Moreover, HSP70 treatment results in an altered immune response as shown by proliferation and a regulatory cytokine profile to both HSP70 and PG. Together these data demonstrate the therapeutic potential of HSP70 in arthritis via the induction of IL-10.

Introduction

Heat shock proteins (HSPs), present in all cellular organisms, both prokaryotic and eukaryotic, function as chaperones in intracellular protein folding, assembly and transport, and are classified into families on the basis of their molecular weight. HSP families are highly conserved throughout evolution (1-3), and some mammalian family members have extensive homologies with bacterial HSPs (4,5). HSPs, also called "stress-proteins", become markedly over-expressed by cells in the synovium during inflammation (4,6-8). A variety of different stressors (e.g. proinflammatory cytokines) is present in the inflamed synovial tissue of rheumatoid arthritis (RA) patients, each of which has the potential to induce HSP upregulation and unleash an HSP-specific immune response (7,9,10). HSPs are highly immunogenic and have the potential to trigger immunoregulatory pathways (11). Increasing evidence suggests that T cells which react against HSPs inhibit immune responses, serving as a feedback loop to suppress an appropriate immune response. More specifically, immunity to HSPs can suppress immune responses that occur in various inflammatory conditions, such as in RA, juvenile idiopathic arthritis (JIA) and related disorders (11-14).

Although HSPs have well described roles as chaperones for intracellular proteins, the significance of the immunoregulatory capacity of HSPs in human arthritis is only now becoming clear (15-17). Much of the evidence for the immunomodulatory capacity of HSPs in inflammatory diseases comes from analysis of experimental models (5,11,18), in vitro study's on HSP60 in patients with autoimmune diseases (14,17) and a few clinical trials in patients with HSP peptides (19,20). Less is known about HSP70, one of the most conserved HSPs (1).

Earlier, we have shown that microbial HSP70 can trigger T cells cross-reactive with self-HSP which then down-regulate adjuvant arthritis via interleukin (IL)-10 release (21,22). More recently, Corrigall et al. (16) demonstrated that, the stress-inducible immunoglobulin binding protein, BiP (member of the HSP70 family) could trigger the production of anti-inflammatory cytokines in RA peripheral blood mononuclear cells (PBMCs). An increased response to BiP was observed in synovial joints of RA patients, indicating attempts of the immune system to regulate the ongoing inflammation (23). Overall, proinflammatory cytokines do promote HSP upregulation (7,9), and such upregulated HSP may promote a counterregulatory, suppressive immune response in PBMCs or synovial fluid derived mononuclear cells. As non-steroid anti-inflammatory drugs (NSAIDs) (24) and gold (25) can also induce the expression of HSP70, these compounds might have added value in promoting HSP-directed immunoregulation. Thus, HSP70 may be a promising target for immunoregulation and may offer opportunities for novel therapies for chronic inflammatory arthritis.

The aim of this study was to further analyze the anti-inflammatory mechanism of HSP70-induced immune regulation in arthritis. We investigated the protective effects of HSP70 in proteoglycan-induced arthritis (PGIA), a progressive T cell-dependent, antibody-mediated autoimmune murine model of RA (26). The progressive character and high incidence of PGIA make this model optimal for testing immunomodulatory agents (26,27). Moreover, in this model, we can induce autoimmune inflammatory arthritis using human cartilage PG (hPG) mixed with synthetic adjuvant dimethyldioctadecylammonium bromide (DDA) instead of complete Freund's adjuvant (CFA) (27). Therefore, we can exclude the role of interfering immune responses induced by microbial HSPs present in CFA.

In the present study we show that HSP70 pretreatment dramatically suppresses the development of inflammation and subsequent tissue damage in PGIA. Moreover, we demonstrate that HSP70 pretreatment results in a regulatory immune response not only to HSP70 but also to hPG.

Materials and Methods

Antigens, animals and immunization

The use of human cartilage from joint replacement surgeries for antigen isolation was approved by the Institutional Review Board of the Rush University Medical Center (Chicago, USA) and the medical ethical regulations of the University Medical Center Utrecht (Utrecht, the Netherlands). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Rush University Medical Center (Chicago, USA) and by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands).

Female BALB/c mice at the age of 16-26 weeks (Charles River, Sulzfeld, Germany) were injected intraperitoneally (IP) with recombinant HSP70 of *Mycobacterium tuberculosis* (Mt) (100 μl of 1 mg/ml; obtained from LIONEX Diagnostics & Therapeutics GmbH, Braunschweig, Germany), or with recombinant enhanced green fluorescent protein (EGFP) (100 μl of 1 mg/ml; control protein produced in *E.coli* in a similar way as HSP70) both mixed 1:1 v/v with the synthetic adjuvant DDA (Sigma, Zwijndrecht, the Netherlands; 20 mg/ml emulsified in phosphate buffered saline (PBS)) or with PBS. HSP70, EGFP or PBS were injected IP 10 days prior to the first immunization for arthritis (day -10). Arthritis was induced with human cartilage proteoglycan (hPG) using a standard immunization protocol as described (27,28). Briefly, the antigen injection (100 μg hPG protein in 100 μl PBS) was given IP with 2 mg of DDA (in 100 μl PBS) on days 0 and 21. Mice were sacrificed on day 35 or in separate experiments on day 63.

Assessment of arthritis

Paws of mice were examined 3 times a week to record arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws as described (27,28). In brief, the degree of joint swelling for each paw (scored from 0 to 4) was used to express a total arthritis score, with a possible maximum severity index of 16 per animal. The first clinical appearance of swelling was recorded as the onset of arthritis (26,28). As above, mice were sacrificed on day 35 following the first hPG/DDA immunization, joints fixed in 10% buffered formalin, decalcified in 0.33 M neutralized EDTA, embedded in paraffin and 5 μm sagittal sections were stained with hematoxylin and eosin.

Measurement of antigen-specific T cell responses

Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates (Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands) at 2 x 10^5 cells per well, in the presence or absence of HSP70 (10 μg/ml), hPG (10 μg/ml) or ovalbumin (OVA; 10 μg/ml). Concanavalin A (ConA; 2 μg/ml) was used as a positive control for T cell proliferation. Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS (Bodinco B.V., Alkmaar, the Netherlands), 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol was used as culture medium. After 96 h, the cells were pulsed overnight with [^{3}H]-thymidine (0.4 μCi per well; Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands), harvested and uptake was measured by liquid scintillation counting (Microbeta, Perkin-Elmer Inc., Boston, MA). The magnitude of the

proliferative response was expressed as delta counts per minute (Δ cpm) calculated by subtracting the cpm of non-stimulated from cpm of stimulated cultures.

In separate experiments for *in vitro* epitope mapping, the responses to the 123 overlapping 15-mer peptides (20 μg/ml), covering HSP70 of *Mycobacterium tuberculosis* (21) were measured in triplicates using the same culturing condition, but using pooled lymph node cells of major draining lymph nodes obtained 12 days after IP immunization with HSP70 in DDA only. The peptides were synthesized via automated simultaneous multiple peptide synthesis developed with a standard autosampler (Gilson 221, Gilson Medical Electronics SA, Villiers le Bel, France) as described previously (29). Briefly, standard Fmoc chemistry with in situ PyBop/NMM activation of the amino acids in a 5-fold molar excess with respect to 2 μmol reactive equivalents per peptide on the PAL-PEG-PS resin (Perseptive Biosystems, Foster City, CA, USA) was employed. Peptides were obtained as C-terminal amides after cleavage with 90-95% trifluoroacetic acid/scavenger cocktails. Peptides were analysed by reversed-phase high-performance liquid chromatography and checked via electrospray ionisation mass spectrometry (LCQ; Thermoquest, Breda, The Netherlands). Purity of the peptides ranged between 50 to 90 %. Sequences of predominantly recognized peptides are shown in Table 1.

Cytokine measurement by multiplex analysis

Supernatants of antigen stimulated spleen cell cultures were collected for cytokine assays after 72 h and analyzed for IL-10 and interferon-gamma (IFNγ) simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA). The LINCOplex assay was performed according to the manufacturer's instructions (Linco Research, Inc., St. Charles, Missouri). In brief, the antibody coated microspheres were incubated with standards, controls, and samples (25 μl) in a 96-well microtiter filter plate overnight at 4ºC. Plates were washed and the mixture of biotinylated IL-10 and IFNγ detection antibodies (Lincoplex) was added for 30 min at room temperature. After repeated washing, streptavidin-phycoerythrin (PE) (Lincoplex) was added for an additional 30 min. Beads were exhaustively washed, resuspended and fluorescence intensity was read on the Luminex model 100 instrument. The concentrations of IL-10 and IFNγ in supernatants were calculated using LMAT software (Luminex Corporation, Austin, TX).

Table 1. Alignment of Mt HSP70 epitope sequences and homologous mouse HSP70 sequences.

HSP70 epitope	Amino Acid Sequence	Mean Δ cpm ± SEM
P5-6 (22-40)†	GDPVVVANSEGSRTTPSIV	4312 ± 2248 / 3727 ± 2475
	|. |.||.||||.|	
Mouse GRP75	KQAKVLENAEGARTTPSVV	
P10-11 (46-65)	GEVLVGQPAKNQAVTNVDRT	3903 ± 1620 / 4831 ± 2142
	|| ||| ||| |||| . |	
Mouse GRP75	GERLVGMPAKRQAVTNPNNT	
P25-26 (121-140)	YFNDAQRQATKDAGQIAGLN	5518 ± 983 / 4832 ± 4159
	||||||||||||| ||||	
Mouse BiP (GRP78)	YFNDAQRQATKDAGTIAGLN	
P90 (445-459)	QIEVTFDIDANGIVH	4167 ± 1798
	||||||||||||||	
Mouse GRP75	QIEVTFDIDANGIVH	
P94-95 (464-484)	DKGTGKENTIRIQEGSGLSKE	4539 ± 3666 / 4553 ± 2675
	||||.| | || ||||.	
Mouse GRP75	DKGTGREQQIVIQSSGGLSKD	
P107-108 (530-549)‡	AEGGSKVPEDTLNKVDAAVA	4409 ± 3449 / 5197 ± 3839
P118 (585-599)‡	AQAASQATGAAHPGG	4242 ± 2367
P120 (596-610)‡	HPGGEPGGAHPGSAD	5250 ± 2048

Alignment of Mt HSP70 (Swissprot. P0A5B9) sequences covering the predominately recognized peptides with the homologous sequences of the mouse HSP70 family members having the highest number of identical residues. Identical amino acids in both sequences are interconnected by lines, and conserved substitutions are interconnected by dots. HSP70 family members GRP75 (Swissprot P38647) and BiP/GRP78 (P20029) showed the highest degree of homology with the peptides recognized by Mt HSP70 immunized mice. Major epitopes and flanking peptides which also induced responses are shown. All other peptides were negative, e.g. had a mean Δ cpm of less than 3600, and data are not shown. The mean background values (responses without antigen) were 7260 ± 2242. † Within brackets residue numbers of Mt HSP70. ‡ No significant homologies were found.

Cytokine measurement by flow cytometry

Spleen cell suspensions of HSP70-pretreated and PBS control mice were cultured in the presence of HSP70 or hPG for 72 h to which for the last 4 h Brefeldin A (Sigma) was added. Cells were washed in PBS with 0.5% BSA and 0.01% sodium azide (FACS buffer) and stained with allophycocyanin (APC)-conjugated anti-CD4 or control mAbs (BD Biosciences Pharmingen, San Diego, CA) for 30 min on ice. Subsequently, cells were fixed (Cytofix/Cytoperm; BD Biosciences Pharmingen, San Diego, CA), permeabilized (Perm/Wash; BD Biosciences Pharmingen, San Diego, CA) and stained with PE-labeled anti-IL-10 and FITC-labeled anti-IFNγ for 30 min on ice. Cells were analyzed using a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences Immunocytometry, San Jose, CA).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the arthritis score and disease incidence were carried out using the Mann-Whitney U test (two-tailed) using Prism software (version 3.00, Graphpad Software Inc., San Diego). The Student's t-test was used for the analysis of antigen-specific proliferation. Significance level was set at ($p < 0.05$).

Results

Disease inhibitory effect of HSP70 on PGIA

The potential for HSP70 to ameliorate PGIA was examined by pretreatment of mice with microbial HSP70 in synthetic adjuvant DDA. Mice were injected with 100 μg HSP70 (group C) 10 days prior to the first hPG immunization (day -10), whereas control groups received EGFP (group B) or PBS (group A). On day 0 and 21, all mice were immunized with hPG in DDA to induce arthritis (Fig. 1A). HSP70 injected mice showed a 2-3-week delayed onset of arthritis and lower incidence compared with EGFP- or PBS-pretreated mice (Fig. 1B). Mean time of onset in HSP70-pretreated animals was at day 52 ± 3.2 compared with day 31 ± 2.0 in EGFP and 30 ± 1.0 in PBS pretreated mice ($p<0.01$). Furthermore, HSP70 significantly reduced severity of PGIA with a maximum arthritis score of 1.1 ± 0.3 compared with a score 4.6 ± 1.6 in the EGFP and 4.4 ± 1.2 in the PBS control mice; $p<0.05$) (Fig. 1C).

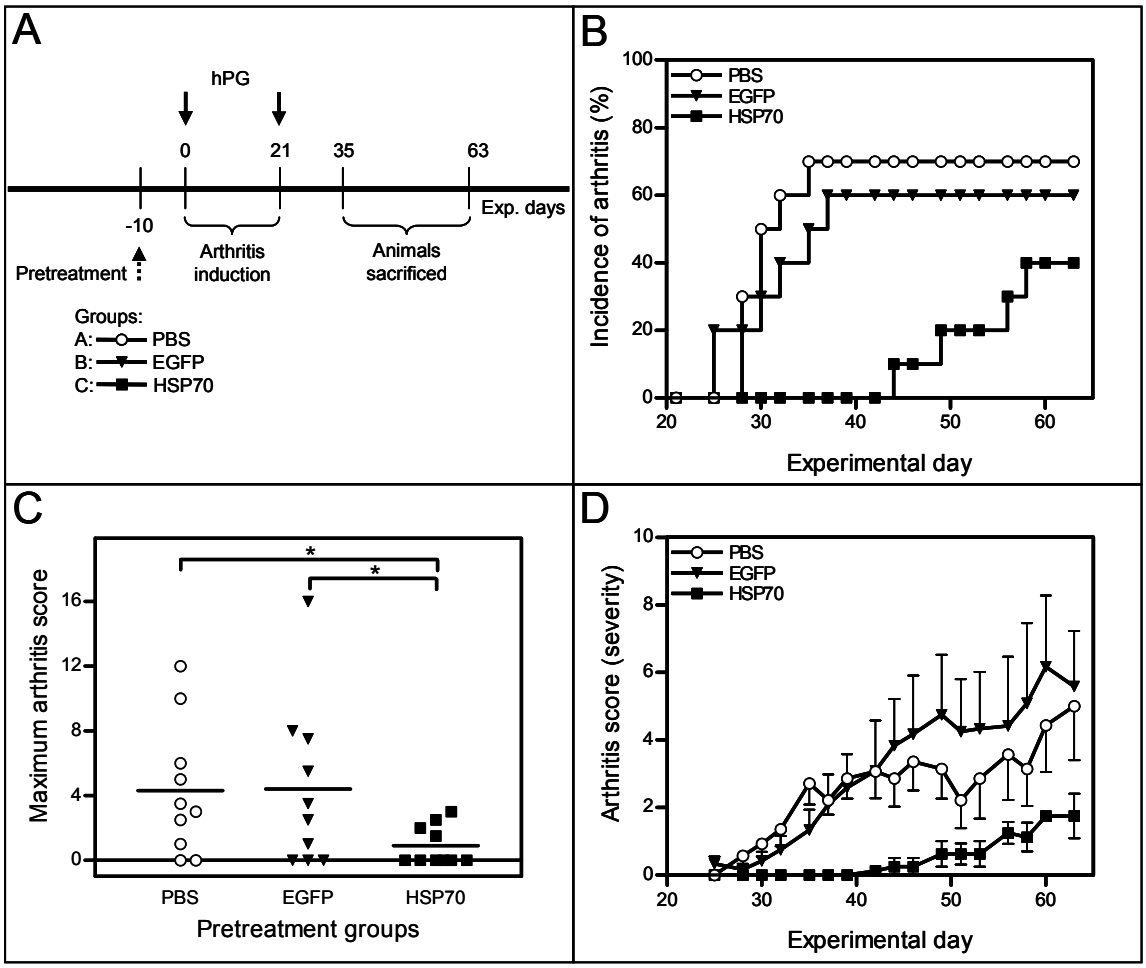

Figure 1. *Schematics of the experimental groups and immunization (**A**), incidence (**B**) and severity (**C-D**) of hPG immunized BALB/c mice. Mice were injected intraperitonally with either 100 μg of recombinant microbial HSP70 (group C) or 100 μg EGFP (group B) both emulsified in synthetic adjuvant DDA or PBS (group A) 10 days prior to the induction of arthritis. Arthritis was induced by two immunizations of hPG in DDA on days 0 and 21 and animals as described in methods. A significantly lower incidence (B) and less severe PGIA (C-D) were found in mice pretreated with HSP70. Incidence of arthritis was expressed as the cumulative percentage of arthritic animals (n = 10 in each group). The severity of the disease is shown as the maximum (highest) score per mouse at any time during disease (C; horizontal lines indicate the mean, significant differences, $p<0.05$ Student's t-test, are indicated by asterisks) and as the mean arthritis score of arthritic animals only (D). Results are presented as the mean ± SEM, and are representative for 3 independent experiments.*

The suppression of arthritis was complete (Fig. 1B and D) until day 42; thereafter some mild clinical symptoms occurred in a few HSP70-pretreated animals (Fig. 1D). In association with low severity of arthritis, joint sections of HSP70-pretreated arthritic mice showed very mild leukocyte infiltration, less reactive synovial cell proliferation, and consequently almost no cartilage damage compared to the joints of control animals (Fig. 2).

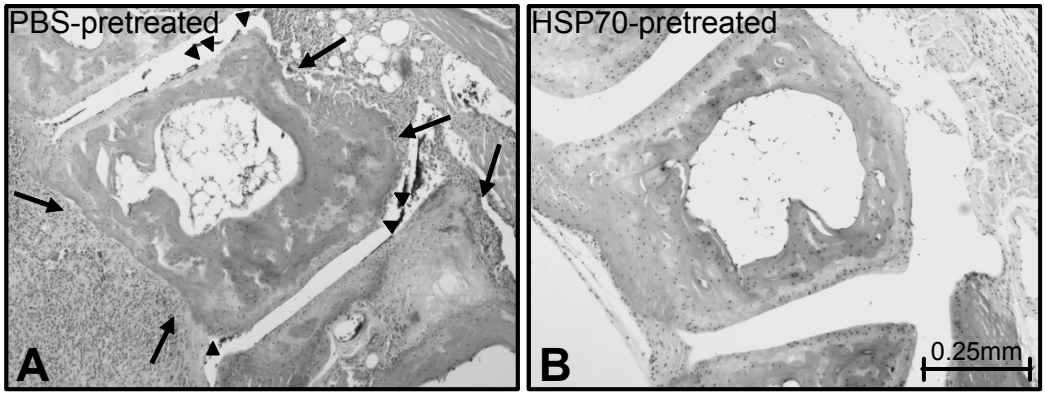

Figure 2. *Histology of tarso-metatarsal joints of BALB/c mice pretreated with PBS (**A**) or with recombinant HSP70 (**B**). Mice received PBS or HSP70/DDA treatment 10 days prior to the immunization with hPG/DDA to induce arthritis. These animals were sacrificed on day 35 after the first hPG/DDA injection for histology. Massive synovial hyperplasia with infiltrating leukocytes, and cartilage (arrow heads) and bone erosions (arrows) can be seen in mice that received only PBS. Synovium of joint sections of HSP70-pretreated mice showed only a very few leukocytes, and no synovial cell proliferation or cartilage destruction can be seen. Sections were stained with hematoxylin and eosin.*

Increased proliferative responses after immunization with HSP70

To analyze the T cell response induced by administration of HSP70 we measured antigen-specific T cell proliferation in the HSP70-pretreated mice (Fig. 1, group C) and the PBS pretreated controls (disease control; Fig. 1, group A). For this purpose, splenocytes were harvested on day 35 and stimulated with ConA, HSP70, hPG or OVA. HSP70 treated animals showed a significantly enhanced response to HSP70, compared to the control group (Fig. 3). Remarkably, in the HSP70 group also a significant increased proliferative response was seen against the arthritis inducing hPG. This increased response was not seen for control antigen OVA or ConA.

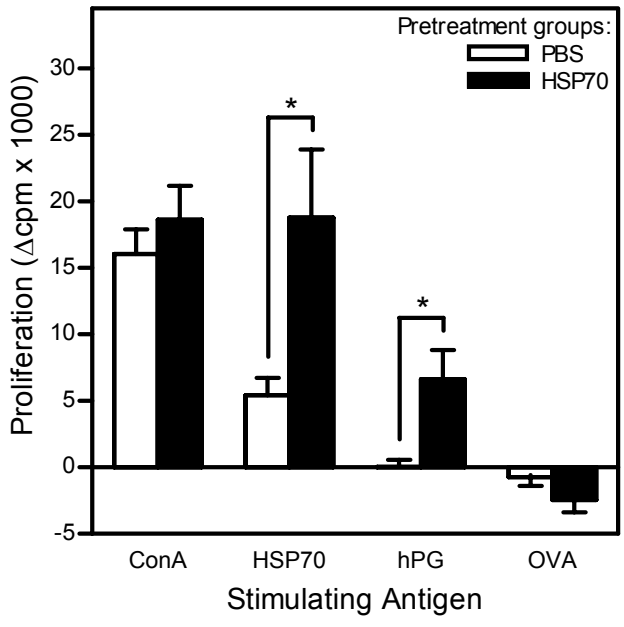

Figure 3. *Antigen-specific proliferation in hPG-immunized BALB/c pretreated with HSP70 or PBS 10 days prior to immunization. Immunization protocol is shown in Figure 1A. Mice for in vitro tests were sacrificed on day 35 (n = 8 in each group). Spleen cells (2x10^5/well) were cultured for 96 hr in the presence of ConA (2 μg/ml), recombinant microbial HSP70 (10 μg/ml), hPG (10 μg/ml) and OVA (10 μg/ml). Proliferation was measured and results are expressed as Δ cpm ± SEM. The mean background values (responses without antigen) were for the PBS control group 8390 ± 2135 and for the HSP70 group 10180 ± 2741. Significant differences (p<0.05; Student's t-test) are indicated by asterisks.*

Antigen-induced cytokine production after immunization with HSP70

Next, we analyzed the antigen-induced production of IL-10 and IFNγ of spleen cells (day 35) from HSP70-pretreated mice and PBS pretreated controls after *in vitro* stimulation with HSP70 or hPG. After 72h of culture, supernatants were collected and the IL-10 and IFNγ secretion was determined using multiplex analysis. Interestingly, not only HSP70-stimulated spleen cells but also cells stimulated with the arthritis inducing hPG were found to produce IL-10 in HSP70-pretreated animals, whereas the IL-10 level in PBS mice was significantly lower (Fig. 4A). Also the amount of INFγ produced by HSP70- and hPG-specific cells was significantly increased in the HSP70-pretreated mice (Fig. 4B). Consistent with the supernatant analysis, intra-cellular staining after 72 h *in vitro* stimulation with HSP70 or hPG, showed that slightly more IL-10 producing cells were present in HSP70-pretreated mice than in PBS-pretreated mice, not only after stimulation with HSP70 but also after hPG stimulation (Fig. 4C). This increased IL-10 expression was especially seen in the $CD4^-$ cell population (1.4%). Also the number of IFNγ producing cells was increased in HSP70-pretreated mice compared to the PBS mice, but this was mainly in the $CD4^+$ cell population (Fig. 4D).

Mapping of the T cell epitopes within HSP70

For the identification of T cell epitopes in *Mycobacterium tuberculosis* HSP70, a panel of 123 overlapping peptides (15 amino acids long) covering the complete sequence of HSP70 was tested for the ability to stimulate splenic (data not shown) and lymph node T cells from HSP70/DDA immunized mice harvested 12 days after immunization. T cells of HSP70 immunized mice predominantly recognized the peptides p5-6, p10-11, p25-26, p90, p94-95, p107-108, p118 and p120 of HSP70 (Table 1). Besides responses to the Mt HSP70-specific peptides (p107-108, p118 and p120) for which no homology with a mouse HSP70 was found, most of the predominantly recognized Mt HSP70 peptides were located especially in regions with a high degree of identity between Mt HSP70 and mouse HSP70 family members (Table 1). Interestingly, in the case of peptide p25 or p26 and peptide p90, 14 and 15 amino acids respectively, in the 15-mer peptide were identical with the mouse HSP70 homologue.

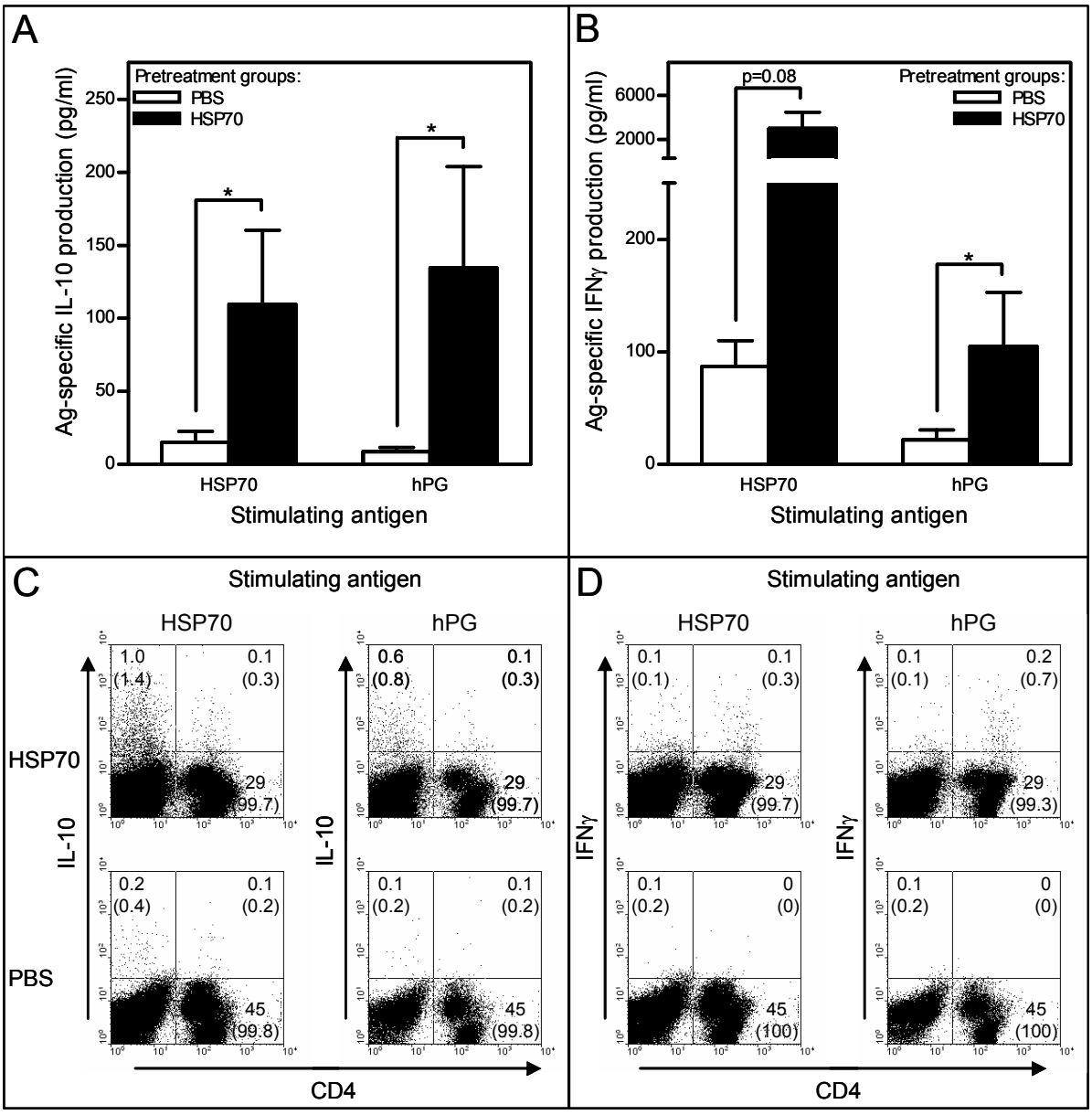

Figure 4. *In vitro cytokine secretion in antigen (HSP70 and hPG)-stimulated spleen cell cultures (**A-B**) and corresponding results of intracellulair cytokine expression (IL-10 and IFNγ). Mice received PBS or recombinant HSP70 pretreatment 10 days prior to hPG/DDA immunizations and were sacrificed on experimental day 35 (as shown in Fig. 1A). Spleen cells were cultured in the presence of HSP70 or hPG and 72-h supernatants were harvested and assayed by multiplex analysis for IL-10 and IFNγ. Values represent the mean ± SEM of cytokine levels (n=8 mice in each group). Significant difference ($p<0.05$, Mann-Whitney U Test) is indicated by the asterisk. For flow cytometry (**C-D**) viable lymphocytes were gated, and IL-10 and IFNγ expressions of $CD4^-$ and $CD4^+$ cells are shown. The percentage of cells in each quadrant is indicated, and the numbers within brackets the percentage of $CD4^-$ and $CD4^+$ cells. Results of a representative experiment are shown.*

Discussion

Microbial HSPs are immunologically dominant antigens, despite the high degrees of sequence identity with mammalian (self)-HSP homologues (30). Recent studies showed that HSPs are critical antigens in the immune regulation of certain chronic inflammatory diseases, and are important in protection from disease (11,31). Initial clinical trials with a peptide of HSP60 in patients with type 1 diabetes (20) and with peptide DnaJp1 (*E. coli* HSP40) in RA (19,32) have indicated that HSPs contain important immunomodulatory epitopes which may be used for immunotherapy.

For further development of HSPs for therapy, it is essential to understand how and which immunomodulatory epitopes may affect chronic inflammatory arthritis. HSP70 might be a candidate antigen for immunoregulation for several reasons. It is a conserved immunogenic protein with the capacity to induce a self-HSP cross-reactive immune response, it is highly induced by stress; it becomes upregulated in the synovium of arthritis patients and it is an immune target in RA (4,7,16,23). While the mechanism of immunoregulation is not exactly known, we propose that increased exposure to microbial HSP70 (in this case by immunization) leads to the activation of self-HSP70-reactive T cells that exert immunoregulatory activities towards self-HSP70-expressing inflamed synovial tissues (11,33).

RA is the result of a complex immunological process that involves both adaptive and innate immunity (34,35). PGIA is a T cell-dependent, antibody-supported chronic autoimmune murine model of RA (26), and can be induced without the use of CFA (27). In this study we demonstrated that HSP70 pretreatment resulted in a significant delay of the arthritis onset and dramatically reduced disease severity both clinically and histologically. *In vitro* tests showed increased proliferation to HSP70 in the HSP70-pretreated animals compared to the control group, indicating that the immunization procedure had been successful. Unexpectedly, in the HSP70-pretreated animals also an increased proliferative response was seen against the arthritis inducing hPG. HSP70 might have interacted with antigen presenting cells resulting in enhanced antigen priming to the subsequent hPG immunization. Srivastava and colleagues discovered that HSP70 potently stimulated Ag-specific T cell responses (36). Microbial HSP70 has been shown to exert a potent adjuvant effect in models of infection and solid tumors (37). In contrast to other studies in which arthritis is induced with avridine or CFA (22,38,39), this is the first time that in a (self)-antigen-induced arthritis model the anti-inflammatory capacity of HSP70 was studied. For this reason the increased proliferative response to the disease inciting antigen has not been noted so far and needs to be analyzed further. Especially that such increased proliferation can coincide with suppression of disease and production of IL-10 (see hereunder) needs further attention.

Pretreatment with HSP70 was seen to lead to production of IL-10 and IFNγ in spleen cells upon *in vitro* restimulation with HSP70. Interestingly, IL-10 and IFNγ were also found after restimulation with hPG in animals that were protected by the administration of HSP70. Similar observations have been made for HSP60 in JIA patients with a remitting form of disease. In these patients responses to HSP60 are consistent with a benign clinical course of this subgroup (14). These HSP60-specific T cells in oligoarticular JIA patients have the phenotype of human Tr1 cells (simultaneously producing IFNγ and IL-10) (40). It was shown that Tr1 clones can suppress the immune responses of other T cells in vitro and in vivo, including inhibiting the development of chronic inflammation and Th1-mediated

autoimmune diseases (41,42). Interestingly, also in human clinical infections, there are first reports of the presence of Tr1-like IL-10 and IFNγ double-producing T cells functioning as regulators of the antiparasite response while preventing hyperinflammation (43). Alternatively, IFNγ is a prototype Th1 proinflammatory cytokine (44). Therefore, the ratio of IL-10 to IFNγ can be seen as a measure for the balance of T-regulatory and Th1 cells. We recorded a raised IL-10 to IFNγ ratio against hPG in HSP70-pretreated mice (ratio in control mice 0.4; ratio in HSP70-pretreated mice 1.3). This suggests that the anti-inflammatory response induced by HSP70 in arthritis was IL-10 mediated. It is possible that HSP70-specific cells as well as hPG reactive cells, displaying a regulatory phenotype, influenced neighboring harmful autoreactive T cells, either directly or indirectly through cytokines; the locally produced anti-inflammatory cytokines may have modified effector T cell cytokine secretion, and may have switched the cytokine profile of hPG-specific cells into a regulatory IL-10 producing phenotype. The possible role of IL-10 in PGIA was indicated by the findings that in IL-$10^{-/-}$ mice the development of PGIA was dramatically more severe as compared to wild-type BALB/c mice (26). Moreover, Guichelaar et al. showed suppression of PGIA, upon *in vivo* transfer, of IL-10 producing hPG-specific T cells (in preparation). Thus, in PGIA IL-10 seems to be a prominent factor in the regulation of the disease and the protective HSP70 pretreatment is also likely to depend on IL-10 production.

In patients with RA, IL-10 is produced by synovial lining cells and high levels of IL-10 can be found in the synovial fluid (45,46). It has been shown that blocking of IL-10 in synovial membrane cultures from RA patients markedly increased the levels of IL-1 and TNFα, suggesting an inhibitory effect of IL-10 on pro-inflammatory cytokines in RA joints (45). It was observed that IL-10 stimulated PG synthesis and reversed cartilage degradation induced by activated mononuclear cells (47). IL-10 also correlated with diminished progression of joint destruction in RA (48). Moreover, IL-10 production has been described as being the most prominent characteristic of a subset of regulatory T cells (Tr1 cells) generated in the presence of IL-10 (41). Also in humans, the importance of IL-10 for the *in vivo* function of regulatory T cells has now positioned IL-10 as a crucial cytokine in the control of immune responses (42). As self-HSP70 is upregulated in joints of RA patients (7,23), the microbial HSP70 activated cells are possibly targeted to the joints on the basis of cross-reactivity with self-HSP70 (11). Increased and targeted HSP-specific immune responses might contribute to disease remission and restore a local balance between protective regulator and disease producing effector cells. This would be in line with what was noted earlier by Kimura et al. (49) that T cells that recognize a microbial HSP70 epitope play a regulatory role in inflammation during Listeria infection via the production of suppressive cytokines including IL-10. Furthermore, Detanico et al. (50) have recently observed that synovia-derived monocytes produce IL-10 in the presence of mycobacterial HSP70. Our present findings confirm previous reports on a protective effect of microbial HSP70 and BiP in rat adjuvant arthritis (21-23,39), and are well in line with the known protective effects of microbial HSP60 and microbial HSP10 (11).

As argued above the immunoregulatory qualities of HSPs may be due to their cross- reactivity between microbial HSPs and self-HSPs (21). Given the presence of T cell responses towards highly conserved or even identical sequences of HSP70 (see results) one may assume that following HSP70 immunization cross-reactive responses to self-HSP70 were induced. Thus, immunization with microbial HSP70 may have activated and expanded self-HSP70-reactive T cells.

Taken together we showed that HSP70 can modulate the development of inflammation in the PGIA model likely through IL-10 dependent mechanisms. The fact that HSP70-mediated preventive and therapeutic immune interventions are effective in animal models of chronic inflammatory diseases suggests the immunotherapeutic potential of HSP70 in patients with inflammatory autoimmune arthritis.

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Chapter 4

Naïve transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation

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Abstract

Proteoglycan (PG)-induced arthritis (PGIA), a murine model for rheumatoid arthritis (RA), is driven by antigen (PG)-specific T and B cell activation. In order to analyze the pathogenic role of antigen-specific T cells in the development of autoimmune arthritis, we have generated a transgenic (Tg) mouse. The $CD4^+$ T cells of this TCR-5/4E8-Tg line express a functional T cell receptor (TCR) composed of the $V\alpha1.1$ and $V\beta4$ chains with specificity for the dominant arthritogenic T cell epitope of human cartilage PG. Adoptive transfer of naïve TCR-5/4E8-Tg cells induced arthritis with severe clinical symptoms in syngeneic immunodeficient BALB/c.$RAG2^{-/-}$ mice. In vivo activation of TCR-5/4E8-Tg $CD4^+V\beta4^+$ cells with cartilage PG seemed to be critical for arthritis induction. Arthritis never developed after transfer of naïve wild-type cells. The arthritis was characterized as a chronic progressive disease with intermittent spontaneous exacerbations and remissions. Inflamed joints showed extensive cartilage damage and bone erosions leading to massive ankylosis in peripheral joints. These PG epitope-specific TCR-5/4E8-Tg mice can be valuable research tools for studying antigen-driven T cell regulation in arthritis, and migration of T cells to the joints. In addition the model may be used for the development of immune modulating strategies in T cell-mediated autoimmune diseases.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that predominantly manifests in synovial joints and affects approximately 1-2% of the human population worldwide. Over time, RA results in significant disability due to persistent inflammation in diarthrodial joints leading to cartilage and bone erosions, and joint deformities. Despite many years of intensive investigation, the etiology and pathogenesis of this multifactorial polygenic autoimmune disease have remained unclear. Accumulating evidence indicates that RA is a T cell-dependent antibody-mediated autoimmune disease in which genetic and environmental factors play crucial roles (1-3). Antigen-specific T cells may extravasate and migrate into the joints. These T cells in the synovium may be reactivated by potentially cross-reactive antigens of joint tissues and/or cytokines, which then may lead to their clonal expansion. Although the presence of T cells in the joint seems to be detrimental and contributes to the destructive joint process (4-7), there is evidence that $CD4^+CD25^+$ T cells of a more benign character can also be found in the joint (8-12). Knowledge about the pathogenic role of T cells in RA, or RA-like diseases, has been derived from corresponding animal models of arthritis (13). In many of these models, T cell responses are central to the initiation and maintenance of the disease, and the arthritis can be transferred via autoreactive T cells to naïve recipients (14,15). Although no animal model appears to be a perfect replica of human RA, they clearly show how T cell involvement in the immune-mediated mechanisms can lead to joint destruction.

In this study, we have used the proteoglycan (PG) aggrecan-induced arthritis (PGIA) model which bears several similarities to the clinical expression, histopathology, and genetics of RA (16). Immunization of genetically susceptible BALB/c mice with human cartilage PG (hPG) induces chronic progressive polyarthritis and ankylosing spondylitis. Immunization with hPG elicits strong T cell responses and specific antibodies to the immunizing antigen, and these PG-specific T cells and antibodies cross-react with the mouse (host) cartilage PG (17,18). Depletion of $CD4^+$ T cells was shown to prevent disease development (19,20) and adoptively transferred PG-specific T cell hybridoma 5/4E8 cells were able to induce arthritis (21). This $CD4^+$ 5/4E8 T cell hybridoma was T cell receptor (TCR)-$\alpha^+\beta^+$, and belonged to the Th1 subset, as the cells secreted IL-2 and IFN-γ, but not IL-4, upon hPG stimulation. The antigen-specific response was MHC class II ($I\text{-}A^d$) restricted (21,22). The 5/4E8 hybridoma recognized an epitope ^{70}ATEGRVRVNSAYQDK$_{84}$ (consensus sequence underlined) in the G1 domain of the core protein of hPG (22,23). Later, this particular sequence was found to be the most dominant arthritogenic epitope in BALB/c (21-23), in HLA-DR4 and -DQ8 transgenic mice (24), and that this epitope could specifically stimulate T cells in a subset of RA patients (25-28). It has also been shown that this 5/4E8 hybridoma was able to cross-react with the mouse homologue of the epitope (ATEGQVRVNSIYQDK) (29). Since the hPG 5/4E8-specific T cells are able to recognize the homologous mouse sequence, despite a two-amino acid difference, we hypothesized that sufficient amounts of mouse epitope from damaged articular cartilage could be released and elicit a T cell response, which would then lead to intra-articular inflammation via a molecular mimicry mechanism (22,30,31).

To gain more insight into the role of antigen-specific T cells in the development of autoimmune arthritis, we generated transgenic (Tg) mice expressing the TCR of hybridoma 5/4E8 specific for hPG. In this study, we have also shown that the adoptive transfer of splenocytes from naïve TCR-5/4E8-Tg donor mice to syngeneic BALB/c. $RAG2^{-/-}$ recipient mice induced autoimmune arthritis. These arthritogenic PG-specific TCR-5/4E8-Tg mice are valuable tools

for further analysis of T cell effector mechanisms in autoimmune arthritis, and potentially developing T cell-directed immune interventions.

Material and Methods

5/4E8 T cell hybridoma

hPG-reactive T cell hybridoma 5/4E8 was generated by the fusion of T cells of mice with acute PGIA and BW5147 thymoma cells as described earlier (21). The 5/4E8 T cell hybridoma specifically recognized a peptide sequence ^{70}ATEGRVRVNSAYQDK$_{84}$ (henceforth: hPG P70-5/4E8 peptide; core sequence underlined) in the G1 domain of hPG and, although weakly, cross-reacted with the mouse homologue sequence (ATEGQVRVNSIYODK; mPG P70).

RNA isolation, oligonucleotides, first strand synthesis, amplification and cloning of the αβ TCR

Total RNA was isolated from 5/4E8 hybridoma cells by TRIzol (Invitrogen B.V., Breda, the Netherlands) extraction, reverse transcribed with the oligo(dT)$_{12-18}$ primer using Superscript Reverse Transcription kit (Invitrogen), and then amplified by polymerase chain reactions (PCR) using either degenerate Vα and Cα primers, or the Vβ4 and the Cβ primers. Oligonucleotide primers and the conditions of the corresponding PCR are listed in Table 1. Both the Vα1- and Vβ4-carrying PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI), introduced in *E. coli* DH5α cells (Invitrogen) and sequenced to determine the TCR usage of the 5/4E8 hybridoma. The TCR of 5/4E8 hybridoma was compressed by Vα1.1 and Vβ4 chains (GeneBank accession nos. **AY823583** and **U19234**, respectively).

Amplification and cloning of the αβ genomic TCR genes

To express the TCR-5/4E8 genes in transgenic mice, we made use of the plasmid pTα and pTβ cassettes obtained from Dr. C. Benoist and Dr. D. Mathis (32). Genomic DNA was isolated from 5/4E8 hybridoma cells to obtain full length rearranged TCRα and β DNA, including leader and intron sequences. 5/4E8-TCR was amplified by PCR using Vα1-*Xma*I and JαTA31-*Not*I primers for Vα1 chain, and the Vβ4-*Xho*I and the Jβ2.5-*Sac*II primers for the Vβ4 chain (Table 1). The two PCR fragments were cloned into the pGEM-T Easy Vector (Promega), introduced in *E. coli* DH5α cells, and sequenced. The *Xma*I- and *Not*I-released DNA fragment containing the 5/4E8-TCRα chain was recloned into the pTα cassette, and the *Xho*I- and *Sac*II-digested and purified 5/4E8-TCRβ fragment inserted into the pTβ cassette (32). Subsequently, XL10-Gold cells (Stratagene, La Jolla, CA) were transfected with the constructs, and sequences were confirmed.

In vitro expression of the αβ TCR

pTα 5/4E8- and pTβ 5/4E8-TCR constructs were tested for their functionality in vitro. The pTα 5/4E8 and pTβ 5/4E8-TCR constructs together with plasmid pcDNA3 (Invitrogen) containing the neomycin resistance gene for positive selection were electroporated into a mouse 58^{-} T cell hybridoma deprived of endogenous functional TCR chains (33). Transfected cells after Geneticin (Invitrogen) treatment were cloned and positive cell lines were selected.

Generation of TCRαβ-5/4E8-Tg (TCR-5/4E8-Tg) mice, and screening of TCR expression

pTα 5/4E8- and pTβ 5/4E8-TCR DNA fragments were linearized with *Sal*I (pTα) and *Kpn*I (pTβ), and then separated from prokaryotic DNA by electroelution. DNA was further purified by phenol extraction followed by ethanol precipitation, and both TCR fragments were injected in equal amounts into the pro-nuclei of fertilized eggs of F1(CBAxC57BL/6) mice (Charles River Laboratories, Sulzfeld, Germany). TCR-5/4E8-Tg founders were identified by PCR analysis of tail genomic DNA (Table 1), and the transgenic expression of the TCR-Vβ4 was confirmed by flow cytometric analysis on peripheral blood lymphocytes.

Table 1. Primers used for TCR Cloning and Analysis

Primer	Sequence 5' to 3' [ab]	Direction	Location	PCR product size (bp)	PCR conditions
Primers for amplification cDNA and identification TCR sequence					
Vα	TGGTACNDVCAGCATCCYGGVGAAGGCC	For	Vα	408	A
Cα	AGCTTTCATGTCCAGCACAG	Rev	Cα		
Vβ4-*Xho*I	CTCGAGCACTGCTATGGGCTCCAT	For	Vβ	466	B
Cβ	CTCTGCTTCTGATGGCTCAAACAAG	Rev	Cβ		
Primers for cloning genomic DNA					
Vα1-*Xma*I	CCCGGGAGAATGAAATCCTTGAGTGTTTCA	For	Lα	628	A
JαTA31-*Not*I	GCGGCCGCTCTCCTGACTAGGGAT	Rev	Jα		
Vβ4-*Xho*I	CTCGAGCACTGCTATGGGCTCCAT	For	Lβ	546	A
Jβ2.5-*Sac*II	CCCAATCCCGCGGAGAAC	Rev	Jβ		
Primers for TCR-Tg genotyping					
TCRα1	TGCTCCAGGCTAATGGTACA	For	α	515	C
TCRα2	CGCTCTCCTGACTAGGGATG	Rev	α		
Vβ4-*Xho*I	CTCGAGCACTGCTATGGGCTCCAT	For	β	546	C
Jβ2.5-*Sac*II	CCCAATCCCGCGGAGAAC	Rev	β		

[a] Vα is a degenerate consensus primer (N=A, C, G or T; D=A, T or G; V=A or C; Y=C or T). [b] Restriction sites for cloning in the TCR cassettes are underlined.

PCR conditions:

A: 94°C for 2 min, 35x (94°C for 15 sec, 62°C for 15 sec, 72°C for 25 sec) + final extension at 72°C for 5 min;

B: 94°C for 2 min, 35x (94°C for 15 sec, 55°C for 15 sec, 72°C for 25 sec) + final extension at 72°C for 5 min;

C: 94°C for 2 min, 35x (94°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec) + final extension at 72°C for 5 min;

Measurement of antigen-specific T cell responses

Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (Bodinco B.V., Alkmaar, the Netherlands), 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol was used as culture medium. Cultures were performed in triplicates in 96-well flat bottom plates

(Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands) in a humidified atmosphere of 5% CO_2 at 37°C. Functionality of the 5/4E8-TCR-transfected and cloned 58$\alpha^-\beta^-$ cells was confirmed by IL-2 secretion in the presence or absence of hPG P70-5/4E8 and control peptides (20 μg/ml) . Peptide-specific IL-2 secretion was determined by bioassay using CTLL-2 cells pulsed with supernatants from 24 h-cultured cells. Peripheral blood mononuclear cells (PBMC; 2 x 10^4 cells per well) of the TCR-5/4E8-Tg founder were tested in a proliferation assay. Assays were done in the presence or absence of hPG P70-5/4E8 (20 μg/ml) and A20 (H-2^d) cells as antigen presenting cells (APCs). An irrelevant peptide (GRVRVNSAYGDKVSL; 20 μg/ml) was used as a negative control peptide for T cell proliferation. Proliferation was measured by adding 0.4 μCi of [^{3}H]-thymidine (Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands) to each well for the last 18 h. [^{3}H]-thymidine uptake was measured using a liquid scintillation counter (Microbeta, Perkin-Elmer Inc., Boston, MA).

hPG isolation

Proteoglycan (aggrecan) was purified from human articular cartilage, removed during knee joint replacement surgery, by 4 M guanidinium chloride extraction as described (34,35). The use of human cartilage was approved by the Institutional Review Board (Rush University Medical Center, Chicago, USA), and animal protocols were approved by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands).

Adoptive transfer of cells

C.129S6(B6)-*Rag2^{tm1Fwa}* N12 mice backcrossed into BALB/c background (BALB/c.RAG2$^{-/-}$; H-2^d) were purchased from Taconic Farm (Doussard, France). All mice were 12-16 weeks old at the beginning of experiments. Cells for transfer were isolated from spleens of TCR-5/4E8-Tg, wild-type (littermate) BALB/c, or arthritic BALB/c mice immunized with hPG. Spleen cell suspensions were prepared, erythrocytes lysed, and cells were washed and suspended in phosphate buffered saline (PBS; 0.14 M NaCl in 0.01 M phosphate buffer, pH: 7.4), and then injected intraperitoneally (IP) into BALB/c.RAG2$^{-/-}$ mice (2 x 10^7 cells per mouse) together with 100 μg of hPG on days 0 and 43 as described for adoptive transfer of PGIA into BALB/c.SCID mice (36,37). Paws of mice were examined 3 times a week to record arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws as described (35).

Histology

Joints of BALB/c.RAG2$^{-/-}$ mice with adoptively transferred arthritis (received naïve TCR-5/4E8-Tg cells) and non-arthritic BALB/c.RAG2$^{-/-}$ mice (received naïve wild-type cells) were fixed in 10% buffered formalin, decalcified in 0.5 M neutralized EDTA, embedded in paraffin and 6 μm sections were stained with hematoxylin and eosin.

Flow cytometric analysis

PBMCs, after erythrocyte lysis using FACS lysing solution (BD Biosciences Pharmingen, San Diego, CA), or popliteal lymph nodes, were washed with PBS containing 0.5% BSA and 0.01% sodium azide (FACS buffer), and then stained

with phycoerythrin (PE)-conjugated anti-Vβ4, anti-CD8 or anti-CD25; FITC-conjugated anti-CD19; anti-CD44; or allophycocyanin (APC)-conjugated anti-CD4 antibodies; or relevant isotype control monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA) for 30 min on ice. Cells were analyzed using a flow cytometer (FACSCalibur) and Cell Quest software (BD Biosciences Immunocytometry, San Jose, CA).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using Student's t test or the Kruskal-Wallis test using Prism software and $p < 0.05$ value was considered statistically significant.

Results

Identification and cloning of the 5/4E8-TCR, and in vitro functional testing of the pTα 5/4E8-TCR and pTβ 5/4E8-TCR transgenic expression vectors

Using mouse Vα and Vβ primers, we first cloned and sequenced the 5/4E8-TCR variable (V), diversity (D) and joining (J) regions out of the mRNA. The 5/4E8-TCR α chain was closely homologous to mouse Vα1.1 and the Jα gene showed highest identity with a mouse TA31 gene. The 5/4E8 β chain was composed of Vβ4, Dβ2 and Jβ2.5 (21). These TCR sequences allowed us to generate new primers to obtain full length TCR variable regions, including the leader sequence and the intron for cloning into the TCR cassettes (32) (Fig. 1).

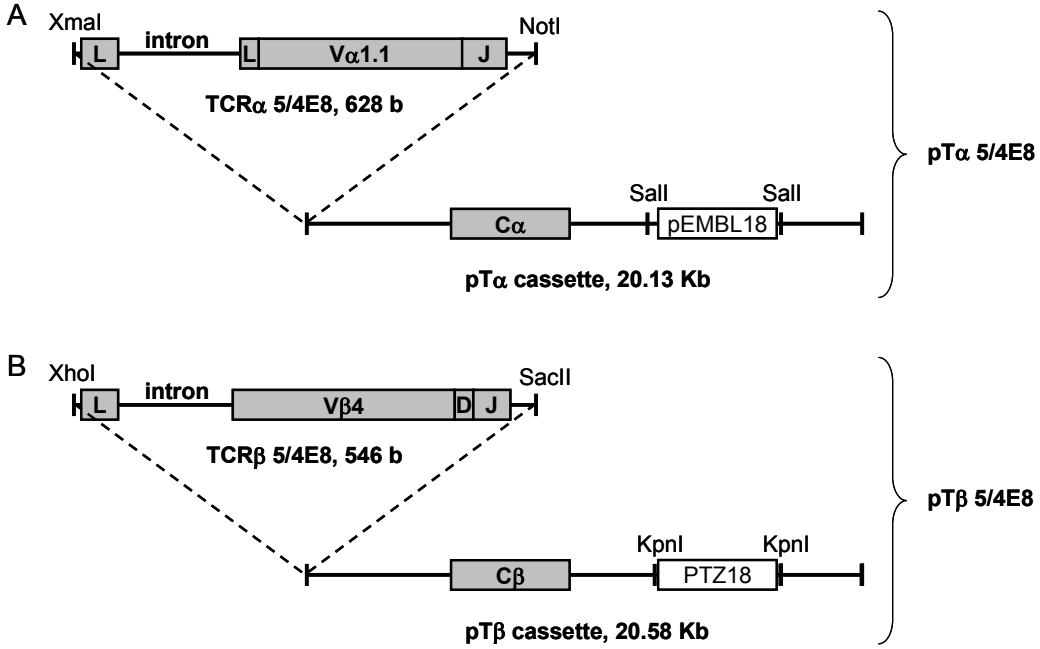

***Figure 1**. Schematic structure of the pTα 5/4E8- and pTβ 5/4E8-TCR transgenic constructs for in vitro and in vivo expression of the hPG P70-5/4E8 epitope-specific TCR. (**A**) The genomic fragment containing the L (leader), V (variable) and J (joining) regions and the intron sequence (as indicated) of the Vα1.1 chain of the 5/4E8 TCR with linkers for cleavage sites for restriction enzymes XmaI and NotI were cloned into the pTα cassette. This construct was linearized with SalI, further purified (removing prokaryotic DNA fragment pEMBL18) for pro-nuclei injection. (**B**) Genomic DNA of the L, V, D (diversity) and J regions of the β chain of the 5/4E8-TCR including the corresponding short intron sequence were cloned into the XhoI and SacII cloning sites of the pTβ cassette, amplified, cleaved with KpnI and then purified (prokaryotic PTZ18 DNA-free). pTα 5/4E8 and pTβ 5/4E8 linearized constructs were electroporated into TCR negative 58αβ- T cell hybridoma for in vitro tests (Fig. 2), or co-injected into the pro-nuclei of fertilized eggs for generation of TCR-5/4E8-Tg mice.*

The pTα 5/4E8-TCR and pTβ 5/4E8-TCR constructs were first tested for the in vitro expression of the TCR chains using TCR-deficient (58α-β-) T cell hybridoma (33). 5/4E8-TCR transfectant expression of the Vβ4 chain was detected by flow cytometry, and the expression of the functionally active TCR was demonstrated by using hPG P70-5/4E8 peptide-specific IL-2 production. Note, flow cytometric analysis of Vα1 chain was impossible due to lack of a specific monoclonal Vα1 antibody. Antigen-specific IL-2 secretion in response to specific (hPG P70-5/4E8) and not to non-specific peptides presented by A20 (H-2^d) APCs was observed by a bioassay using CTLL-2 cells (Fig. 2).

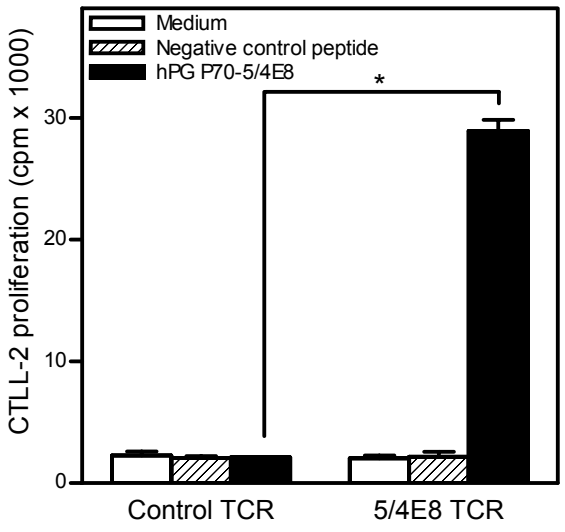

Figure 2. *In vitro functional analysis of the transgenic constructs (pTα 5/4E8-TCR and pTβ 5/4E8-TCR). TCR-negative 58$\alpha^-\beta^-$ T cell hybridoma was co-transfected with both transgenic constructs and with pcDNA3 vector for positive selection as described in Materials and Methods. The peptide-specific T cell response (IL-2 production) was measured by CTLL-2 bioassay. Viable 58$\alpha^-\beta^-$ T cells (1 x 10^5), transfected only with pTα 5/4E8-TCR construct and pcDNA3 vector (left-side columns), or simultaneously with both pTα and pTβ constructs (right-side columns), were co-cultured with 2 x 10^5 irradiated A20 APCs in the presence or absence of 20 μg/ml positive hPG P70-5/4E8 peptide (ATEGRVRVNSAYQDK) or negative control peptide (GRVRVNSAYGDKVSL). IL-2 was measured in 24 h-culture supernatants. The results are expressed as the mean (cpm) of triplicate wells ± SD, and significant difference ($p < 0.0001$) is indicated by an asterisk.*

Generation of 5/4E8 $\alpha\beta$ TCR$^+$ transgenic (TCR-5/4E8-Tg) mice

Linearized α and β 5/4E8-TCR fragments were co-injected into the germline of cell-stage fertilized eggs of CBAxC57BL/6 F1 hybrids (H-2^b) by microinjection. Transgenic founders were identified by PCR using genomic DNA (Table 1), and by flow cytometric analysis of the Vβ4 chain transgene expression of blood lymphocytes. Two $\alpha\beta$-positive founders and two β- positive founders were identified. A proliferation assay using PBMCs of $\alpha\beta$-positive TCR-5/4E8-Tg founder showed that the cells were functionally active and able to respond to the specific hPG P70-5/4E8 peptide (Fig. 3). As CD4$^+$ hybridoma 5/4E8 was of BALB/c (H-2^d) origin and MHC class II restricted, T cells of the TCR-5/4E8-Tg founder (H-2^b) could respond to the specific hPG P70-5/4E8 peptide only in the presence of H-2^d APCs (Fig. 3). Besides the alloreactive reaction, however, there was a significantly increased response to the hPG P70-5/4E8 peptide, which was antigen-specific because the TCR-5/4E8-Tg T cells failed to proliferate in response to a negative control peptide (Fig. 3). In contrast, hPG P70-5/4E8 peptide-specific response was not detected in cultures of PBMCs from naïve wild-type (littermate) mice. As soon as the TCR-5/4E8-Tg founders were identified, they were backcrossed into BALB/c background. Normal mating of transgenic males with BALB/c females was used for the first five backcrosses, and 5/4E8-TCR$^+$ transgenic mice selected by PCR. Later, after selecting TCR$^+$ Tg males, a marker assisted approach (18) was used to identify males with the most advanced BALB/c background, and these males were used for the next backcross. After 5 additional backcrosses, all TCR-5/4E8-Tg mice have a BALB/c background (>97%) as confirmed by 140 microsatellite markers (24,38). Throughout these backcrosses of TCR-5/4E8-Tg mice, co-expression of Vα1.1 and Vβ4 chains was always detected. Therefore, it is very likely that either both of the TCR transgenic constructs underwent homologous integrations in one of the transgenic lines or were incorporated together into the mouse genome and without segregation in the offspring.

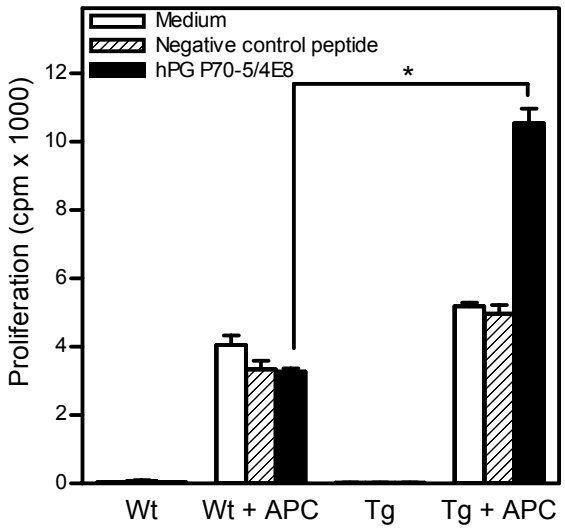

Figure 3. *In vitro proliferation of TCR-5/4E8-Tg (Tg) founder cells. PBMCs (4 x 10^4 cells/well) of TCR-5/4E8-Tg founder and wild-type (Wt) littermates were cultured in the presence or absence of 1 x 10^5 irradiated A20 APCs/well without or with 20 μg/ml positive (hPG P70-5/4E8) or 20 μg/ml negative control peptides as indicated. The sequences of negative and positive peptides are described in Figure 2 legend. As the PBMCs were isolated from TCR-5/4E8-Tg founder and wild-type littermate mice, both carrying the H-2^b allele, a relatively high background (alloreactive reaction) can be detected in the presence of A20 APCs carrying H-2^d allele. The results are expressed as the mean (cpm) of triplicate wells ± SD, and the level of significance ($p < 0.01$) is indicated by an asterisk.*

TCR-5/4E8-Tg cell transfer to BALB/c.RAG2$^{-/-}$ mice

To study whether PG-specific T cells were able to induce arthritis in syngeneic immunodeficient RAG2$^{-/-}$ mice we have adoptively transferred TCR-5/4E8-Tg cells to BALB/c.RAG2$^{-/-}$ mice. Splenocytes of naïve TCR-5/4E8-Tg donor mice or wild-type littermate mice were injected IP together with hPG (without adjuvant) into syngeneic BALB/c.RAG 2$^{-/-}$ recipient mice. Splenocytes from acutely arthritic BALB/c mice (PGIA), known to be capable of inducing arthritis in BALB/c.SCID mice, were used as positive control cells (36,39). A mild but progressive, eventually chronic arthritis with extensive ankylosis in peripheral joints developed in mice injected with spleen cells of naïve TCR-5/4E8-Tg cells. The clinical symptoms (redness and swelling) were comparable to arthritic joints of mice injected with cells from acutely arthritic BALB/c mice with PGIA. However, the onset of arthritis was delayed 2-3 weeks in BALB/c.RAG2$^{-/-}$ mice injected with naïve TCR-5/4E8-Tg cells, when compared to those that received spleen cells from arthritic BALB/c mice. None of the BALB/c.RAG2$^{-/-}$ mice injected with wild-type cells developed arthritis. Figure 4 summarizes these results showing the maximum arthritis scores in BALB/c.RAG2$^{-/-}$ mice with transferred spleen cells. The mean arthritis score was identical in BALB/c.RAG2$^{-/-}$ mice that received naïve (non-immunized) TCR-5/4E8-Tg spleen cells and those that received spleen cells from arthritic BALB/c mice (Fig. 4).

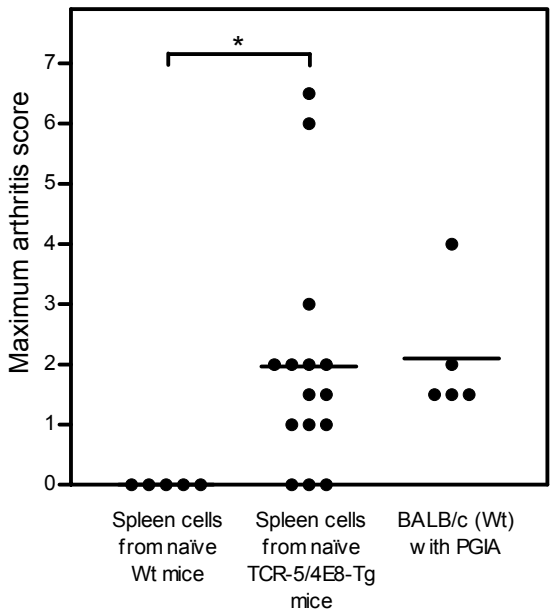

Figure 4. *The maximum (highest) arthritis scores after adoptive transfer. Spleen cells from naïve (non-immunized) TCR-5/4E8-Tg mice, wild-type (Wt) littermates, or BALB/c mice with PGIA (positive control) were transferred into BALB/c.RAG2$^{-/-}$ mice. Freshly isolated 2 x 10^7 donor spleen cells/recipient with 100 μg of hPG protein were injected intraperitoneally as described earlier for adoptive transfer into BALB/c.SCID mice (36,37). BALB/c.RAG2$^{-/-}$ mice received cells and hPG on days 0 and 43. Arthritis is depicted as the highest score per mouse at any time during disease. Statistically significant differences ($p < 0.05$) are indicated (Kruskal-Wallis Test).*

The clinical appearance and histopathological features of arthritis were similar in both groups, and identical with the primary form of PGIA in BALB/c mice (16,34). Joint inflammation was progressive and characterized by intermittent spontaneous exacerbations and remissions resulting in extensive cartilage damage and bone erosions, leading to ankylosis and deformities of the peripheral joints (Fig. 5). The synovium was hyperplastic with massive cellular infiltration. In summary, in vivo activation of arthritogenic epitope-specific 5/4E8-TCR expressed by $CD4^+$ cells seems to be sufficient to induce arthritis in syngeneic immunodeficient animals.

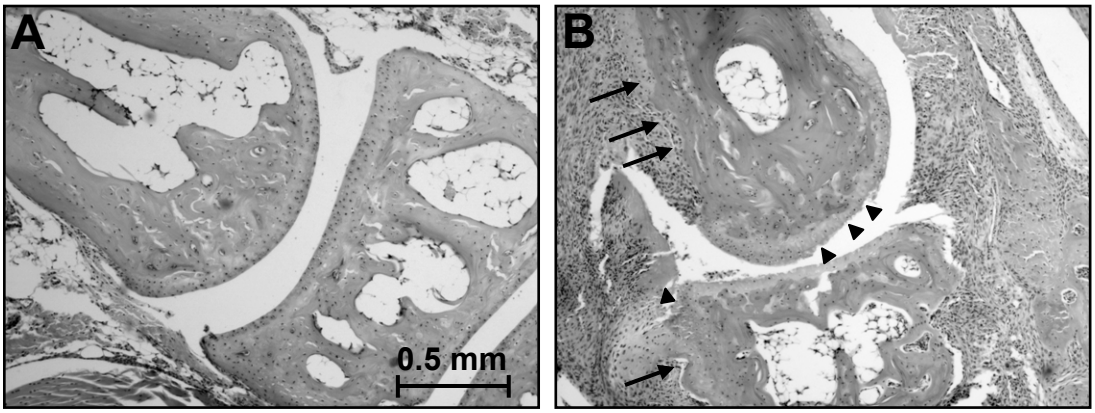

***Figure 5**. Histology of tarso-metatarsal joints of BALB/c.RAG2$^{-/-}$ mice transferred with Wt cells (**A**) or 5/4E8-TCR Tg cells (**B**). BALB/c.RAG2$^{-/-}$ recipient mice received twice 2 x 10^7 spleen cells (on days 0 and 43) from naïve (non-immunized) Wt or naïve TCR-5/4E8-Tg mice. BALB/c.RAG2$^{-/-}$ mice were sacrificed on day 70 after the first cell transfer. Massive synovial hyperplasia with infiltrating mononuclear cells, and cartilage (arrow heads) and bone erosions (arrows) can be seen in mice that received naïve T cells from TCR-5/4E8-Tg mice. Sections were stained with hematoxylin and eosin.*

Characterization of transferred cells in BALB/c.RAG2$^{-/-}$ mice

The cell recovery and reconstitution of (auto)immune homeostasis in BALB/c.RAG2$^{-/-}$ mice after adoptive transfer using naïve TCR-5/4E8-Tg or wild-type spleen cells was analyzed by flow cytometry. The ratios of $CD4^+V\beta4^+$ donor T cells from wild-type and TCR-5/4E8-Tg mice are shown in Figure 6A. On days 41 and 70, cells were isolated from peripheral blood from BALB/c.RAG2$^{-/-}$ mice and analyzed for CD4/CD19 and CD4/TCR Vβ4 surface expression (Figs. 6B and 6C). On day 70, popliteal lymph node (PLN) cells were also analyzed for T and B cell reconstitution, and T cell activation (Fig. 6D). In BALB/c.RAG2$^{-/-}$ mice, which received TCR-5/4E8-Tg cells, the $CD4^+V\beta4^+$ cells were detectable and expanded significantly accordingly to the progression of arthritis. The number of $CD19^+$ B cells in peripheral blood was undetectable (Fig. 6B) after the first transfer with TCR-5/4E8-Tg T cells, but increased after the second transfer (Fig. 6C), and transferred cells migrated to the PLN. The T/B cell ratio in the joint-draining PLNs was comparable in BALB/c.RAG2$^{-/-}$ mice having either transferred TCR-5/4E8-Tg or wild-type cells, but the number of $CD4^+V\beta4^+$ T cells was significantly higher in arthritic animals (Fig. 6D). Moreover, these $CD4^+V\beta4^+$ T cells seemed to be activated in the PLNs of arthritic animals as shown by the expression of high levels of CD25 and CD44, whereas the expression of these activation markers were low on the $CD4^+$ PLN cells of animals that received wild-type cells. In summary, PG-specific TCR-5/4E8-Tg cells expanded and became activated in joint-draining PLNs of BALB/c.RAG2$^{-/-}$ mice with arthritis.

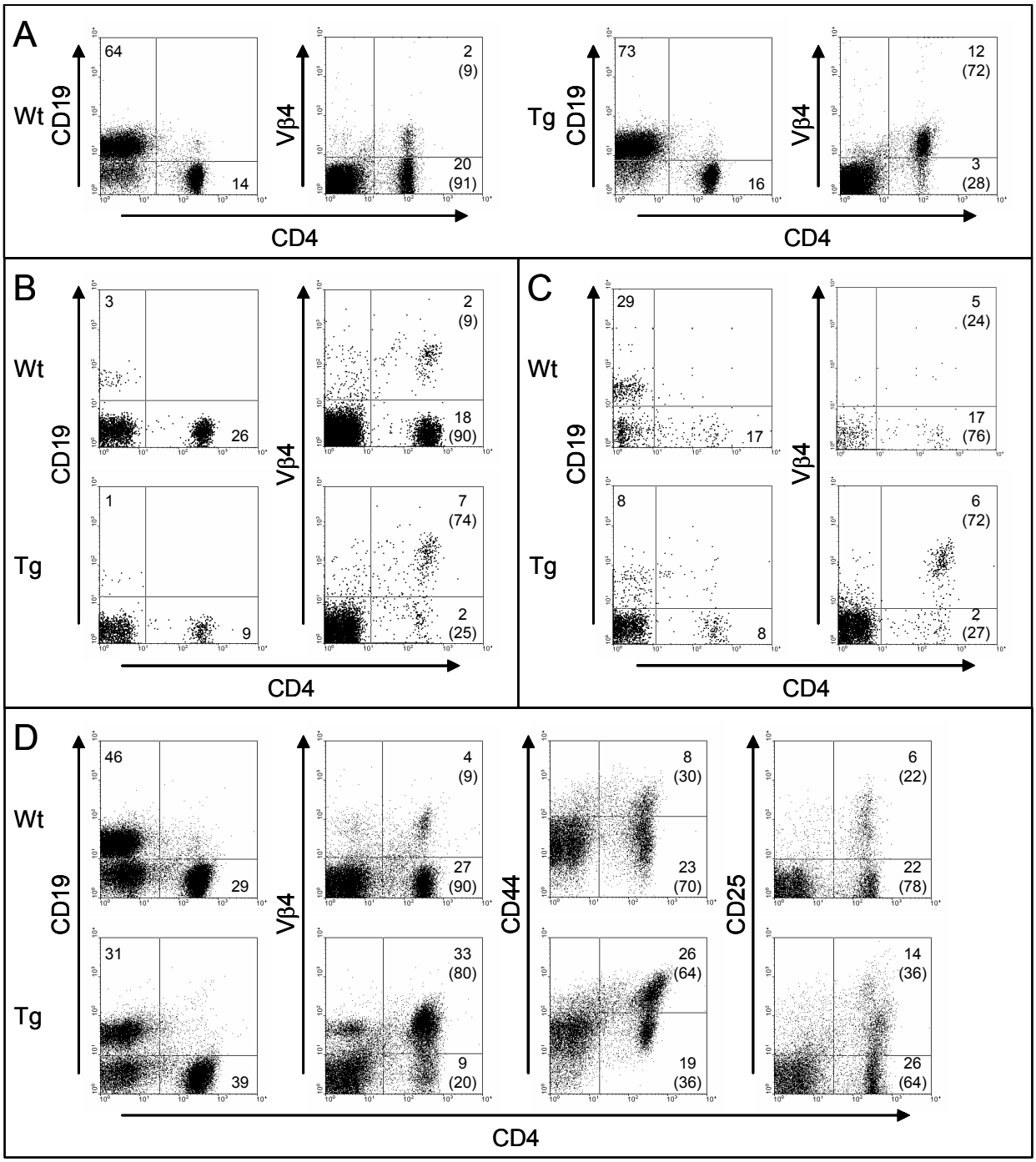

***Figure 6**. Flow cytometric analysis of lymphocytes repopulating BALB/c.RAG2$^{-/-}$ mice after adoptive transfer. Freshly isolated spleen cells from naïve TCR-5/4E8-Tg mice (2 x 10^7 cells/recipient), their wild-type (Wt) littermates, and from BALB/c mice immunized with cartilage PG for PGIA (positive control group) along with 100 μg of hPG protein were injected IP into BALB/c.RAG2$^{-/-}$ mice on days 0 and 43. Donor spleen cells were analyzed prior to the transfer (**A**), and then cells were isolated from peripheral blood on day 41 (**B**) and day 70 (**C**). The bottom panel shows the flow cytometry results of popliteal lymph node cells on day 70 (**D**). The percentage of cells per quadrant is indicated, and the numbers within brackets the percentage of CD4$^+$ T cells.*

Discussion

We have generated TCR αβ Tg mice with Vα1.1 and Vβ4 TCR chains obtained from T cell hybridoma 5/4E8 with MHC restricted specificity for hPG (consensus sequence of ^{73}GRVRVNSAY). In these TCR-5/4E8-Tg mice the transgenic T cells appear to have been positively selected in the thymus and exported to the periphery. Several observations provide compelling evidence that "arthritogenic" transgene-encoded α and β chains were co-expressed on the surface of a significant number of transgenic peripheral CD4$^+$ T cells. First, PCR of genomic DNA revealed only a product when both the Vα1.1 transgene and Vβ4 transgene were in the genome. Second, specific in vitro T cell proliferation to hPG P70-5/4E8 peptide in the presence of H-2^d APCs without priming provided further evidence that functional 5/4E8 TCR had been generated in the transgenic mice (Fig. 3). This also showed that, despite the fact that hPG-specific TCR-5/4E8-Tg T cells also recognize the homologous mouse sequence and could therefore cross react with self PG, TCR-5/4E8-Tg T cells in the transgenic mice were not tolerized in the periphery by either clonal deletion or anergy. This is consistent with other reports, showing that potentially self-reactive T cells exist in the peripheral T cell pool. Osman et al. showed in type II collagen (CII)-TCR-Tg mice that peripheral T cells were not tolerant to CII, and responded to CII stimulation in vitro (40). Finally, adoptive transfer of naïve TCR-5/4E8-Tg donor cells, given together with hPG, could induce arthritis in syngeneic BALB/c.RAG2$^{-/-}$ recipient mice (Fig. 4), which never occurred after the transfer of wild-type cells.

The clinical appearance of arthritis in BALB/c.RAG2$^{-/-}$ mice was similar to the primary form of PGIA in BALB/c mice, and to the arthritis in BALB/c.SCID mice adoptively transferred with acutely arthritic BALB/c spleen cells (36,37,39). Inflamed joints of BALB/c.RAG2$^{-/-}$ mice transferred with TCR-5/4E8-Tg cells showed extensive cartilage degradation and bone erosions (Fig. 5) similar to that seen in arthritic joints of human patients with RA. Because this arthritis transfer model makes use of naïve TCR-5/4E8-Tg cells with one defined specificity, it provides a straightforward arthritis model for studying the role of joint antigen-specific T cells in arthritis.

Our analysis of the recovered TCR-5/4E8-Tg cells after the adoptive transfer in BALB/c.RAG2$^{-/-}$ mice showed a repopulation and a marked increase of CD4$^+$ transgenic T cells (Fig. 6B and C). Repopulation and T cell activation (Fig. 6D) coincided with arthritis induction. The process responsible for focusing autoimmune destruction to the joints in BALB/c.RAG2$^{-/-}$ mice that received TCR-5/4E8-Tg cells seems to be the presence of the large number of arthritogenic joint-specific T cells with a high expression level of joint (cartilage PG)-specific TCRs. These data clearly underline the critical function of CD4$^+$ T cells in arthritis (41). However, previously it has been shown that there is also a role for antibodies to mouse (self) PG, and/or B cells in the pathogenesis of PGIA (19,29). PG-specific B cells appeared to play a major role in antigen presentation (29). Nevertheless, neither anti-PG Abs nor PG-specific B cells alone were able to induce arthritis (19,42). An antigen-specific T cell population seems to play a central role in the mechanism of murine PGIA. Most likely, highly specific cooperation between antigen-primed CD4$^+$ Th1 and B cells is needed for the development of the disease.

As the PG-specific T cell response is rather confined to the joint-draining lymph nodes (43), it is very likely that an autoantigen-driven mechanism of joint inflammation becomes local and self-sustaining by PG (cartilage) degradation. Autoreactive T cells can migrate to, and proliferate in, the synovium and joint draining lymph nodes (42), where the self-peptides are present in relatively high concentrations as a result of the normal turnover of the cartilage matrix

and, even more, as a consequence of increased PG degradation in inflammatory joint diseases. There is growing evidence that at least a subset of patients with RA exhibits antigen-specific T cell responses to hPG (25-28,44-47). Moreover, the core protein of human/mouse cartilage PG has been mapped in HLA-DR4- and HLA-DQ8-humanized transgenic mice (24)

This is the first report of a TCR transgenic mouse generated to a hPG epitope having a dominant/arthritogenic function in BALB/c mice. $CD4^+$ T cells harboring TCR $\alpha\beta$ transgenes comprised the vast majority of peripheral $CD4^+$ T cells. The finding that naïve TCR-5/4E8-Tg mice enabled the transfer of arthritis to BALB/c.$RAG2^{-/-}$ recipient mice is direct evidence that circulating PG-specific T cells can be activated in vivo, and these cells may migrate to joint-draining lymph nodes and synovial joints. These TCR-5/4E8-Tg mice offer a valuable tool for investigating the role of antigen specific T cells in the development of autoimmune arthritis and studying immunopathogenic and modulatory mechanisms of RA.

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Chapter 5

Increased arthritis susceptibility in cartilage proteoglycan-specific T cell receptor (TCR) transgenic mice

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Abstract

Objective. To better understand the role of antigen (arthritogenic epitope)-specific T cells in the development of autoimmune arthritis.

Methods. A transgenic (Tg) mouse expressing T cell receptor (TCR) Vα1.1 and Vβ4 chains specific for a dominant arthritogenic epitope (designated 5/4E8) of human cartilage proteoglycan aggrecan (hPG) was generated. This TCR-5/4E8-Tg mouse was backcrossed into the PG-induced arthritis (PGIA)-susceptible BALB/c strain, and tested for arthritis incidence and severity.

Results. $CD4^+$ TCR-5/4E8-Tg T cells carried functionally active TCR specific for a dominant arthritogenic epitope of hPG. T cells of naïve TCR-5/4E8-Tg mice were in an activated stage as they responded *in vitro* to hPG or peptide stimulation with IFNγ and IL-4 production. TCR-5/4E8-Tg mice, without exception, uniformly developed severe and progressive polyarthritis after a single hPG injection, even without adjuvant. Inflamed joints showed extensive cartilage degradation and bone erosions, similar to that seen in arthritic joints of wild-type BALB/c mice with PGIA. Spleen cells from either naïve or hPG-immunized arthritic TCR-5/4E8-Tg mice could adoptively transfer arthritis to syngeneic severe immunodeficient (BALB/c.SCID) recipients.

Discussion/Conclusion. TCR-5/4E8-Tg BALB/c mice display increased arthritis susceptibility and develop aggravated disease upon *in vivo* antigen stimulation. This TCR-5/4E8 transgenic BALB/c mouse is a novel and valuable research tool for studying mechanisms of antigen (arthritogenic epitope)-driven regulation of arthritis, and understanding how T cells recognize autoantigen in the joints. These mice could also be used to develop new immunomodulatory strategies in T cell-mediated autoimmune diseases.

Introduction

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases characterized by chronic inflammation of the synovium of diarthrodial joints. Although the etiology is unknown, accumulating evidences indicate that RA is a T cell-mediated and auto- antibody dependent disease in which both genetic and environmental factors play crucial roles(1-3). The RA synovium is infiltrated with $CD4^+$ T cells of the Th1 phenotype (4,5), and antibodies are also involved in the pathological mechanisms of joint inflammation and progression of the disease (6,7). Therapeutic efficacy of agents that block T cell activation (8) or deplete B cells (7) in RA patients has confirmed the critical role of the adaptive immune system. Among the candidate autoantigens, the cartilage proteoglycan (PG) aggrecan is one of the target autoantigens in RA joints (9-14). PG is a complex macromolecule consisting of a large core protein (>2,200 amino acids) to which more than 100 glycosaminoglycan and oligosaccharide side chains are covalently attached (15,16). The core protein of aggrecan is heavily degraded by proteases released during either degeneration or inflammatory processes, resulting in the loss of the normal function of articular cartilage (17).

Immunization of BALB/c mice with human cartilage PG (hPG) induces chronic progressive polyarthritis (18). This PG-induced arthritis (PGIA) shows many similarities to human RA, as indicated by clinical assessments, radiographic analyses, scintigraphic bone scans, laboratory tests and histopathology of the peripheral joints (18-21). The development of the disease is based upon the development of T and B cell responses cross-reactive between the immunizing human and self (mouse) cartilage PG (mPG). This cross-reactivity, most likely achieved through epitope spreading, could explain why these T cells home to mouse joints to initiate arthritis (22,23).

Several lines of evidence are indicative of T cell involvement in the pathogenesis of PGIA. For example, as $CD4^+$ T cells selectively proliferate in response to hPG (24,25), arthritis can be prevented when $CD4^+$ T cells are depleted either *in vivo* (26) or *in vitro* prior to adoptive transfer to naïve mice (27,28) a PG-specific T cell hybridoma clone 5/4E8 can induce arthritis in BALB/c mice (29), $CD4^+$ T cells from arthritic animals are resistant to activation induced cell death (30), and susceptibility to PGIA is associated with MHC class-II ($H\text{-}2^d$ haplotype in BALB/c) (20). In addition, immunization of BALB/c mice with PG induces a dominant Th1 T cell response, and treatment of arthritic mice with IL-4 can prevent disease development by inducing a switch from the originally Th1-polarized to a Th2-polarized response (20,31). The importance of $CD4^+$ Th1-cells was further underlined by the observation that IL-4-deficient BALB/c mice developed a significantly more severe form of the disease than wild-type BALB/c mice (32).

The arthritogenic 5/4E8 T cell hybridoma has a $CD4^+$ Th1 phenotype and expresses T cell receptor (TCR)-$\alpha\beta^+$ chains (29). These hybridoma cells secrete IL-2 and IFNγ, but not IL-4, upon hPG stimulation, and the antigen specific response is MHC class II ($I\text{-}A^d$) restricted (29). The epitope recognized by 5/4E8 cells is located in the G1 domain of hPG, and has been identified as an immunodominant, and possibly the most arthritogenic, T cell epitope of hPG in previous mapping studies (16,29). The 5/4E8 hybridoma shows cross-reactivity with a homologous epitope of mPG (16). In this study, to gain more insight into the role of antigen-specific T cells in the development of autoimmune arthritis, we generated transgenic (Tg) mice expressing the TCR of the 5/4E8 hybridoma. We found that a single PG injection provoked a severe form of PGIA in TCR-5/4E8-Tg BALB/c mice. Splenocytes from even naïve TCR-5/4E8-Tg mice, after *in vivo* activation, or cells from hPG-immunized arthritic TCR-5/4E8-Tg mice adoptively transferred arthritis into syngeneic severe combined immunodeficient (SCID) recipient mice.

Materials and Methods

Isolation, amplification, and cloning of the αβ chains of TCR-5/4E8

T cell hybridoma 5/4E8 (29), a CD4+ Th1 cell line, recognizes the most dominant arthritogenic peptide sequence ^{70}ATEGRVRVNSAYQDK$_{84}$ (henceforth: hPG P70-5/4E8 peptide; core sequence underlined) in the G1 domain of hPG, and cross-reacts with the mouse homologue sequence (ATEGQVRVNSIYODK; mPG P70). T cell hybridoma 5/4E8 carries Vα1.1 and Vβ4 chains of the TCR (GeneBank accession numbers AY823583 and U19234, respectively).

Genomic DNA was isolated from 5/4E8 hybridoma cells to obtain full length rearranged TCRα and TCRβ DNA, including leader and intron sequences and the corresponding regions were amplified by polymerase chain reaction (PCR). The two PCR fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), introduced in *E. coli* DH5α cells (Invitrogen B.V., Breda, the Netherlands), and sequenced. The *Xma*I- and *Not*I-released DNA fragment containing the TCR-5/4E8α chain was recloned into the pTα cassette; and the *Xho*I- and *Sac*II-digested and purified TCR-5/4E8β fragment was inserted into the pTβ cassette (33). Both in vivo expression plasmid constructs (pTα and pTβ) were generous gifts from C. Benoist and D. Mathis. Subsequently, XL10-Gold cells (Stratagene, La Jolla, CA) were transfected with the constructs, and the correct sequences confirmed.

Generation of TCR-5/4E8αβ-Tg (TCR-5/4E8-Tg) mice

Linearized pTα TCR-5/4E8 and pTβ TCR-5/4E8 DNA fragments were purified by electroelution, phenol extraction, and ethanol precipitation, respectively. Both TCR fragments were co-injected in equal amounts into the pronuclei of fertilized eggs of F1(CBAxC57BL/6) mice (Charles River Laboratories, Sulzfeld, Germany). TCR-5/4E8-Tg founders were identified by PCR analysis of tail genomic DNA. TCRα1 chain was genotyped by PCR using forward primer 5'-TGCTCCAGGCTAA TGGTACA-3' and reverse primer 5'-CGCTCTCCTGACTAGGGATG-3'; the Vβ4-chain was detected by using forward primer 5'-CTCGAGCACTGCTATGGGCTCCAT-3' and reverse primer 5'-CCCAATCCCGCGGAGAAC-3'). The expression of TCR-Vβ4 was confirmed by flow cytometric analysis on blood lymphocytes. Unfortunately, flow cytometric analysis of Vα1.1 chain was impossible due to lack of Vα1-specific antibody. Since the PGIA was restricted to the susceptible BALB/c strain and the CD4+ hybridoma 5/4E8 was of H-2^d MHC class II (BALB/c origin), the TCR-5/4E8-Tg founders were backcrossed onto BALB/c (H-2^d). A marker-assisted genome screening process was used (20) until the pure BALB/c genomic background was achieved (backcross 8). This was confirmed by using 244 simple sequence length polymorphic markers as described (20). Throughout the backcross processes of TCR-5/4E8-Tg mice, the co-expression of Vα1.1 and Vβ4 chains was always detected in one transgenic line by genotyping, and all heterozygous TCR-5/4E8-Tg mice were tested for PGIA susceptibility at each backcross level (see below). Finally, heterozygous TCR-5/4E8-Tg BALB/c males and females were intercrossed to select homozygous offspring.

Antigens, animals, immunization, and experimental groups

The use of human cartilage from joint replacement surgeries for PG isolation was approved by the Institutional Review Board (Rush University Medical Center, Chicago, USA). All animal experiments were approved by the Institutional Animal Care and Use Committee (Rush University Medical Center) and by the Animal Experiment Committee of Utrecht University (Utrecht, the Netherlands). hPG was used for immunization of 16-26-week-old TCR-5/4E8-Tg or their wild-type littermates, and age matched female BALB/c (National Cancer Institute: NCI, Kingston colony, NY) mice using a standard immunization protocol described earlier (19,21). Briefly, the antigen injection (100 µg hPG protein in 100 µl phosphate-buffered saline; PBS, pH 7.2) was given intraperitoneally (IP) with or without 2 mg of the synthetic adjuvant dimethyldioctadecylammonium bromide (DDA) on days 0 and 21. hPG-immunized TCR-5/4E8-Tg and wild-type littermate mice were sacrificed within 3-9 days after the onset of primary PGIA. Spleen cells were isolated for transfer experiments and *in vitro* tests, and sera were collected for antibody and cytokine measurements. Severe combined immunodeficient (SCID) female mice of BALB/c background (NCI/NCrC.B-17-scid/scid; henceforth designated BALB/c.SCID) were purchased from the NCI and maintained under germ-free conditions.

Transfer of arthritis using spleen cells from naïve, and from hPG-immunized wild-type or TCR-5/4E8-Tg BALB/c mice with arthritis

Prior to adoptive transfer experiments (summarized in Table 1), approximately 60 BALB/c.SCID mice were used to optimize cell number, time interval between injections (if repeated), and the dose of peptide (designated hPG P70-5/4E8) or hPG. For adoptive transfer experiments, BALB/c.SCID mice received unseparated spleen cells (30 or 15 x 10^6) IP from either naïve or hPG-immunized arthritic mice. Spleen cells were injected first with 100 µg hPG P70-5/4E8 peptide or 100 µg hPG, or injected without antigen (see Table 1) as described earlier (28).

Assessment of arthritis

The paws of mice were examined daily to record abnormalities due to arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of the paws (19,21). The degree of joint swelling for each paw (scored from 0 to 4) was used to express a cumulative arthritis score of 4 paws, with a possible maximum severity index of 16 per animal. The first day of the clinical appearance of paw swelling was recorded as the onset of arthritis. At the end of the experiments, the limbs of arthritic and non-arthritic mice were dissected, fixed, decalcified, sectioned, and the tissue sections were stained with hematoxylin and eosin for histopathological examination.

Flow cytometric analysis

Single cell suspensions of thymus and spleens of naïve or hPG-immunized arthritic wild-type and TCR-5/4E8-Tg mice were separated and washed with PBS containing 0.5% bovine serum albumin and 0.01% sodium azide (FACS buffer). Cells were stained with phycoerythrin (PE)-conjugated anti-Vβ4, anti-CD8 or anti-CD25, fluorescein

isothiocyanate (FITC)-conjugated anti-CD19, anti-CD44, or anti-Vβ4, allophycocyanin (APC)-conjugated anti-CD4 or peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 monoclonal antibodies (mAb), or identically-labeled relevant IgG isotypes as controls (BD Biosciences Pharmingen, San Diego, CA) for 30 min on ice. After incubation, cells were washed twice with FACS buffer and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry, San Jose, CA).

Measurement of antigen-specific T cell responses

Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (Bodinco B.V., Alkmaar, the Netherlands), 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 x 10^{-5} M 2-mercaptoethanol was used as culture medium. Single-cell suspensions of spleens were cultured in triplicates in 96-well flat bottom plates (Corning B.V. Life Sciences, Schiphol-Rijk, the Netherlands) at 2 x 10^5 cells per well, in the presence or absence of hPG P70-5/4E8 peptide (0.1 µg/ml), hPG (10 µg protein/ml) or control mPG P70 peptide (50 µg/ml). Proliferation was determined by overnight incorporation of [^{3}H]-thymidine (0.4 µCi per well; Amersham Biosciences Europe GmbH, Roosendaal, the Netherlands) and measured using a liquid scintillation counter (Microbeta, Perkin-Elmer Inc., Boston, MA). The magnitude of the proliferative response was expressed as delta counts per minute (Δ cpm) calculated by subtracting the cpm of non-stimulated cultures from cpm of stimulated cultures.

Cytokine analysis

Supernatants were collected for cytokine assays after 72 h of culture and analyzed for IL-4 and IFNγ simultaneously using the Luminex 100 system (Becton Dickinson, Mountain View, CA) according to the method of Carson et al. (34). Briefly, fluoresceinated microbeads coated with enzyme-linked immunosorbent assay (ELISA) capture antibodies (BD Biosciences Pharmingen) were added to 50 µl of culture supernatant. Beads were centrifuged after a 45 min incubation period at room temperature, and then washed twice. Biotinylated detection antibodies (BD Biosciences Pharmingen) were added, incubated for 15 min, washed, and subsequently PE-labeled streptavidin was added. Cytokines were measured using a Luminex model 100 (Luminex Corporation, Austin, TX), and results were analyzed using LMAT software (Becton Dickinson).

Measurement of antigen (PG)-specific antibodies

PG-specific antibodies were measured by ELISA as described (19,28). ELISA 96-well plate (Corning) was coated overnight with hPG (0.1 µg protein/well) or mPG (0.15 µg protein/well), and the free binding sites were blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and both total anti-PG antibodies and isotypes of PG-specific antibodies were determined using peroxidase-conjugated goat anti-mouse IgG (Accurate Chemical & Scientific Corp., Westbury, NY) or rat mAbs to mouse IgG1 or IgG2a (BD Biosciences Pharmingen) as secondary antibodies (28). Serum antibody levels were calculated relative to the corresponding mouse IgG isotype (IgG1 or IgG2a) standards (all from BD Biosciences Pharmingen) or mouse serum immunoglobulin fractions (Sigma-Aldrich, St. Louis, MO) (19,28).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the arthritis score at different time points and *in vitro* tests were carried out using the nonparametric Mann-Whitney U test using Prism software. Significance level was set at ($p<0.05$).

Results

In vivo expression of the transgenic TCR

As compared to wild-type littermates, TCR-5/4E8-Tg thymocytes showed an increase in $CD4^+CD8^-$ population (Fig. 1A), which was expected due to the 5/4E8 hybridoma being a $CD4^+$ T cell clone. Importantly, almost all thymocytes (~86%) from TCR-5/4E8-Tg mice displayed the transgenic TCR Vβ4 chain (Fig. 1A). Spleen cells in naïve TCR-5/4E8-Tg and wild-type (littermate) mice were also analyzed for surface marker expression. The CD4/CD8 ratio in the spleen of TCR-5/4E8-Tg mice was significantly higher (9:1) than in wild-type animals (2:1) (Fig. 1B).

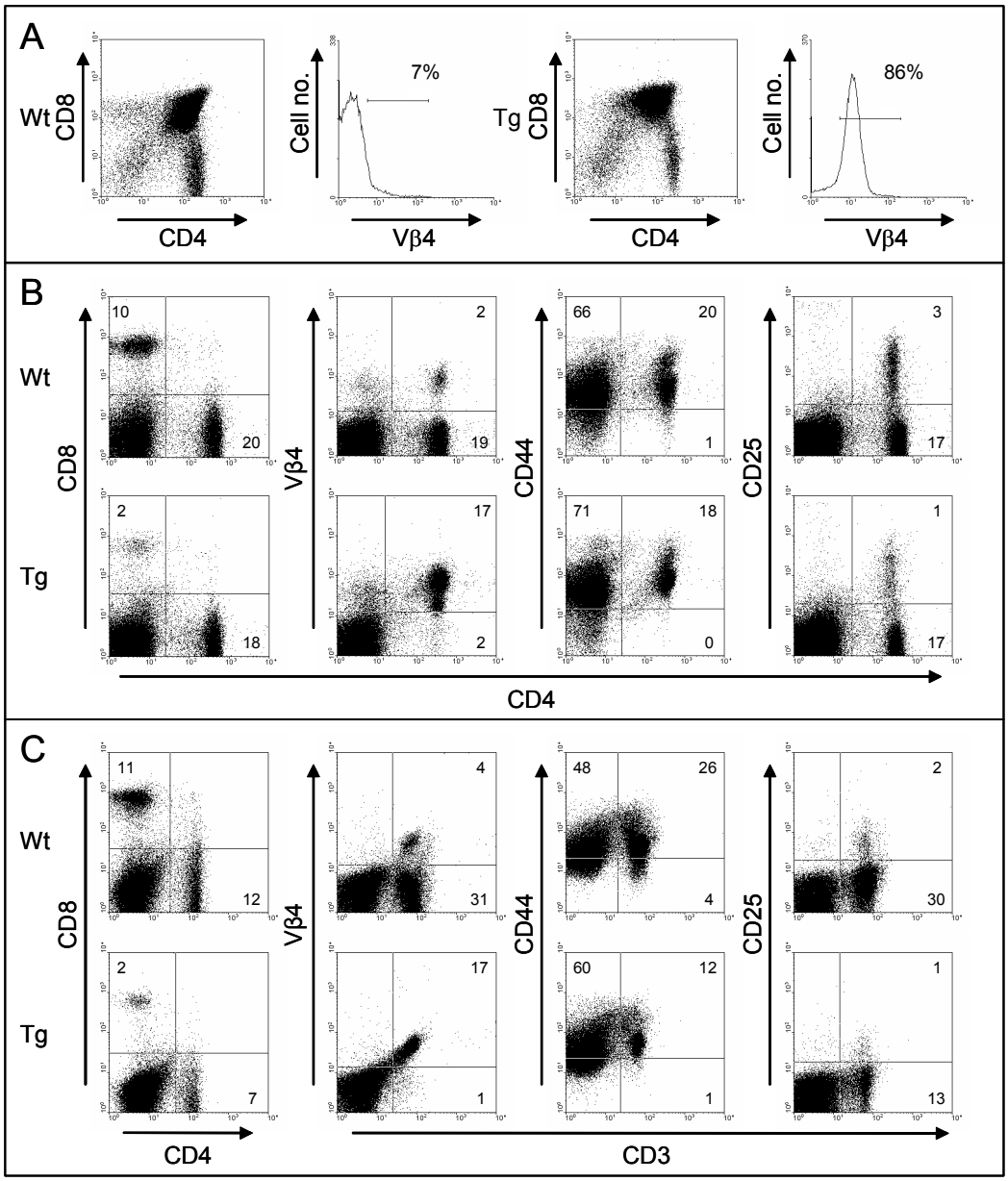

Figure 1. *Flow cytometric analysis of the expression of cell surface molecules in TCR-5/4E8-Tg mice. Cells were isolated from the thymus (**A**) and spleen (**B**) of naïve (non-immunized) mice, or spleens (**C**) of hPG-immunized arthritic TCR-5/4E8-Tg mice and their wild-type (Wt) littermates. The percentages of single or double positive cells are indicated in the quadrants of the scatter plots or in the histograms.*

Nearly all CD4+ T cells in the transgenic mice expressed the Vβ4 chain, whereas the CD4/Vβ4+ cell number was low in wild-type littermates (Fig. 1B). The T/B cell ratio measured by CD4/CD19 expression was comparable in TCR-5/4E8-Tg and wild-type mice (data not shown). Also, the expression levels of CD25 or CD44 were comparable in either spleen (Fig. 1B) or popliteal lymph node (data not shown) cells of TCR-5/4E8-Tg and wild-type mice, and the Vβ4 chain was present in the majority of TCR-5/4E8-Tg CD3+ T cells (Fig. 1C).

Arthritis in TCR-5/4E8-Tg mice

Since the TCR-αβ-5/4E8 was expressed already in heterozygous TCR-5/4E8-Tg mice, and because these CD4+ T cells were hPG-reactive (without priming) and also cross-reacted with mPG, we were interested in determining whether the peripheral T cells in transgenic mice were 'spontaneously' activated by endogenous mPG. Spontaneous activation of the self-reactive T cells might cause autoimmune arthritis in TCR-5/4E8-Tg mice. Indeed, during the backcrossing process to BALB/c (H-2^d), some TCR-5/4E8-Tg mice spontaneously developed arthritis (<15%). However, the majority of TCR-5/4E8-Tg mice, even on a BALB/c background, remained symptom-free.

We injected TCR-5/4E8-Tg and wild-type BALB/c mice with hPG in DDA IP on day 0 and, if necessary, on day 21, using a standard immunization protocol (19,21). At the time of relatively early (5th-7th) backcross level into BALB/c mice, approximately 30% of the TCR-5/4E8-Tg mice developed arthritis after the first hPG injection, which occurred only 6-7 days after the second hPG/DDA injection in wild-type littermates (Fig. 2).

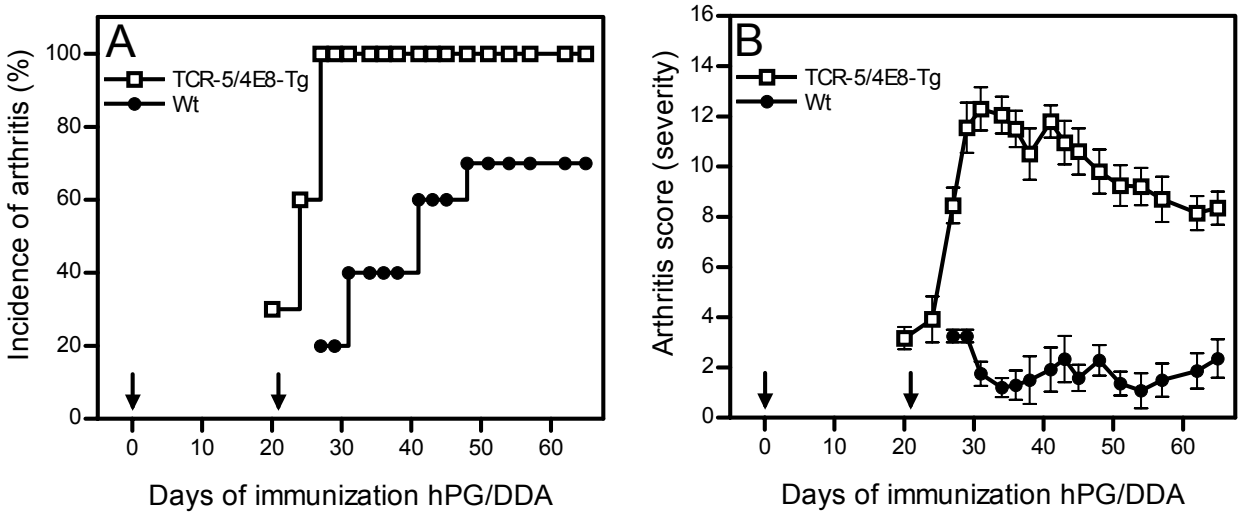

Figure 2. *Incidence and severity of arthritis in TCR-5/4E8-Tg and wild-type (littermate) BALB/c mice. Mice were immunized with hPG in DDA on days 0 and 21 (arrows). The open squares represent the heterozygous TCR-5/4E8-Tg and the black circles represent the wild-type littermates (Wt). (**A**) Incidence of arthritis is expressed as the percentage of arthritic animals relative to the total number of TCR-5/4E8-Tg or wild-type mice (n = 10 in each group). (**B**) Disease severity is expressed as the mean of cumulative arthritis score in arthritic animals only. Results are the mean ± SEM of 3 independent experiments using heterozygous TCR-5/4E8-Tg mice and their littermates after 5-6 backcrosses to BALB/c. Severity of arthritis was significantly different between TCR-5/4E8-Tg and wild-type mice (p<0.0001) from day 27 till the end of the experiment (day 67).*

Arthritis progressed and, by day 30-45 after the first hPG injection, all peripheral joints of all TCR-5/4E8-Tg mice became inflamed and/or deformed, and a second hPG injection was needed only to accelerate the onset of arthritis. When negative TCR-5/4E8-Tg mice and their wild-type littermates were boosted on day 21 (Fig. 2), the incidence of arthritis increased quickly and reached 100% within a few days in TCR-5/4E8-Tg mice with more severe arthritis than in wild-type littermates (Fig. 2A-B). Up to 70% of the wild-type littermates also developed arthritis after the second hPG/DDA injection, but with later onset and milder clinical symptoms than TCR-5/4E8-Tg mice (Fig. 2).

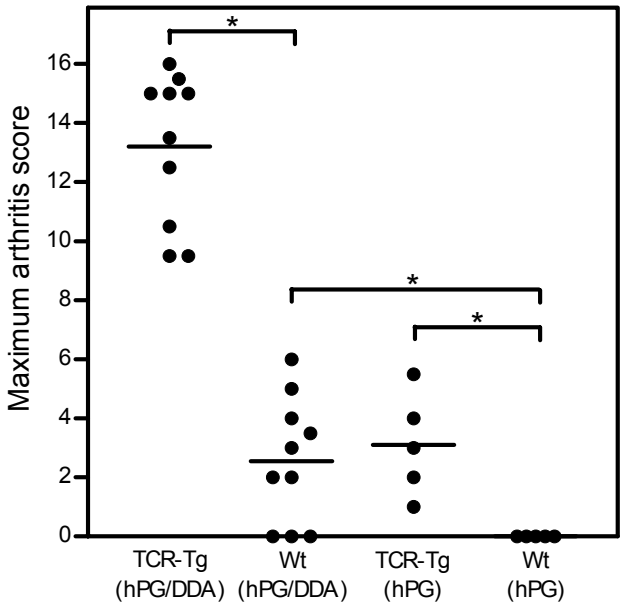

Figure 3. *Immunization of TCR-5/4E8-Tg (TCR-Tg) mice with hPG (without adjuvant DDA) produced mild arthritis, but no inflammation was observed in wild-type (Wt) littermates. TCR-5/4E8-Tg and their wild-type littermates (at backcross level 8) were also immunized with hPG and adjuvant DDA (hPG/DDA). The maximum arthritis score, achieved at any time-point after the immunization, is shown. Significant differences ($p<0.05$) are indicated by the asterisks.*

We also tested whether hPG alone (without adjuvant) could induce arthritis in TCR-5/4E8-Tg mice. These experiments indicated that hPG without adjuvant could induce arthritis in TCR-5/4E8-Tg mice, but not in wild-type animals (Fig. 3). Although disease severity was lower in adjuvant-free hPG-injected than in hPG/DDA-injected TCR-5/4E8-Tg mice, our results suggested that constitutive (over)expression of the TCR-αβ-5/4E8 partially bypassed the requirement for adjuvant in arthritis induction. The clinical appearance and histopathological features of arthritis in TCR-5/4E8-Tg mice with comparable scores and onset (Fig. 4B) were very similar to the primary form of PGIA in wild type BALB/c mice (18,20). Joint inflammation resulted in pannus formation, extensive cartilage and bone erosions, leading to massive ankylosis and deformities of the peripheral joints in TCR-5/4E8-Tg mice (Fig. 4B), as reported for PGIA in wild-type BALB/c mice (18-20).

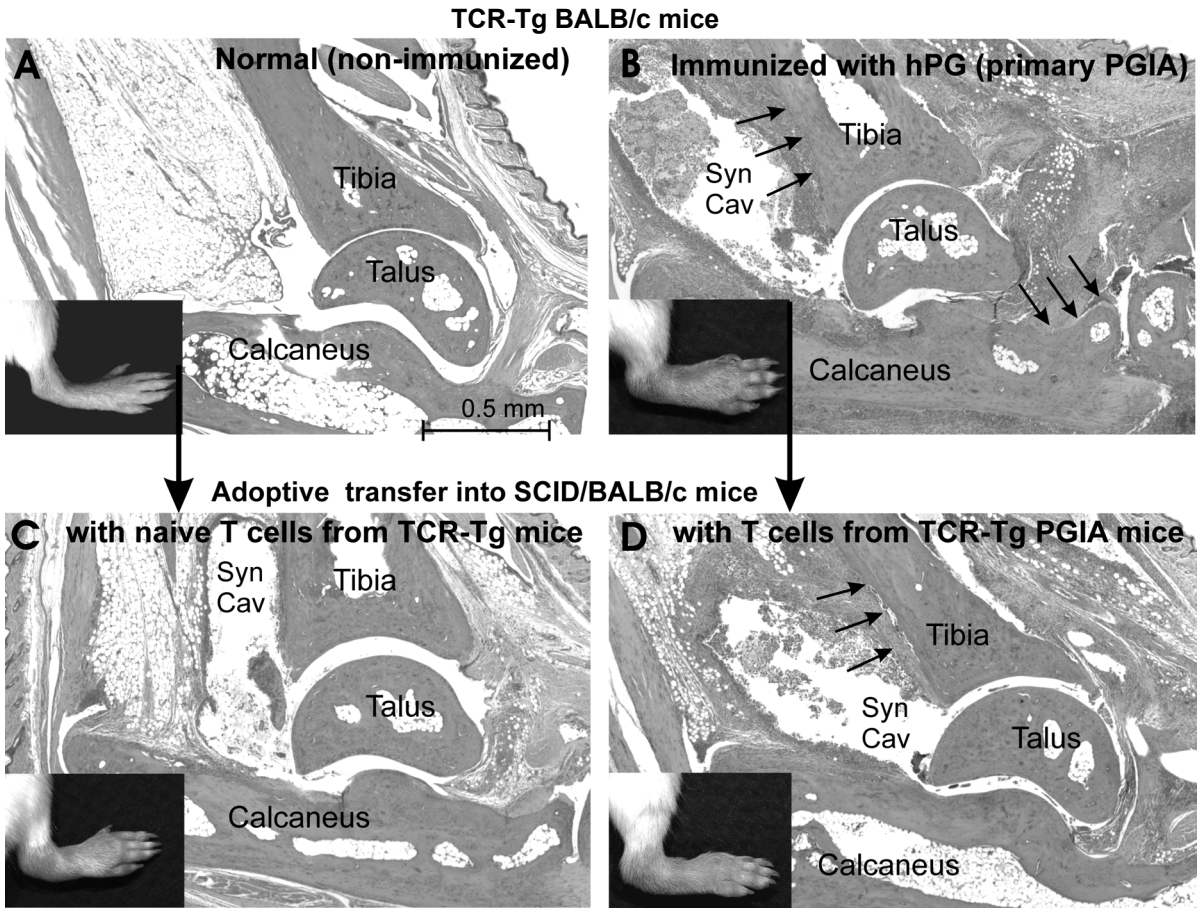

Figure 4. *Histological analysis of TCR-5/4E8-Tg BALB/c mice. Histology of ankle joints of non-immunized (**A**), human PG (hPG)-immunized TCR-5/4E8-Tg BALB/c mice (**B**), and after adoptive transfer into BALB/c.SCID mice (**C-D**). These BALB/c.SCID mice received spleen cells IP (30 x 10^6 and 15 x 10^6 cells one week apart) from naïve (non-immunized) TCR-5/4E8-Tg mice (C) or from hPG-immunized arthritic TCR-5/4E8-Tg mice (D). The first cell injection was given along with 100 μg of hPG. Arthritic mice were sacrificed for histology 6-7 days after the onset of arthritis, when the joints showed massive cartilage and bone erosions (arrows). SynCav indicates expanded synovial cavity filled with a large volume of synovial fluid, and inflammatory cells. Synovial hyperplasia accompanied by infiltrating cells and pannus formation is evident in all arthritic joints (B-D). The overall histopathology of joint inflammation and tissue destruction in a BALB/c.SCID mouse receiving spleen cells from naïve TCR-5/4E8-Tg mice (C) is similar, but the joint damage is less extensive than in another BALB/c.SCID mouse that received spleen cells from arthritic TCR-5/4E8-Tg mice (D). Sections are stained with hematoxylin and eosin.*

Recognition of hPG P70-5/4E8 peptide and hPG by peripheral T cells of TCR-5/4E8-Tg mice

Peripheral T cells of transgenic mice were examined for *in vitro* responses to the hPG P70-5/4E8 peptide and hPG, respectively. Spleen cells from naïve TCR-5/4E8-Tg mice proliferated vigorously when stimulated with the P70-5/4E8 peptide or hPG (Fig. 5A), but did not respond to the control peptide (data not shown). hPG specific response was not detected in spleen cell cultures from naïve wild-type littermates (Fig. 5A). Although T cell responses to hPG or hPG P70-5/4E8 peptide were higher in naïve TCR-5/4E8-Tg mice than in hPG-immunized and arthritic transgenic or wild-type mice, T cell proliferation in the presence of self mPG P70 peptide (5/4E8 epitope homologue) was detectable only in cell cultures of arthritic mice (Fig. 5A).

IL-4 and IFNγ production was detected in naïve TCR-5/4E8-Tg mice after *in vitro* stimulation with hPG P70-5/4E8 peptide, which then dramatically shifted toward the Th1 bias (IL-4 < IFNγ) at the time of onset of arthritis (Fig. 5B). In contrast, the production of IFNγ after *in vitro* stimulation was much higher in hPG/DDA-immunized arthritic TCR-

5/4E8-Tg mice. These data indicate that T cells from naïve transgenic mice were not tolerized, instead, they appeared to be differentiated *in vivo* because they responded vigorously to the hPG P70-5/4E8 peptide by proliferation, as well as by IFNγ and IL-4 production.

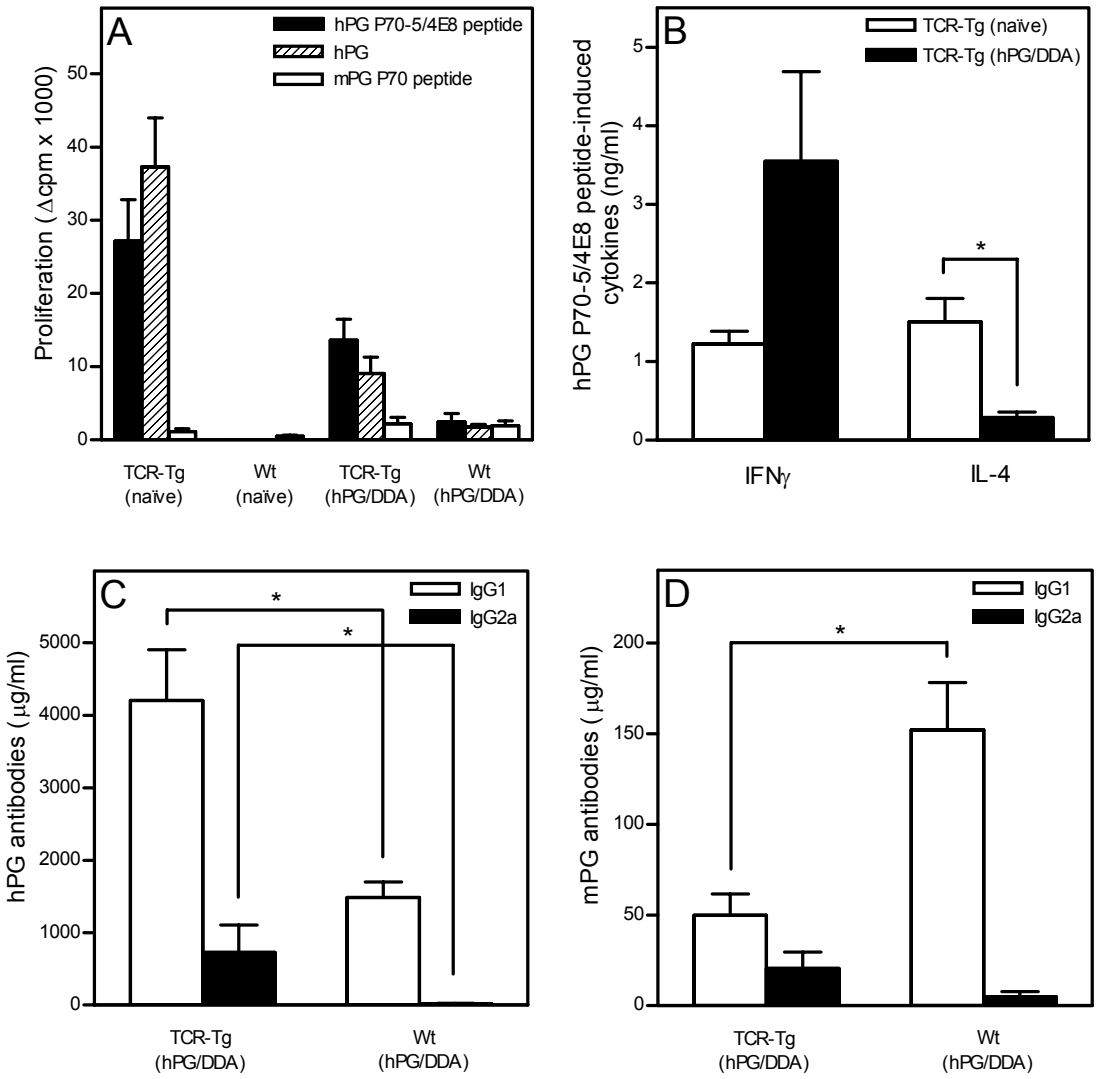

Figure 5. *Analysis of antigen-specific T- and B-cell responses in naïve and arthritic TCR-5/4E8-Tg mice. hPG-immunized (hPG/DDA) arthritic TCR-5/4E8-Tg and wild-type mice were sacrificed 9 days after the onset (~ day 31). Naïve TCR-5/4E8-Tg and wild-type (Wt) mice were used as controls. Values are mean ± SEM (n = 4-10). Levels of significance (*p<0.01) are indicated. (**A**) Proliferation assays: spleen cells (2 x 10^5/well) were cultured in the presence of P70-5/4E8 peptide (0.1 μg/ml; closed bars), hPG (10 μg/ml; dashed bars) or mPG P70 peptide (50 μg/ml; open bars). The results are expressed as Δ cpm ± SEM. (**B**) Cytokine production: Supernatants of hPG P70-5/4E8 peptide-stimulated spleen cell cultures of naïve and hPG-immunized (arthritic) TCR-5/4E8-Tg were harvested after 72 h and assayed by Luminex analysis for IFNγ and IL-4. Values of naïve TCR-5/4E8-Tg mice are indicated by open bars and values of hPG/DDA immunized TCR-5/4E8-Tg mice by closed bars. (**C-D**) Serum anti-PG antibodies: PG-specific antibodies to immunizing hPG (C) and mPG (D) in the sera of arthritic TCR-5/4E8-Tg and wild-type mice (IgG1, open bars; IgG2a, closed bars) are shown.*

PG-specific antibodies could not be detected in non-immunized mice but were present at high levels in hPG-immunized wild-type and TCR-5/4E8-Tg mice. Most of the antibodies were specific for the immunizing hPG (Fig. 5C), although there was a clear antibody response against self-PG (mPG) (Fig. 5D). The amount of hPG-specific IgG1

and IgG2a antibodies were significantly higher in TCR-5/4E8-Tg than in wild-type mice ($p<0.01$). While antibodies of the IgG1 isotype were predominant against either hPG (Fig. 5C) or mPG (Fig. 5D) in both TCR-5/4E8-Tg and wild-type BALB/c mice, the IgG2a/IgG1 ratios of antibodies to either hPG or mPG were about 10 times higher in transgenic mice than the ratios in wild-type control mice.

Adoptive transfer to BALB/c.SCID mice

To test whether spleen cells of TCR-5/4E8-Tg mice could transfer arthritis adoptively, we injected unseparated spleen cells from naïve and arthritic TCR-5/4E8-Tg, and from arthritic wild-type (BALB/c) mice into syngeneic BALB/c.SCID recipient mice. The results of these transfer experiments are summarized in Table 1. When SCID mice were injected with naïve TCR-5/4E8-Tg cells alone (without activation), no arthritis was adopted. However, when these naïve TCR-5/4E8-Tg cells were injected together with either hPG P70-5/4E8 peptide or hPG, some of the recipient animals developed arthritis (Table 1). The onset of arthritis was relatively late (~ day 27), with a maximum incidence of 57% (Table 1). In contrast, spleen cells from arthritic TCR-5/4E8-Tg mice could induce arthritis upon a single transfer, which was even faster and more severe when cells from arthritic mice were co-injected with either hPG P70-5/4E8 peptide or hPG. Remarkably, in this case only one cell transfer was sufficient to induce arthritis in BALB/c.SCID recipient mice (Table 1), which can not be easily achieved using spleen cells from arthritic wild-type BALB/c mice (28). The clinical appearance of the disease and the histopathology of acutely inflamed joints of adoptively transferred arthritis from TCR-5/4E8-Tg and wild-type mice were similar (Figs. 4C-D), and indistinguishable from those described in primary PGIA (18,20). Synovial hyperplasia accompanied by infiltrating cells and pannus formation was evident in all arthritic joints. The overall histopathology of joint inflammation and tissue destruction in BALB/c.SCID mice that received spleen cells from naïve TCR-5/4E8-Tg mice was somewhat less extensive than in the BALB/c.SCID mouse that received spleen cells from arthritic TCR-5/4E8-Tg mice (Figs. 4C-D).

Table 1. Summary of adoptive transfer experiments using spleen cells from wild-type and TCR-5/4E8-Tg mice into syngeneic BALB/c.SCID mice

Source of donor spleen cells	Number of cells (x 10^6) per transfer*	Challenging antigen (peptide or hPG)*	Arthritic/ total number of animals	Incidence (%)	Onset (day)		Maximum severity† (arthritis score ± SD)
					Earliest	Mean (± SD)	
Naïve TCR-Tg Mice	up to 4x 30	None	0/8	0	NA	NA	NA
	30 + 15	P70-5/4E8	2/10	20	24	28.0 ± 5.7	3.25 ± 1.77
	30 + 15	hPG	8/14	57	22	26.3 ± 4.0	5.43 ± 2.99
Arthritic TCR-Tg mice (PGIA)	30 + 15	None	7/10	70	14	18.4 ± 4.6	4.14 ± 4.68
	30	P70-5/4E8	8/9	89	16	18.6 ± 2.8	9.75 ± 4.98
	30	hPG	13/15	87	6	9.8 ± 2.9	8.85 ± 2.94
Arthritic wild-type (BALB/c) mice (PGIA)	30 + 15	None	1/15	6.7	42	42	3
	30 + 15	P70-5/4E8	1/9	11	36	36	4
	30 + 15	hPG	15/17	88	7	12.1 ± 3.3	9.53 ± 4.63

**Spleen cells (30 x 10^6) were injected IP alone or with 100 µg hPG P70-5/4E8 peptide (ATEGRVRVNSAYQDK) or 100 µg hPG. A second spleen cell transfer (15 x 10^6 cells), if indicated, was given one week later without peptide or hPG as described in the Methods section. The first group received 4 times 30 x 10^6 spleen cells from naïve (non-immunized) TCR-5/4E8-Tg mice.*
†Animals were scored daily for arthritis symptoms, and all were sacrificed on days 49-52 after the first transfer. Adoptive transfer experiments were performed after the backcrossing process into BALB/c background was completed. NA = not applicable, hPG = human proteoglycan.

Discussion

PGIA is a T cell-dependent and antibody (B cell)-mediated autoimmune model of RA (20). Here, we describe a novel and exaggerated model of PGIA, wherein TCR-5/4E8 transgenic T cells, mostly $CD4^+$, respond only to a single arthritogenic epitope of human cartilage PG. These TCR-5/4E8-Tg mice represent a unique source of naïve antigen (arthritogenic epitope)-specific T cells that are capable of inducing progressive chronic arthritis. Histological analysis of inflamed joints showed extensive cartilage and bone erosions similar to those seen in arthritic joints of wild-type BALB/c mice, and was reminiscent of the histopathological appearance of RA-affected joints. Arthritis onset, however, is much faster and the disease is more severe in TCR-5/4E8-Tg mice compared to wild-type littermates.

$CD4^+$ T cells of the TCR-5/4E8-Tg mice carrying $V\alpha1.1$ and $V\beta4$ TCR chains with MHC-II restricted specificity for the consensus sequence of ^{73}GRVRVNSAY of hPG (16) were positively selected in the thymus and exported to the periphery where they constituted the vast majority of T cells (Fig. 1). The dominant arthritogenic hPG P70-5/4E8 peptide (the consensus sequence flanked with 3 amino acids at both sides) induced T-cell proliferation (Fig. 5A) indicating that a functional TCR was indeed generated in the TCR-5/4E8-Tg mice. In contrast to the classical (original) form of PGIA in wild-type BALB/c mice that required multiple immunizations with hPG in adjuvant (18,20,21), a single dose of hPG, even in the absence of adjuvant, produced disease in TCR-5/4E8-Tg mice, whereas the injection of the hPG P70-5/4E8 peptide or adjuvant DDA alone did not induce arthritis. Altogether, this new model of PGIA is much more efficient in the sense that it is epitope-restricted. Moreover, as ~90% of $CD4^+$ T cells carries hPG P70-5/4E8-specific TCRs (Fig. 1A), this transgenic model offers an excellent opportunity to test T cell activation events via a single epitope-specific TCR.

Flow cytometric analysis of cells from TCR-5/4E8-Tg mice showed a marked reduction in the $CD8^+$ thymocyte population (Fig. 1A). This reduction, however, was expected based on the fact that the 5/4E8 epitope was class II-restricted. Shrinkage of the $CD8^+$ T cell pool has also been observed in other MHC class II-restricted TCR-Tg mice expressing TCR specific for self antigens such as type II collagen (35-37) or myelin basic protein (38). Besides the reduced number of $CD8^+$ cells and the expression of TCR-$V\beta4$ on almost all $CD4^+$ T cells in TCR-5/4E8-Tg mice, the expression of all other surface markers tested on naïve T cells was comparable between TCR-5/4E8-Tg and wild-type mice. *In vitro* studies (Fig. 5) however, showed extensive proliferation of TCR-5/4E8-Tg T cells in response to either hPG P70-5/4E8 peptide or hPG.

Both IL-4 and IFNγ cytokine-producing cells were present, in either naïve or hPG-immunized transgenic mice prior to the onset of inflammation (data not shown). However, the IL-4:IFNγ ratio shifted significantly toward a Th1 dominance at the time of onset of arthritis, illustrating a dynamic polarization during *in vivo* T cell activation. The presence of antibodies to both hPG and mPG in sera of arthritic TCR-5/4E8-Tg mice demonstrated that $CD4^+$ transgenic T cells were capable of providing sufficient help *in vivo* for PG-specific B cells (39). Autoantibodies, such as those produced against mPG, could play a role in initiating inflammation in the joints by binding to the cartilage surface (40), and inducing chemokine and complement-dependent leukocyte recruitment (41).

A remarkable observation of this study was that we could transfer the disease into syngeneic BALB/c.SCID recipients using splenocytes from naïve TCR-5/4E8-Tg mice, upon activation of these cells not only with hPG, but also with hPG P70-5/4E8 peptide. Moreover, spleen cells of arthritic TCR-5/4E8-Tg donor mice were able to induce arthritis in

recipient BALB/c.SCID mice without exogenous hPG or specific peptide, which was not possible using splenocytes from arthritic wild-type donor BALB/c mice (20,27,28). In addition, adoptive transfer of PGIA was achieved by the injection of cells from arthritic TCR-5/4E8-Tg mice (Table 1). It is conceivable that T cells from arthritic TCR-5/4E8-Tg mice migrate to the joints upon adoptive transfer and become reactivated by mPG in the mouse joint where self-peptides are released during the normal turnover of the cartilage matrix. Production of mPG peptides might be increased when PG degradation occurs in the inflamed joint, thus amplifying the inflammatory response.

Therapeutic efficacy of agents that can block T cell stimulation or deplete B cells has confirmed the pathogenic role of the adaptive immune system in RA (1,6-8). Adaptive immune responses seem to play an important role in PGIA as well (20). Although the critical function of $CD4^+$ T cells was implicated in arthritis induction (29,39,40,42), both autoreactive T cells and B cells, and possibly autoantibodies, are required for the development of severe arthritis (20,43), indicating that B cell-mediated effector pathways contribute significantly to PGIA. Cooperation between antigen-primed $CD4^+$ Th1 T cells and B cells appears to be essential for the development of a chronic progressive disease in this model (20,39); similar mechanisms may be involved in the pathogenesis of RA (3,44,45).

T and B cell responses against cartilage matrix components do occur in RA, at least in a subset of patients, although the pathogenic role of these autoimmune responses is controversial (1,46,47). Among these matrix components, hPG could be a candidate autoantigen in humans. Relevant to this, we showed earlier that several predicted epitopes within the core protein of hPG were recognized by T cells in context with human MHC class II in HLA-DR4 and HLA-DQ8 transgenic mice (48). Collectively, the results of the present and earlier studies suggest that hPG, especially the 5/4E8 epitope, plays a critical role not only in PGIA, but more importantly, this epitope may become a target of disease associated T cell responses in patients with RA (12-14).

Based upon the clinical and histopathological features and the autoimmune aspects shared between PGIA and RA (20), we believe that these TCR-5/4E8-Tg mice are a valuable tool for further analysis of the mechanisms associated with the initiation and pathogenesis of autoimmune arthritis; more specifically, for the analysis of the role of antigen specific T cells in disease development. Our TCR-Tg model also supports the hypothesis that antigen-specific T cells play a critical role in the initiation of arthritis, as disease could be induced in the absence of adjuvant. Given the paucity of relevant animal models for RA, this novel TCR-Tg mouse model is a valuable addition to the arthritis research armamentarium. Furthermore, these TCR-5/4E8-Tg mice may be useful for developing new immunomodulating agents, which might provide further insights into the immunopathogenic mechanisms of RA.

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Chapter 6

Cartilage proteoglycan-specific T cell response in rheumatoid arthritis and osteoarthritis

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Abstract

Objective. Antigen-specific T cell responses to cartilage matrix proteins (among which proteoglycan (PG) aggrecan) are present in a subset of patients with rheumatoid arthritis (RA). Potential T cell epitopes of the core protein of human PG have been mapped in HLA-DR4- and HLA-DQ8-humanized transgenic mice. The objective of this study was to determine whether these epitopes are recognized by peripheral blood lymphocytes from patients with RA or osteoarthritis (OA), and healthy controls.

Methods. Peptide-specific T cell proliferation in response to 14 human PG-specific peptides was measured in 60 RA, and 32 OA patients and 32 healthy controls. In addition, the release of 26 cytokines/chemokines (determined by multiplex immunoassay) was determined in responding patients.

Results. PG-specific peptides in the amino acid region 16-39 and region 263-282 of the G1 domain of human PG were most frequently recognized by T cells and induced significant T cell proliferation in a subset of human patients with RA or OA. PBMCs from RA and OA patients showed increased production of proinflammatory cytokines in response to the PG peptides. PG region 263-282 had a striking sequence homology with Yersinia Yop protein and therefore, the homologous bacterial (Yersinia) peptide was also tested. Remarkably, RA and OA patients responding to the Yersinia peptide also responded to the PG peptide, suggesting a possibility of molecular mimicry in these patients.

Conclusion. These results indicate that PG-specific peptides, located in the G1 domain of human PG, can induce autoantigenic T cell responses *in vitro*, suggesting that this might be involved in RA pathomechanisms.

Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovium of diarthrodial joints leading to the destruction of cartilage and bone erosions. Although the pathogenesis is not completely understood, antigen-driven autoimmune responses are proposed to mediate joint pathology. As the primary target organ is the synovial joint, candidate autoantigens include type II, IX, and XI collagens (1), human cartilage glycoprotein-39 (HC-gp39) (2), link protein (3,4) and proteoglycan aggrecan (PG) (5,6). However, there is no clear evidence yet that any macromolecule of cartilaginous tissues, bone or synovium would serve as a preferential autoantigen (7).

Cartilage is one of the immunologically privileged tissues in the body as it is essentially avascular and therefore not subjected to immunological surveillance. Cartilage PG aggrecan, a major structural component of the extracellular matrix of cartilage, is a complex macromolecule. The core protein contains the globular domains (G1, G2 and G3) and two glycosaminoglycan attachment regions (8). The presence of the large number of glycosaminoglycan side chains (over 120 per PG molecule), which otherwise are ubiquitous components in all tissues of all mammalian species, may mask the antigenic sites of the core protein, thus intact PG is a relatively weak antigen. Some structural changes, such as partial deglycosylation (9) or cleavage of the core protein of PG by various metalloproteinases (10,11), occur *in vivo* during the normal turnover of cartilage PG (aging) and these processes are more extensive in inflammatory conditions (12). Fragments released during PG degradation may trigger and/or maintain local immune reactions in the synovial joints in arthritis-susceptible animals, and perhaps in humans as well.

Indeed, immune responses to human cartilage PG aggrecan (henceforth designated hPG) have occasionally been detected in patients with RA, juvenile idiopathic arthritis (JIA), osteoarthritis (OA) and ankylosing spondylitis (AS) (4,13-17) supporting the hypothesis that, among other candidate autoantigens, cartilage PG might be an autoimmune target in RA. Subsequently, autoreactive T cells to the G1 domain of cartilage PG were detected in peripheral blood and synovium of RA patients (17), and soon after, a few T cell epitopes of the G1 domain have been identified using overlapping synthetic peptides (18).

Defining T cell epitopes of hPG recognized in RA is essential for the understanding of the role of PG-specific immune responses in the autoimmune process of RA. A general problem with epitope mapping studies in humans is the extremely high genetic variation within the human population. In addition, the large core protein (~2,400 amino acids) of hPG is another major limit of epitope mapping studies. Therefore, to determine the epitope repertoire of hPG is a tedious task. In support of human studies, we have generated human leukocyte antigen (HLA)-humanized transgenic mice in an arthritis-susceptible genetic background expressing the most predictive major histocompatibility (MHC) alleles in RA patients, such as DR4 and DQ8, but lacking their own MHC (H-2^d) alleles (19). A total of 143 predicted T cell epitopes were tested (19). This experimental system identified T cell epitopes of hPG that were presented by RA-predisposing HLA molecules and thus are potential targets of autoreactive T cells in RA.

In this study we tested the T cell responses to the positive (pre-selected) peptide sequences in RA and OA patients, and in healthy individuals to address the question whether the epitopes found in HLA transgenic mice (19) were recognized by human lymphocytes *in vitro* using peptide-stimulated peripheral blood mononuclear cells (PBMCs). Indeed, some of these hPG epitopes were frequently recognized by lymphocytes of RA and OA patients and induced

proinflammatory cytokine production, thus underscoring that T cell response to these epitopes may be involved in the pathogenesis.

Materials and Methods

Participants

Heparinized blood samples were collected from RA patients (n=60), OA patients (n=32) and healthy controls (n=32). The patients were visiting the outpatient clinic of the departments of Rheumatology & Clinical Immunology and Orthopedic Surgery of University Medical Center Utrecht (Utrecht, The Netherlands). The RA patients fulfilled diagnostic criteria of the American College of Rheumatology criteria for RA (20). The demographic and clinical characteristics of the patients and healthy controls are shown in Table 1. The RA patients formed a transversal group of patients with longstanding RA.

Table 1. Demographic and clinical characteristics of patients and healthy controls.

	RA	OA	HC
Number	60	32	32
Sex (male/female)	17/43	12/20	15/17
Age (years ± SD)	60.5 ± 13.4	59.4 ± 11.7	36.9 ± 9.8
*Disease duration (years ± SEM)	13.2 ± 1.4	6.3 ± 1.3	NA
Radiographic changes (non-erosive/erosive)	11/49	1/31	ND
RF (negative/positive)	17/41	ND	ND
ESR (mm/hr ± SEM)	26.4 ± 2.9	ND	ND
Treatment (no DMARD/DMARD)	5/55	ND	ND

HC, healthy controls; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; DMARD, disease-modifying anti-rheumatic drug; NA, not applicable; ND, not determined.
**Years from the date of established diagnosis*

Peptide selection and synthesis

Peptide sequence selection was based upon theoretical MHC-binding motifs (21) and T cell epitopes identified in HLA-DR4 and DQ8 transgenic mice (19). Based on this selection, a total of 14 hPG core protein-specific peptides (located in 3 different regions within the G1 domain and 1 region within the G3 domain) were synthesized by the pin method (Chiron Mimotopes Pty., Raleigh, NC) (22). These peptides were used for initial screening of T cell proliferation. Peptide sequences that elicited T cell responses were further fine-tuned using shifted sequences based on predicted DR4 and DR1 binding scores (Table 2) and synthesized in mg quantities with amide-blocked C terminus for large-scale studies (Research Genetics, Inc., Huntsville, AL; or Alpha Diagnostic International, San Antonio, TX). Peptides were dissolved (10 mg/ml), diluted to 1 mg/ml in phosphate buffered saline (PBS), and stored at -20^{o}C. Preliminary experiments were performed to identify the optimal peptide concentration for the lymphocyte proliferation assay. Dose-response curves (10-50 μg/ml) showed that approximately 50 μg/ml achieved an optimal and reproducible peptide concentration in *in vitro* tests (data not shown) and therefore this concentration was used for all epitope-screening studies.

Table 2. Human cartilage PG peptide sequences.

Peptide code†	Amino Acid Sequence	DR1 Binding score	DR4 Binding score
p25-39	GTSLTIPCYFIDPMH	12	8
p16-31	QPSPLRVLLGTSLTIP	35	20
p21-36	RVLLGTSLTIPCYFID	14	14
p30-45	IPCYFIDPMHPVTTAP	21	22
p61-76	KEKEVVLLVATEGRVR	27	20
p69-85	VATEGRVRVNSAYQDKV	14	12
p76-90	RVNSAYQDKVSLPNYP	14	26
p268-282	YLAWQAGMDMCSAGW	18	16
p263-280	TTGHVYLAWQAGMDMCSA	30	26
p272-289	QAGMDMCSAGWLADRSVR	25	14
p278-295	CSAGWLADRSVRYPISKA	17	22
p280-294	AGWLADRSVRYPISK	17	3
p283-300	LADRSVRYPISKARPNCG	17	14
p2379-2394	LQKRSSRHPRRSRPST	15	6
p263-282	TTGHVYLAWQAGMDMCSAGW	30/18	26/16
pYersinia 68-82	QKQLGWQAGMDEART	22	8
	* ******		

hPG peptide sequences containing T cell epitopes within the core protein of PG were revealed from mapping studies in HLA-Tg mice (19). Four sequence regions, p16-45, p61-90, p263-300 and p2379-2394 were tested for T cell responses (proliferation and cytokine production) in RA and OA patients and healthy controls. The amino acid sequences of the peptides most positive in HLA-Tg mice (underlined) (19) were extended with their flanking amino acids based on theoretical binding scores given by motif-based prediction algorithm in SYFPEITHI database (http://www.syfpeithi.de) (36), until the peptides with the extended flanking region showed high, occasionally even higher scores, then the positive peptides in HLA-Tg mice. hPG peptides p16-45 and p263-282 sequences were searched for homologies in protein genbank databases. Alignment of hPG p263-282 revealed homology with Yop translocation protein L of various Yersinia subspecies e.g. Yersinia enterocolitica (Genbank accession number Q01253, gi:267573). Identical amino acids are indicated by asterisks. † Residue numbers of the amino acid position in human cartilage PG (Genbank accession no. NP_001126, gi:4501991).

Measurement of antigen-specific T cell responses

Antigen-specific proliferative activity of lymphocytes from patients with RA was compared with PBMCs obtained from OA patients and healthy controls. PBMCs were isolated by Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation of heparinized blood. RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin and streptomycin (Gibco BRL, Gaithersburg, MD, USA), and 10% AB-positive human serum (Sanquin Blood Bank, Utrecht, the Netherlands) was used as culture medium. Cells were cultured (2 x 10^5 cells in 200 µl per well) in triplicate in round-bottom 96 well plates (Nunc, Roskilde, Denmark) for 96 h at 37°C in 5% CO_2 with 100% relative humidity, in the absence or presence of 50 µg/ml hPG peptides. Tetanus Toxoid (5 Lf/ml; RIVM Bilthoven, Netherlands) was used as positive control. A synthetic ovalbumin (OVA) peptide 323-339 (50 µg/ml) was used as

irrelevant (negative) control and a peptide (p2379-2394) of the G3 domain of hPG turned out to be a hPG-specific negative control. After 96 h, the cells were pulsed overnight with [^{3}H]-thymidine (1 μCi per well; ICN Biomedicals, Amsterdam, the Netherlands) and uptake was measured using a liquid scintillation counter (Betaplate, Wallac, Turku, Finland). The magnitude of the proliferative response was expressed as stimulation index (S.I.), which is calculated as the mean counts per minute of cells cultured with antigen (peptide) divided by the mean counts per minute of cells cultured without antigen. Stimulation indices $\geq$ 1.8 were defined as a positive response.

Multiplex cytokine analysis

For analysis of antigen-specific cytokine production, PBMCs of all patient groups were cultured as described above. Culture supernatants were collected after 72 h and stored at -80^{o}C. Concentrations of the following cytokines and chemokines, eotaxin, interferon-gamma (IFNγ), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, interferon-inducible protein-10 (IP10; CXCL10), monocyte chemoattractant protein (MCP-1; CCL2), macrophage-derived chemokine (MDC; CCL22), macrophage migration inhibitory factor (MIF), monokine induced by interferon-gamma (MIG; CXCL9), macrophage inflammatory protein (MIP-1α; CCL3), osteoprotegerin (OPG), oncostatin M (OSM), regulated upon activation normal T cell expressed and secreted (RANTES), soluble receptor activator of nuclear factor-kappa B ligand (sRANKL), thymus- and activation-regulated chemokines (Tarc; CCL17) and tumor necrosis factor-alpha (TNFα), were determined using a multiplex immunoassay (MIA; LabMAP technology, Luminex Corporation Austin TX, USA) as previously described (23). In brief, the antibody coated microspheres were incubated with standards, controls, and samples (25 μl) in 96 well 1.2 μm filter plate (Millipore, Amsterdam, the Netherlands) for 60 min at room temperature. Plates were washed and the cocktail of biotinylated detection antibodies was added for 60 min at room temperature. After repeated washings, streptavidin-phycoerythrin (PE) was added for an additional 10 min. Beads were washed twice, resuspended and fluorescence intensity was measured. Measurements and data analysis of all assays were performed using the Bio-Plex system in combination with the Bio-Plex Manager software version 3.0 using five parametric curve fitting (Bio-Rad Laboratories, Hercules CA, USA). Based on initial results (data not shown), we focused on IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ, MCP-1 and MIP-1α.

Cytokine analysis by flow cytometry

The stimulation method to measure intracellular cytokines represents a modification of the protocols previously described (24,25). PBMCs were cultured in the presence of 0.5 μg/ml anti-CD28 and 0.5 μg/ml anti-CD49d (both from Sanquin, Amsterdam, the Netherlands) with or without a hPG-peptide (50 μg/ml) for 6 h, Brefeldin A (10 μg/ml; Sigma, Zwijndrecht, the Netherlands) was added for the last 4.5 h of the stimulation. Cells were washed in ice-cold PBS, incubated in PBS containing 2mM ethylenediaminetetraacetic acid for 10 min at 37^{o}C and stained with peridinin-chlorophyll-protein (PerCP)-labeled anti-CD4 and allophycocyanin (APC)-labeled anti-CD69 or control mAbs (BD Biosciences Pharmingen, San Diego, CA) for 30 min at 4^{o}C. Subsequently, cells were fixed (Cytofix/Cytoperm; BD Biosciences Pharmingen), permeabilized (Perm/Wash; BD Biosciences Pharmingen) and

stained with PE-labeled anti-IFNγ and fluoroscein isothiocyanate (FITC)-labeled anti-TNFα (BD Biosciences Pharmingen) for 30 min at 4°C. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences Immunocytometry, San Jose, CA).

Statistical analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean (SEM). Statistical analyses of the proliferative responses were carried out using the nonparametric Mann-Whitney U test (two-tailed) using Prism software (version 3.00, Graphpad Software Inc., San Diego). Significance level was set at ($p<0.05$).

Results

PG peptide-specific proliferation in RA and OA patients

To investigate whether human cartilage PG epitopes identified in HLA transgenic mice (19) were recognized by human lymphocytes, we tested peptide-induced *in vitro* response of PBMCs of patients with RA or OA, and healthy individuals (negative controls). The demographic and clinical characteristics of patients and healthy controls are summarized in Table 1, peptide sequences and their locations in the core protein of hPG are presented in Table 2, and all proliferation results in Table 3. As determined in a set of preliminary experiments (data not shown), we considered the T cell proliferation to be positive if the stimulation index (S.I.) was higher than 1.8. We extended the pre-selected T cell epitope sequences (p25-39 and p268-282) with their flanking amino acids, because the shifted or extended sequences with various lengths of the flanking region still showed high binding scores by T cell prediction methods (Table 2).

Table 3. Results T cell proliferation assay.

Peptide code†	RA		OA		HC	
	median*	% ≥ 1.8	median	% ≥ 1.8	median	% ≥ 1.8
p25-39	1.4	29.4 (15/51)	1.1	3.1 (1/32)	1.0	7.1 (2/28)
p16-31	2.0	54.5 (12/22)	1.7	42.9 (9/21)	1.0	5.9 (1/17)
p21-36	1.7	45.0 (9/20)	1.6	42.9 (9/21)	1.1	17.6 (3/17)
p30-45	1.6	35.0 (7/20)	1.3	9.5 (2/21)	1.2	17.6 (3/17)
p61-76	1.6	35.0 (7/20)	1.3	19.0 (4/21)	1.1	12.5 (2/16)
p69-85	1.2	5.0 (1/20)	1.2	4.8 (1/21)	1.0	11.8 (2/17)
p76-90	1.0	7.7 (3/46)	0.9	0 (0/32)	1.0	5.6 (2/31)
p268-282	2.0	61.7 (29/47)	1.2	20.0 (3/15)	1.3	25.9 (7/27)
p263-280	3.4	100 (22/22)	4.0	80.1 (17/21)	1.6	35.3 (6/17)
p272-289	1.5	25.0 (5/20)	1.7	45.0 (9/20)	1.2	23.5 (4/17)
p278-295	1.2	15 (3/20)	1.2	5.0 (1/20)	1.0	5.9 (1/17)
p280-294	1.0	0 (0/15)	0.9	0 (0/11)	0.8	0 (0/15)
p283-300	1.1	5.0 (1/20)	1.1	10.0 (2/20)	1.0	0 (0/17)
p2379-2394	1.0	4.3 (1/23)	0.9	0 (0/11)	1.0	0 (0/15)

*Fourteen PG peptides were tested for recognition in RA and OA patients and healthy controls (HC). The table summarizes proliferation data, showing the median stimulation indices (S.I.) and the frequency of positive responders. A S.I. higher than 1.8 was recorded as a positive response. The numbers of responding individuals of the total number of tested individuals are shown in parenthesis. † Residue number of the amino acid position in human cartilage PG (Genbank accession no. NP_001126, gi:4501991). Human cartilage PG peptides sequences revealed from mapping studies in HLA-Tg mice (19) are underlined. * Individual T cell responses to positive or control peptides are shown in Fig. 1.*

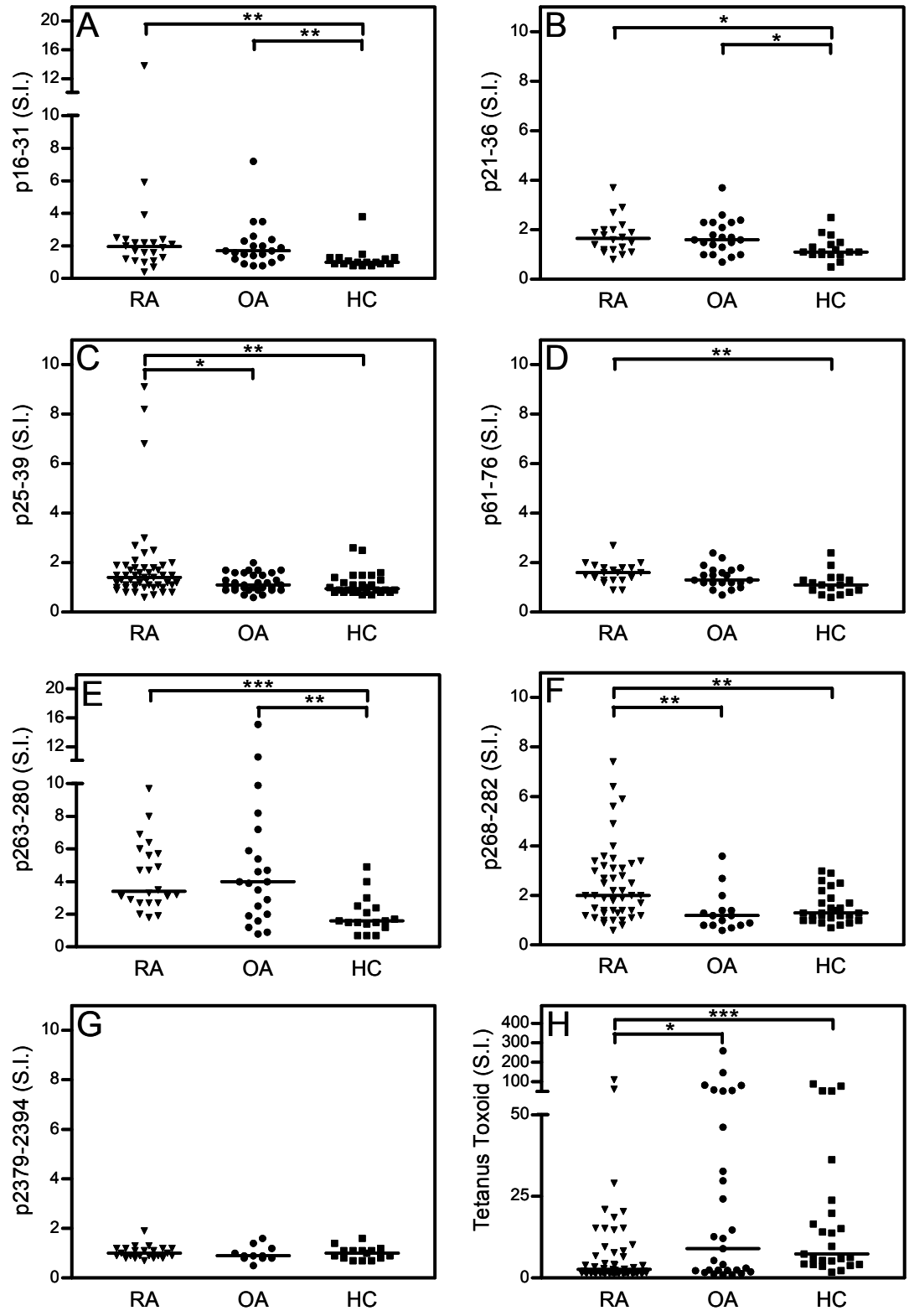

Figure 1. *Peptide-induced T cell proliferation of PBMCs from patients with RA, OA and healthy controls (HC). Only positive results with hPG peptides listed in Table 2 are shown. PBMCs were stimulated with PG p16-31 (**A**), p21-36 (**B**), p25-39 (**C**), p61-76 (**D**), p263-280 (**E**), p268-282 (**F**), p2379-2394 (**G**) or Tetanus Toxoid (**H**). Proliferation is expressed as stimulation index (S.I.) Horizontal lines show the median. Significant differences in responses between patients and controls are indicated as follows: *=p< 0.05, **=p<0.01, ***=p<0.001.*

As summarized in Fig. 1 (positive results), six of the 14 tested peptides (p16-31, p21-36, p25-39, p61-76, p263-280 and p268-280) induced significant T cell proliferation in RA patients when compared to normal healthy individuals. These six peptides represented three different regions in the G1 domain of human cartilage PG (Table 2): two were selected from a complex study of epitope-mapping, immune response and their association with arthritis in HLA-humanized mice (19), and the third one (p61-90) from an extensive G1 peptide-mapping study in RA patients (hPG p65-82) (18). This latter peptide sequence contains the most arthritogenic T cell epitope in BALB/c (H-2^d) mice (19,26). Interestingly, three (p16-31, p21-36 and p263-280) of the six peptides were also positive in OA patients (compared to healthy controls), whereas the other three peptides (p25-39, p61-76 and p268-280) induced no or non-significant T cell responses in OA patients (Fig. 1). These observations indicate that hPG epitopes may be the target of autoreactive T cells in RA and OA, and suggests that such T cells may be involved in the inflammatory processes leading to cartilage destruction in RA and OA.

Table 4. Patients showing pYersinia-specific proliferation also responded to hPG p263-282.

No.	Patient	pYersinia response (S.I.)	p263-282 response (S.I.)
1	RA	1.9	4.0
2	RA	3.8	3.5
3	RA	2.9	5.7
4	RA	7.6	2.7
5	RA	1.8	2.0
6	RA	1.8	5.6
7	OA	3.3	8.2
8	OA	2.0	4.0

p263-282 sequence was blasted with the protein genbank and showed identical residues with a Yersinia peptide derived from Yop translocation protein L appearing in various Yersinia subspecies e.g. Yersinia enterocolitica (Table 2). RA and OA patients showing pYersinia-specific proliferation did also respond to hPG peptides p263-280 and p268-282. S.I. = stimulation index.

One of the most unexpected results was that peptide sequences covering an amino acid region of 263-282 in the core protein of hPG were frequently positive not only in RA and/or OA patients, but also in some healthy controls (Table 3 and Fig. 1E-F). An extensive blast search explored a significant homology of this region with YscL translocation protein L required for Yersinia outer protein (Yop) secretion of various Yersiniae subspecies e.g. *Yersinia enterocolitica* (Table 2). Therefore, we tested all patients simultaneously with the two peptides and, indeed, those patients who showed T cell proliferation to the Yersinia peptide also responded to hPG region p263-282 (Table 4), indicating the possibility of molecular mimicry. In addition, pYersinia peptide (Table 2) induced not only T cell proliferation, but also cytokine secretion (3 RA and 1 OA patients were tested), which was especially high for MCP-1 level (data not shown).

Both control peptides (hPG peptide p2379-2394, Fig. 1G and OVA peptide p323-339) were uniformly negative in each individual (RA, OA or healthy controls); the median responses were 1.0 ± 0.1 and 1.3 ± 0.3 respectively. In contrast, Tetanus Toxoid induced a high proliferative response in each group, being the lowest in patients with RA (Fig. 1H). The difference was especially impressive, when the RA patients were compared to healthy controls ($p<0.001$) but also compared to OA patients ($p<0.05$). Reduction of proliferative responses *in vitro* to Tetanus Toxoid has been described in RA patients with established disease (27), although this difference may also be a result of the age difference.

RA patients show more production of proinflammatory cytokines

Culture media were collected and the cytokine/chemokine levels in the supernatants of the two main hPG peptides, p25-39 and p268-282 based on proliferation data and pre-selection in HLA-humanized mice (Table 3 and Fig. 1), were measured using a multiplex immunoassay system developed in our laboratory (23). This panel simultaneously measures 26 cytokines/chemokines. As shown in Fig. 2, cytokine/chemokine values of IL-1α, IL-1β, IL-2, IL-6, IL-10, TNFα, MCP-1, MIP-1α were much higher in RA patients compared to the OA or healthy control groups even without a peptide-specific *in vitro* activation. This underlines the inflammatory state in RA. Indeed other studies showed that plasma and synovial fluids from RA patients contain significantly higher levels of proinflammatory cytokines compared to controls (28,29). IL-10 levels were low in all groups. IFNγ and IL-4 production were below detection limit in all culture media.

After stimulation with p25-39, IL-1α and TNFα amounts decreased slightly in RA patients although the levels were still higher compared to the OA patients and healthy controls. IL-1β, IL-2, IL-6, MCP-1 and MIP-1α levels which were much higher in the medium of unstimulated PBMCs of RA patients, stayed high after stimulation with p25-39 or increased slightly in the case of IL-6 and MIP-1α (Fig. 2). OA patients showed no induction of cytokines after stimulation with p25-39. After stimulation with p268-282 the cytokine levels were about the same in RA and OA patients and significantly higher compared to healthy controls (Fig. 2). The production of IL-6 and MIP-1α increased in both RA ($p=0.07$ for both cytokines) and OA patients ($p<0.001$) (Figs. 2D and 2H), and MCP-1 levels (Fig. 2G) were significantly higher in all groups in response to p268-282.

To briefly summarize these cytokine assays, RA patients had much higher cytokine levels than the OA patients, and especially higher than healthy controls even without activation. In response to stimulation with hPG peptide p268-282, proinflammatory cytokines IL-6 and MIP-1α were higher in RA and OA patients. In addition to the increased levels of IL-6 and MIP-1α, OA patients showed significantly higher levels of IL-1β and TNFα. Stimulation with p25-39 didn't show significantly elevated cytokine production.

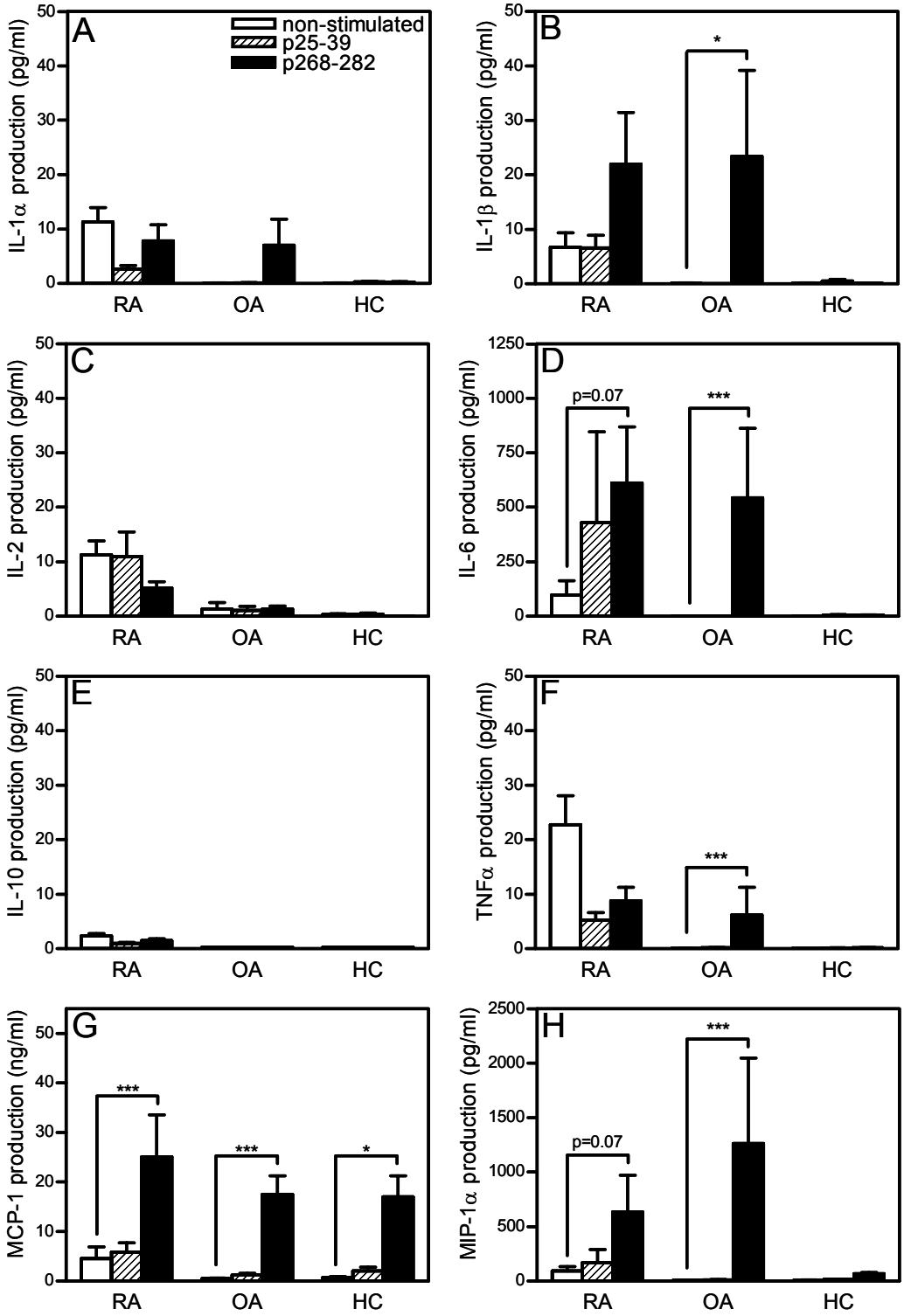

Figure 2. *hPG peptide p25-39 and p268-282-induced cytokine production in patients with RA, OA and healthy controls (HC). Cytokines (indicated on the y-axis scales) IL1α (**A**), IL1-β (**B**), IL-2 (**C**), IL-6 (**D**), IL-10 (**E**), TNFα (**F**), MCP-1 (**G**) and MIP-1α (**H**) were measured in 72-h culture media of non-stimulated (background; open bars), hPG peptide p25-39 stimulated (dashed bars) and hPG peptide p268-282 (closed bars) stimulated cultures using multiplex analysis. RA patients: n=14-16, OA patients: n=7 and HC: n=7. Values are expressed as the mean ± SEM. Significant differences in responses between patients and controls are indicated as follows: *=p< 0.05, ***=p<0.001.*

Intracellular analysis of IFNγ and TNFα

Although the two selected peptides (p25-39 and p268-282) elicited increased T cell proliferation in RA patients (Fig. 1), we were unable to detect significantly elevated levels for most cytokines/chemokines in the culture media after stimulation with the peptides compared to non-stimulated cells (Fig. 2). Many of the 72-h culture media of PBMCs derived from RA patients contained relatively high levels of various cytokines/chemokines without peptide stimulation, which were never detected in supernatants of OA patients or healthy individuals (Fig. 2). Thus, there was compelling evidence that these cells of RA patients were already in an activated stage, i.e., the high cytokine/chemokine "background" overlayed the peptide-specific response. To test this hypothesis, we selected a few RA patients for intracellular cytokine detection. We decided to measure the IFNγ, which was under detection level in all peptide-stimulated cultures (data not shown), and TNFα, which was even less in peptide-stimulated cultures (Fig. 2F).

We could demonstrate increased intracellular amounts of IFNγ and TNFα producing $CD4^+$ T cells after stimulation with positive peptides. A representative example of intracellular IFNγ and TNFα production by $CD4^+$ T cells in PBMCs of a RA patient in response to hPG p263-280 peptide stimulation is depicted in Fig. 3. A clear increase of CD69 expression on $CD4^+$ cells is seen, which indicates that these cells were indeed activated by stimulation with the hPG peptide p263-280. Moreover, an increased frequency of IFNγ producing cells (4.01%) was detected after stimulation with the peptide in the $CD69^+$ fraction of the $CD4^+$ cells, when it was compared to non-peptide stimulated cells (0.83%) (Fig. 3). Similarly, the frequency of TNFα producing $CD4^+CD69^+$ cells also increased from 0.89% to 4.03% after peptide stimulation (Fig. 3). Thus, the increased production of proinflammatory cytokines in response to e.g., hPG epitope p263-280 stimulation was confirmed by intracellular cytokine analysis.

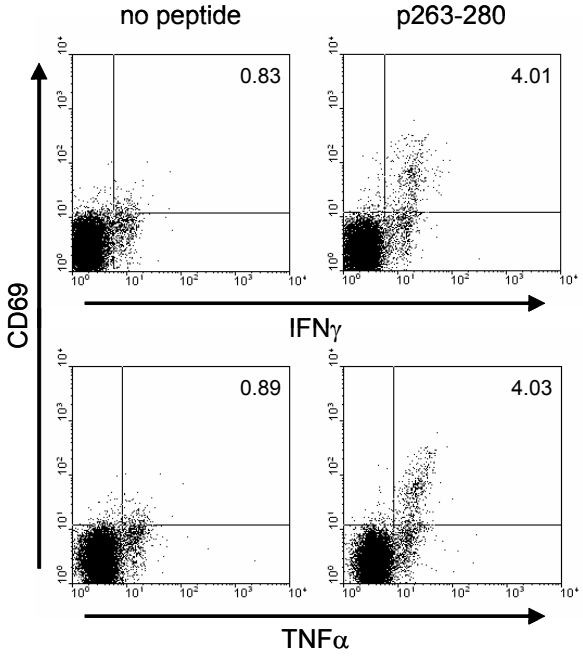

Figure 3. *Representative example of a peptide-specific intracellular cytokine response in a RA patient. PBMCs of a RA patient were cultured for 6 h in the presence of anti-CD28 and anti-CD49d monoclonal antibodies (0.5 μg/ml). IFNγ or TNFα production of $CD69^+$ T cells was measured after antigen-specific in vitro stimulation with hPG p263-280 in comparison with stimulation without antigen (medium). $CD4^+$ cells were gated. The number indicates the percentage of IFNγ and TNFα producing $CD4^+CD69^+$ cells.*

Discussion

The etiology of RA is unknown, and both environmental and genetic factors are thought to be involved in the pathogenesis. While the MHC is the most contributing factor for RA, an "appropriate" non-MHC-dictated genetic background is also critical. In this study, we selected a set of peptides of the human cartilage PG to test T cell responses in RA patients. These peptide sequences showed high MHC-binding scores to RA-predictive HLA alleles using a software-assisted (*in silico*) prediction method (21), and appeared to be the most dominant antigenic/arthritogenic T cell epitopes in HLA-humanized DR4 and DQ8 transgenic mice (19). We showed that T cell responses to amino acid regions 16-39 and 263-282 of hPG were significantly enhanced in a relatively large percent of RA patients. Interestingly, proliferative responses to the PG regions 16-36 and 263-280 were also significantly increased in OA-affected individuals. Although OA is not considered to be an autoimmune disease, cartilage degradation and loss are the major features of OA and, as a consequence, matrix molecules might become available for the immune system which seems to be not tolerant for these molecules. Thus, based on the experimental epitope mapping studies in HLA-Tg mice, we could identify two new hPG epitope targets of autoreactive T cells in RA and OA patients.

Interestingly, as found in this study and by others, T cells of RA patients are often hyporesponsive to stimulation, showing a peptide specific reduced T cell proliferation in response to either hPG or Tetanus Toxoid (27,30). This observation underscores the importance of the proliferative responses to hPG peptides in RA patients, as it appears that in RA patients despite the general hyporesponsiveness there is a selective increase in PBMC responsiveness to certain hPG epitopes.

The overall proinflammatory cytokine profile measured *in vitro* was high in supernatants of PBMCs of RA patients even without stimulation. Another keynote message of this study is that PBMCs of RA patients carry T cells being in an activation phase, probably due to the ongoing inflammation in peripheral joints. However, a peptide-specific cytokine production and/or a further activation of T cells still could be detected by measuring intracellular cytokine levels. Higher amounts of proinflammatory cytokines were also seen in OA patients after specific stimulation with p268-282. It is known that proinflammatory cytokines reduce matrix synthesis and induce the production of matrix metalloproteinases, leading to cartilage degradation (31).

Immune responses to human cartilage PG core protein have occasionally been found in patients with RA, JIA, OA and AS (4,13-18). Thus, our data are consistent with other studies showing $CD4^+$ T cell responses to several epitopes within the G1 domain of hPG. Peptide regions p16-39 and p263-282 of the core protein of hPG are recognized as T cell epitopes. These observations suggest that cartilage PG-reactive T cells might play a role in the pathogenesis of RA and possibly OA as well. It remains unclear, however, whether the reactivity against these hPG epitopes plays a primary role resulting in the induction of a joint-specific disease due to a lack of tolerance towards certain cartilage epitopes, or more likely, is a secondary phenomenon induced by availability of cartilage matrix molecules. In other words, known or unknown etiological factors, or aging processes lead to a more extensive degradation of cartilage matrix molecules, and some of these degradation products, as potential (auto)epitopes, may become accessible to a malfunctioning immune system. In this case, however, either a certain (probably a critical)

amount of antigenic challenge, or sufficient duration of the disease is needed before hPG-reactive T cells could be involved in perpetuating chronic inflammation and/or further destruction of cartilage.

The autoreactive T cell response to cartilage PG raised the question whether this could also be the result of molecular mimicry with e.g. bacterial proteins in a subset of patients. Peptides sequences used in this study were aligned to protein sequence databases and hPG peptides p263-280 and p268-282 showed extensive homology with Yop translocation protein L of various Yersinia subspecies e.g. *Yersinia enterocolitica*. A number of RA and OA patients showed a T cell response to this Yop-derived peptide. Reciprocally, all the patients responding to the pYersinia peptide also showed a T cell response to the homologous hPG 263-280 and/or 268-282 peptide. Coincidentally, increased titers of antibodies to Yersinia Yop proteins of different serogroups were also found in sera and synovial fluids of patients with arthritis (32,33). Whether there is an association between the arthritogenic capability of *Y. enterocolitica* and the homology with hPG p263-282 remains to be determined in future studies, especially in patients with a history of reactive arthritis.

Although the reasons for the loss of self tolerance to cartilage PG remain elusive, modulating of the immune response to hPG by induction of a tolerogenic response may lead to a more effective, yet hypothesized, treatment of positive (i.e. hPG reactive) RA patients avoiding toxicities associated with nonselective bioactive or immunosuppressive therapies. In PG-induced arthritis, nasal administration of hPG exerted a strong suppressive effect on both the incidence and severity of arthritis by reducing responsiveness towards the immunizing hPG antigen (34). Recently, the dominant/arthritogenic epitopes of hPG in BALB/c mice have been determined (35), and by using these peptides for nasal tolerance we could achieve a dramatic suppressive effect on the PG-induced arthritis (authors personal observation; manuscript in preparation).

Taken together, this study shows that T cells of the majority of RA and some of the OA patients can recognize one or more epitopes in the G1 domain of human cartilage PG. The identification of T cell epitopes is crucial for the understanding of the immune responses in autoimmune diseases and suggests that autoimmunity to PG might be involved in RA pathomechanisms.

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Chapter 7

Summary and General discussion

Summary

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

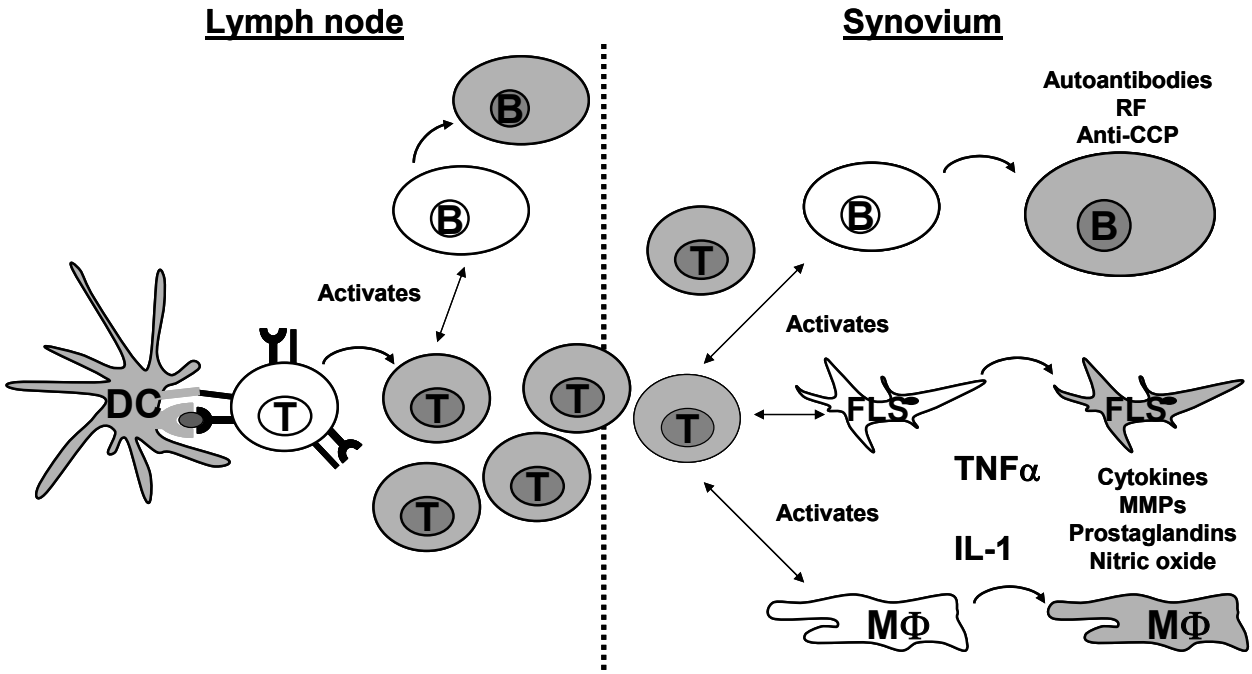

Figure 1. *This schematic figure summarizes the most important participants in RA pathogenesis. The left side illustrates the interaction between antigen presenting cell (dendritic cell) and T cell in the lymph node, which then can activate other T and B cells, and cause proliferation of a T cell population.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

In order to study arthritis, we made use of the proteoglycan-induced arthritis (PGIA) model in BALB/c mice, which is a T cell-dependent, autoantibody-mediated progressive relapsing autoimmune model resembling many characteristics of RA. Originally PGIA was induced by four PG immunizations of which the first and fourth injection were in complete Freund's adjuvant (CFA) and the second and third PG injection in incomplete Freund's adjuvant (IFA). In chapter 2 we described that by replacing the Freund's adjuvants by dimethyldioctadecylammonium bromide (DDA), arthritis can be induced by only two PG immunizations. Although the clinical and immunological features of the "classic" form of PGIA are preserved, the onset of the disease is faster and the severity is increased. An additional advantage is

that hereby all undesired side effects of the Freund's adjuvants, such as the role of interfering immune responses induced by microbial HSPs present in CFA, are eliminated. DDA exerted a strong stimulatory effect via the activation of innate immunity and forced the immune response toward Th1 dominance (chapter 2). In the PGIA model using DDA as adjuvant, we explored the anti-inflammatory capacity of immune responses to HSP70 in arthritis and searched for the mechanisms involved (chapter 3). HSP70 pretreatment dramatically suppressed the development of inflammation and subsequent tissue damage in PGIA. Moreover, HSP70 pretreatment resulted in a regulatory immune response not only to HSP70 but also to PG. To better understand the role of T cells in arthritis we generated a T cell receptor transgenic (TCR-Tg) mouse (chapter 4 and 5). The T cells of this mouse are specific for an epitope (designated 5/4E8) in the G1 domain of human cartilage PG (amino acids 70-84). These TCR-5/4E8-Tg mice are a unique source of naïve antigen-specific T cells that are capable of inducing progressive chronic arthritis, both spontaneously (<15%), and after immunization with PG (100%) and upon adoptive transfer to BALB/c.SCID mice. Based upon the clinical and histopathological features and the autoimmune aspects shared between PGIA and RA, we believe that these TCR-5/4E8-Tg mice are a valuable tool for further analysis of the mechanisms associated with the initiation and pathogenesis of autoimmune arthritis; and more specifically, for the analysis of the role of antigen specific T cells in disease development.

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

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

General discussion

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

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

PGIA

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

The use of the synthetic lipophilic quaternary amine adjuvant DDA accelerated the development of a more severe arthritis via a more potent activation of the innate immunity. DDA acts as an adjuvant through enhancing interactions with both antigen and components of the host immune system (9). In PGIA induced with PG in DDA the overall immune responses (antibody production and antigen-specific T cell responses) were highly comparable with arthritis induced with PG in CFA. However, smaller doses of cartilage PG (2x instead of 3-4x) and use of crude cartilage extract from osteoarthritic cartilage (instead of highly purified PG), which has only a suboptimal arthritogenic effect when injected in CFA, were very effective in provoking arthritis when mixed with DDA. While the overall effects of DDA and CFA on the T cell response were highly comparable, PG-specific IFNγ:IL-4 ratios in spleen cells or peripheral lymph node cells were significantly different and clearly shifted toward Th1 in the DDA/PGIA model. In the

peritoneal cavity, at least a 2-4-fold increase in macrophage influx, accompanied by more activated ($CD11c^+$) dendritic cells was observed in DDA-injected mice compared to CFA-injected mice.

A critical role for innate immunity in arthritis induction is consistent with the findings of studies that used only adjuvants (nonspecific stimulators) in genetically susceptible strains of rodents (10,11). Thus, nonspecific activation of the immune system is probably an important component of the disease pathological mechanism. Our observations using DDA as adjuvant support this important role of innate immunity, in addition to the inducing adaptive immune responses (PG-specific Th1 T cells). Macrophages and dendritic cells involved in innate immune responses were stimulated more potently by DDA compared to CFA resulting in an accelerated onset of arthritis and more severe arthritis. In PGIA, a rapid accumulation of PG-specific Th1 cells in the synovium appears to be the most critical component of the development of arthritis (5). Autoantibodies to PG contribute to the severity of disease possibly by specifically binding to the cartilage surface, thus inducing more extensive cartilage damage (12). It seems that DDA has stimulated both the adaptive immunity (significantly higher ratios of PG-specific IFNγ to IL-4 were found in PG/DDA immunized mice) and the innate immunity. Most components of innate immunity play an important role in perpetuating inflammation by their interactions with the cognate immune cells, and they also can induce subsequent tissue damage (13). This could have contributed to the loss of cartilage, resulting in the increased release of mouse PG fragments which may have further stimulated the PG-specific immune responses and amplified the inflammatory process.

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

TCR-5/4E8-Tg

Several lines of evidence indicate that the effector mechanism, which initially attacks small joints, is T cell driven. One way of gaining more insight into the role T cells in PGIA was by establishing transgenic mice expressing a TCR specific for one of the immunodominant epitopes of human PG, namely, the epitope recognized by T cell hybridoma 5/4E8. This specific epitope of human PG differs only in 2 amino acids from the homologous mouse PG sequence. TCR-5/4E8-Tg mice supply us with a unique source of naïve antigen (arthritogenic epitope)-specific $CD4^+$ T cells that are capable of inducing progressive chronic arthritis. Only 15% of the TCR-Tg mice after several backcrossings to the arthritis susceptible BALB/c background developed spontaneous arthritis, but severe arthritis was quickly induced after immunization with PG in DDA. Interestingly, also immunization with PG in the absence of adjuvant resulted in arthritis indicating the importance of T cells in the initiation of arthritis in this model.

In addition to arthritis development in the TCR-5/4E8-Tg mice, adoptive transfer of spleen cells from arthritic TCR-Tg induced arthritis in BALB.SCID mice. Arthritis was induced by this transfer without adding exogenous PG or specific peptide, which was not possible using splenocytes from arthritic wild-type donor BALB/c mice (20). We hypothesize that T cells from arthritic TCR-5/4E8-Tg mice migrate to the joints upon adoptive transfer and become further reactivated (or maintain their activated shape) by mouse PG in the mouse joint where self-peptides are released during the normal turnover of the cartilage matrix. As a consequence of the inflammatory processes in the joint the production of mouse PG peptides might be increased, which could amplify the autoimmune response.

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

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

During the progression of arthritis, cartilage has been shown to be damaged by the invasion of pannus. The degradation of the cartilage matrix by various metalloproteinases upregulated by TNFα and/or IL-1, and by apoptosis of chondrocytes, dominates the clinical and histopathological picture of RA (24-26). The cartilage degradation releases matrix components, such as type II collagen (CII) and PG fragments, which then may be exposed to the immune system. PG (aggrecan) or CII within healthy cartilage normally is not subjected to immune surveillance, due to the avascular structure of hyaline cartilage. However, potential antigenic determinants might be generated during degenerative or inflammatory processes, and then these (auto)antigenic components can trigger immune reactions. Although the target organ of RA is the synovial joint, there is no clear evidence that any macromolecule of cartilaginous tissues, bone, or synovium, is a preferential disease-inducing autoantigen (27). However, T cell epitopes on matrix molecules may be critical in the pathogenesis of arthritis. Several studies have demonstrated that patients with joint diseases show immune responses to various cartilage matrix molecules, such as to CII (28,29), human cartilage glycoprotein 39 (HC gp-39) (30) and PG (31-34).

Recently, the epitope repertoire of the human cartilage PG was determined in HLA-DR4-humanized and HLA-DQ8-humanized mice and it was shown that these transgenic mice immunized with human cartilage PG developed arthritis, but only when these class II MHC molecules were present on the arthritis-susceptible (BALB/c) genetic background (35). Two highly immunogenic T cell epitopes, both in the G1 domain of human PG could be identified, and were both associated with arthritis. We tested these human PG-epitopes recognized in HLA-Tg mice for T cell recognition in patients with RA and OA, and healthy controls. In this study we demonstrated that T cell responses to cartilage PG epitopes occur in RA with a pro-inflammatory cytokine profile. This might highlight that PG is a relevant joint-specific autoantigen in the pathogenesis of RA. The reasons for the loss of self tolerance to this major cartilage component still remain elusive. In addition, it has raised questions as to whether the the PG autoreactivity is causal, an early disease-driving event, mediates the joint pathology or merely is a consequence of cartilage destruction. (36). Recently, in this respect Burkhardt *et al.* have been provided first evidence that citrullinated derivatives of well conserved immunodominant native CII epitope might be targets of an autoantibody response at very early stages of RA (37). They suggested that as B cell tolerance is more easily broken, this could result in breakdown of tolerance of the better controlled T cells. Autoimmunity to cartilage-specific modified self components might be a critical intermediate step that helps to lower the threshold for the final breakage of tolerance to other joint proteins in chronic inflammatory joint disease (37). Thus, PG (or CII) autoimmunity in RA may be a secondary phenomenon, induced following inflammation in the joints and might play a role in the persistence of the disease, rather than in the actual induction of arthritis.

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

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

Animal models of arthritis can be used to understand elements of the arthritic process in patients, although final proof of concept must come from clinical studies (46). In order to benefit from using an animal model, we must understand and utilize the common (shared) immune-pathogenic components of the human disease (RA) and a corresponding

animal model (PGIA). Our findings support the use of the PGIA model to extrapolate to human diseases; as T cell epitopes of PG that were defined in HLA-DR4 and HLA-DQ8 transgenic mice stimulated T cells from human RA and OA patients.

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

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

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

Here, we studied the capacity of immune responses to HSP70 to prevent or arrest inflammatory damage in PGIA and searched for the anti-inflammatory mechanism. HSP70 pretreatment resulted in a significant delay of the arthritis onset and dramatically reduced disease severity; less inflammation and almost no synovium cell infiltration, cartilage damage or bone erosions were detectable. The *in vivo* protective effect of HSP70, however, was accompanied not only with increased HSP70-specific proliferation but also with increased *in vitro* T cell proliferation to PG. This increased PG-specific T cell proliferation seems to be contradictory to *in vivo* results (suppressed PGIA). On the other hand, HSP70 has an adjuvant effect as well, known from work from Srivastava and others (54,55). As this is the first time the anti-inflammatory effect of HSP70 is investigated in an (self)-antigen-induced arthritis model, this increased PG-specific response could not have been noticed so far, thus this observation will need further attention. This effect might be part of the regulatory mechanism, because, not only after *in vitro* restimulation with HSP70, but also restimulation with PG showed enhanced IL-10 and IFNγ production in mice that were otherwise protected by the administration of HSP70. A simultaneous production of IFNγ and IL-10 is a characteristic of T regulatory 1 (Tr1) cells (56). In JIA patients with a remitting form of disease (oligoarticular JIA) responses to HSP60 are consistent with a benign clinical course and these HSP60-specific T cells have the phenotype of human Tr1 cells (57). On the other hand, IFNγ has also been described as a prototype Th1 proinflammatory cytokine (58) and the production of IFNγ in response to immunization with PG is an important contributor to the induction of PGIA (5). The IL-10:IFNγ ratio, however, can be an indicator of the balance of Tregs and Th1 cells. We recorded an increased IL-10:IFNγ ratio in

response to PG in HSP70-pretreated mice. IL-10 plays an important role in the immunoregulatory mechanisms that control inflammatory responses (59,60). IL-10 has the capacity to down-regulate monocyte-derived proinflammatory cytokines such as IL-1, IL-6, and TNFα (61). IL-10 production seems to be protective in RA patients, especially against progression of joint destruction (62). HSP70-specific T cells as well as PG-reactive T cells, displaying a regulatory phenotype, may have been influenced by the neighboring harmful autoreactive T cells. Thus, these Tr1 cells can switch the proinflammatory cytokine profile of PG-specific cells into an anti-inflammatory (regulatory) IL-10 producing phenotype, thereby modulating the development of inflammation in PGIA.

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

Conclusion

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

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Nederlandse samenvatting

Inleiding

Reuma is een verzamelnaam voor een groot aantal ernstige aandoeningen van het bewegingsapparaat, waarbij gewrichtsklachten een belangrijke rol spelen. Reumatoïde artritis (RA) is een vorm van reuma die relatief vaak voorkomt. Ongeveer 1 op de 100 mensen heeft hier last van. De ziekte wordt gekenmerkt door chronische (langdurige) ontstekingen van de gewrichten (artritis). Ze raken gezwollen en stijf en bewegingen zijn pijnlijk. RA treedt het meest op in de gewrichten van de handen, polsen en voeten, maar ook andere gewrichten in het lichaam kunnen worden aangetast. De ontstekingen veroorzaken schade aan het kraakbeen en de botten van de gewrichten. Dat kan op langere termijn tot aanzienlijke vervormingen leiden wat functiebeperkingen tot gevolg heeft. Bij RA gaat weliswaar veel aandacht uit naar de gewrichtsafwijkingen, maar in feite is RA een systeemziekte. Dat wil zeggen dat de ziekte op het hele lichaam ingrijpt. Ook klachten als moeheid, gewichtsverlies, koorts en stoornissen van organen komen voor.

De precieze oorzaak van RA is onbekend. Wel is duidelijk dat het afweersysteem (immuunsysteem), dat het lichaam gewoonlijk beschermt tegen lichaamsvreemde stoffen zoals virussen, een belangrijke rol speelt. RA is een zogenaamde auto-immuunziekte. De afweerreactie is gericht tegen gezonde lichaamseigen weefsels waarbij de gewrichten worden beschadigd. In de gewrichten van RA patiënten treft men flinke hoeveelheden ontstekingscellen (o.a. T cellen) aan. Ook produceert het lichaam veel antistoffen (antilichamen) en ontstekingsmediatoren (cytokines). Door de voortdurende ontstekingsreacties raken de gewrichten beschadigd. De behandeling van RA richt zich erop de ontstekingen te bestrijden en de geactiveerde ontstekingscellen tot rust te brengen. Dit lukt helaas vaak maar deels met therapieën of medicijnen en de behandeling kan bijwerkingen opleveren. Eenmaal ontstane kraakbeenschade en boterosies zijn onherstelbaar. RA kan (nog) niet worden voorkomen of genezen.

Dit proefschrift

RA is een complexe ziekte. Vele cellen van het immuunsysteem spelen een rol in de ontstekingsprocessen. Een beter begrip van die ontstekingsreacties die tot schade aan kraakbeen en bot van de gewrichten leiden, is een belangrijke stap om tot betere behandelmethoden te komen. Het doel van dit proefschrift is om de kennis van de ontstekingsprocessen te vergroten en er meer inzicht in te krijgen. Om deze specifieke complexe mechanismen beter te kunnen begrijpen, maar ook om te bestuderen hoe we bepaalde ontstekingsprocessen kunnen beïnvloeden, zijn diermodellen noodzakelijk. Mechanismen die tot ontstekingen leiden, komen voor een groot deel overeen tussen mensen en dieren. Maar dieren zijn natuurlijk geen mensen, dus om RA beter te kunnen begrijpen is het cruciaal om een diermodel te kiezen waarin de karakteristieke kenmerken van RA (zoals de specifieke aanval van de gewrichten, het chronische op en neer gaande karakter etc.) gewaarborgd zijn.

Proteoglycaan-geïnduceerde artritis; een muizenmodel voor RA

In de studies beschreven in dit proefschrift hebben we gebruik gemaakt van het proteoglycaan-geïnduceerde artritis model in muizen. Proteoglycaan (PG) is een kraakbeen eiwit. Door muizen te immuniseren met humaan PG in combinatie met een stimulerende stof (adjuvant) ontwikkelen ze artritis. Het ontstaan van deze artritis is gebaseerd op een kruisreactieve immuunreactie (afweerreactie) tussen het humane PG waarmee geïmmuniseerd wordt en het muizen eigen PG in de gewrichten. De artritis in deze muizen lijkt in vele opzichten op RA bij mensen, zowel de klinische vertoning van zwelling en stijfheid van gewrichten als ook de gewrichtsbeschadigingen op röntgenfoto's, maar ook wat betreft de ontstekingsprocessen en de cellen die daarbij een rol spelen. Nadeel van dit model was dat het relatief lang duurde voordat de muizen artritis kregen en dat het adjuvant vervelende bijwerkingen had.

In hoofdstuk 2 is getest of vervanging van het oorspronkelijke adjuvant (Freund's adjuvant) door een ander adjuvant het ziekteproces kon beïnvloeden. Hiertoe hebben we PG immunisaties in het oorspronkelijke complete/incomplete Freund's adjuvant vergeleken met PG immunisaties in dimethyldioctadecylammonium bromide (DDA). De klinische en immunologische karakteristieken van de artritis van de oorspronkelijke vorm van PG-geïnduceerde artritis bleven bewaard. Maar het ontstaan van de ziekte was veel sneller; er waren maar 2 in plaats van 3 tot 4 immunisaties nodig. Daarnaast was de ziekte ernstiger. Het gebruik van DDA versterkte de immuunreactie tegen PG. Door het gebruik van DDA zijn de bijwerkingen en de nadelen van het oorspronkelijke adjuvant geëlimineerd. Dit is een groot voordeel omdat er hierdoor minder muizen en humaan PG nodig zijn, en experimenten sneller verlopen. Dit bij elkaar maakt PG-geïnduceerde artritis een nog aantrekkelijker en interessanter model voor het bestuderen van artritis.

Zoals eerder al genoemd spelen ontstekingscellen, waaronder T cellen, een belangrijke rol in zowel RA als ook in PG-geïnduceerde artritis. In de hoofdstukken 4 en 5 hebben we geprobeerd meer inzicht te krijgen in de rol van de T cel in de ontstekingsprocessen bij artritis. Daarvoor hebben we een T cel receptor transgene muis gemaakt. De T cellen van deze muis zijn specifiek voor PG. Deze PG-reactieve T cellen zijn in staat progressieve artritis te induceren. Ongeveer 15% van deze T cel receptor transgene muizen krijgt spontaan artritis en 100% ontwikkelt artritis na immunisatie met PG. Cellen uit deze T cel receptor transgene muis zijn ook in staat artritis te veroorzaken

als ze worden getransfereerd naar immuun gecomprimeerde muizen. Dit benadrukt de belangrijke rol van T cellen in proteoglycaan-geïnduceerde artritis.

Gezien de klinische en pathologische overeenkomsten tussen PG-geinduceerde artritis en RA, denken we dat deze transgene muizen belangrijk kunnen zijn voor het verder analyseren van de ontstekingsprocessen en de cellen betrokken bij het ontstaan en het voortschrijden van de artritis. In het bijzonder kunnen ze helpen bij het onderzoeken van de rol van de T cel in artritis.

Afweerreacties tegen proteoglycaan in RA patiënten

Gedurende artritis wordt het kraakbeen van de gewrichten beschadigd door ontstekingscellen en allerlei mediatoren. Kraakbeenafbraak leidt ook tot het vrijkomen van kraakbeen eiwitten zoals PG uit de gewrichten. Normaal gesproken vindt er geen afweerreactie (immuunreactie) tegen kraakbeen eiwitten plaats omdat het niet beschikbaar is voor het afweersysteem in een gezond persoon. Maar door het ontstekingsproces en de versnelde afbraak van kraakbeen in RA zouden afweerreacties tegen kraakbeen eiwitten kunnen worden opwekt.

In hoofdstuk 6 hebben we gekeken of RA patiënten afweerreacties tegen bepaalde PG peptiden vertonen. We hebben gekeken of T cellen uit het bloed van RA patiënten reageren op PG door middel van proliferatie en analyse van ontstekingsmediatoren (cytokinen). Hierbij konden we aantonen dat veel RA patiënten inderdaad een afweerreactie tegen een aantal PG peptiden vertonen. In reactie op die PG peptiden produceerden ze ook voornamelijk pro-inflammatoire (ontstekingsbevorderende) cytokinen. Dit zou kunnen betekenen dat PG een relevant gewrichtsspecifiek eiwit is in het ziekteproces van RA.

Waarom er nu een afweerreactie tegen PG plaats vindt is niet duidelijk. Het roept ook de vraag op of deze reactie primair een rol speelt bij het ontstaan van de artritis of, meer waarschijnlijk, een gevolg is van het ontstekingsproces en de kraakbeenschade. Hoe dan ook selectieve beïnvloeding van die afweerreacties tegen PG zou van belang kunnen zijn bij behandeling van artritis. In het PG-geïnduceerde artritis model is gebleken dat het deactiveren van PG-specifieke afweerreacties een sterk effect had op de incidentie en de ernst van de artritis in de muizen. Meer onderzoek is nodig om verder uit te zoeken wat de betekenis van de PG-specifieke afweerreacties voor het ziekteproces van RA is.

Heat-shock eiwitten en het mogelijke gebruik daarvan als therapeutische behandeling

Heat-shock eiwitten (HSPs) vormen een groep van eiwitten die bij ongunstige omstandigheden allerlei cellulaire processen beschermen. Er komt steeds meer bewijs dat immuunreacties (T cellen) gericht tegen HSPs als een feedback loop kunnen functioneren en ontstekingsreacties kunnen remmen. HSPs zouden een aantrekkelijke kandidaat voor therapie kunnen zijn om meerdere redenen: 1) HSPs zijn aanwezig in alle organismen. 2) HSPs zijn in de evolutie sterk geconserveerd gebleven, dat wil zeggen dat HSPs van de mens sterk overeen komen met HSPs van bijvoorbeeld bacteriën. Dit zou kunnen leiden tot kruisherkenning. 3) HSPs zijn dominante eiwitten, er vinden immuunreacties tegen plaats. 4) HSPs zijn aanwezig op de plaats van de ontsteking; ze worden in grote hoeveelheden geproduceerd door de cellen in de gewrichten.

In hoofdstuk 3 hebben we de capaciteit van HSP70 bestudeerd in het voorkomen of remmen van de ontstekingsreactie die schade aanricht in PG-geïnduceerde artritis. Daarnaast hebben we geprobeerd te achterhalen welke mechanismen bij die ontstekingsremmende werking betrokken zijn. Dus op welke manier HSP70 kan beschermen tegen artritis. HSP70 werd vooraf toegediend en daarna volgden de immunisaties met PG in DDA om de artritis te induceren in de muizen. Muizen die de behandeling met HSP70 hadden gehad, kregen geen of pas veel later artritis. Deze artritis was ook minder ernstig dan in de controle groep die geen HSP70 van tevoren had gehad. Dit effect van HSP70 was niet alleen klinisch zichtbaar (minder zwelling), maar was ook zichtbaar in coupes van de muizenpootjes. In de coupes van dieren die waren behandeld met HSP70 waren veel minder ontstekingscellen aanwezig en er was nauwelijks kraakbeen schade. Het ontstekingsremmende effect was geassocieerd met een versterkte immuunreactie niet alleen tegen HSP70, maar ook tegen het artritis inducerende eiwit PG. Deze versterkte immuunreactie ging samen met een verhoogde productie van de cytokinen interleukine-10 en interferon-gamma. Gelijktijdige productie van interleukine-10 en interferon-gamma is een karakteristiek van regulatoire cellen. Dit zijn cellen die in staat zijn ontstekingsbevorderende cellen te remmen in hun activiteit. Samengevat hebben we dus aangetoond dat immuunreacties tegen HSP70 ziektesymptomen kunnen verminderen.

Het exacte mechanisme dat betrokken is bij de regulatie door HSPs moet nog verder onderzocht worden. We denken dat verhoogde blootstelling aan microbieel HSP70 (in dit geval door immunisatie) leidt tot het activeren van T cellen die kruisreageren tussen microbieel en humaan HSP70. We hebben aangetoond dat immuunreacties tegen HSP70 vooral gericht zijn tegen die delen van HSP70 die grotendeels hetzelfde zijn in bacteriën en mensen. Deze kruisreagerende T cellen hebben een ontstekingsremmend effect. Dus het opwekken van meer kruisreagerende immuunreacties tegen HSP70, bijvoorbeeld door immunisatie met HSP70, zou een mogelijke vorm van ontstekingsremmende therapie voor artritis kunnen zijn.

Conclusies

RA is een complexe ziekte waarvan de precieze oorzaak niet duidelijk is. De aandoening is het resultaat van een samenspel tussen vele cellen van het immuunsysteem, omgevingsfactoren en genetische componenten. Een succesvolle behandeling is afhankelijk van een vroegtijdige diagnose en het beter begrijpen van de ontstekingsprocessen om gewrichtsschade zoveel mogelijk te voorkomen. Het PG-geïnduceerde artritis model maakt het mogelijk verschillende van deze ziekmakende processen en hun rol in de ziekte te onderzoeken. Het bestuderen van PG en HSPs, beide aanwezig op de plek van ontsteking, heeft meer inzicht gegeven in de ontstekingsprocessen in artritis en suggereert dat beide eiwitten potentiële targets voor modulatie (therapie) kunnen zijn via verschillende mechanismen.

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Curriculum Vitae

Suzanne E. Berlo werd op 26 augustus 1975 geboren in Sint Michielsgestel. In 1994 behaalde zij het Atheneum diploma aan Scholengemeenschap Durendael in Oisterwijk. Ze studeerde Hoger Laboratorium Onderwijs, afstudeerrichting medische microbiologie, aan de Fontys Hogeschool in Eindhoven. In 1998-1999 volgden twee onderzoeksstages bij Roche in Penzberg, Duitsland. De eerste stage betrof de ontwikkeling van diabetes type 1 gerelateerde anti-IA-2 humane monoklonale antilichamen (begeleider Dr. J. Endl, afdeling celbiologie). De tweede stage betrof het kloneren en tot expressie brengen van verschillende tumor markers voor immunologische testen (begeleider Dr. A.M. Engel, afdeling genetica).

In oktober 1999 startte zij als assistent in opleiding in dienst van de Universiteit Utrecht, faculteit Diergeneeskunde bij de hoofdafdeling infectieziekten en immunologie, divisie immunologie. Het promotieonderzoek beschreven in dit proefschrift werd verricht onder begeleiding van Dr. C.P.M. Broeren† en Dr. A.B.J. Prakken (afdeling pediatrische immunologie, UMC Utrecht) en supervisie van Prof. Dr. W. van Eden. Gedurende 9 maanden verbleef ze bij Prof. Dr. T.T. Glant (Section of Molecular Medicine, Department of Orthopedic Surgery, Rush University Medical Center, Chicago, USA) om daar een deel van het onderzoek uit te voeren en alle ins en outs van het proteoglycaan-geïnduceerde artritis model te leren. Sinds 2005 is zij lid van de Wetenschappelijke Adviesraad Reumafonds, ingesteld ten behoeve van de advisering omtrent de wetenschappelijke kwaliteit en maatschappelijke relevantie van ingediende onderzoeksvoorstellen.

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* Authors were equally involved in this work