Predominant cellular immune response to the cartilage autoantigenic G1 aggrecan in ankylosing spondylitis and rheumatoid arthritis

J. Zou¹, Y. Zhang³, A. Thiel², M. Rudwaleit¹, S.-L. Shi³, A. Radbruch², R. Poole³, J. Braun¹,⁴ and J. Sieper¹,²

Objectives. Based on their HLA association, both ankylosing spondylitis (AS) and rheumatoid arthritis (RA) seem to be T-cell-driven diseases in which the autoantigens remain to be defined. One possible autoantigen is the G1 domain of aggrecan, the major cartilage proteoglycan. In BALB/c mice immunized with this protein, spondylitis and erosive polyarthritis have been reported. Immune reactivity to the G1 has been described in patients with RA and AS in an earlier study. Using novel and more sensitive techniques and relevant controls we sought to define the role of G1 as an autoantigen more precisely and to extend the specific analyses to the peptide level.

Methods. Peripheral blood (PB) mononuclear cells (MNC) from 47 AS patients, 22 RA patients and 20 healthy normal controls were exposed in vitro for 6 h to the cartilage-derived autoantigens G1, human cartilage (HC) gp-39 and collagen II. Synovial fluid (SF) MNC from seven AS and four RA patients were similarly analysed. Furthermore, PB MNC of 15 AS and 10 RA patients were examined with overlapping 18-mer peptides covering the whole G1 protein to identify the immunodominant epitopes. T cells were stained by monoclonal antibodies directed against the surface markers CD4, CD69 and against the intracellular cytokines interferon-γ (IFNγ), tumour necrosis factor-α (TNFα), interleukin 4 (IL-4) and IL-10. The percentage of reactive T cells was quantified by flow cytometry.

Results. After antigen-specific stimulation with the G1 protein, the CD4+ T cells of 30 AS patients (61.7%) and of 12 RA patients (54.5%) secreted significant amounts of IFNγ and TNFα, while, in contrast, only 10% of the normal controls showed a response (P < 0.05). The synovial CD4+ T cells of five AS (71.5%) and of all four RA patients showed antigen-specific responses to the G1. In contrast, stimulation with HC gp-39 and collagen II showed no significant IFNγ and TNFα secretion of MNC in all groups. Several G1-derived T-cell epitopes were identified as immunodominant in PB MNC of AS and RA patients and were partly overlapping.

Conclusions. These data show that a cellular immune response to G1 is present in most AS and RA patients. G1-immunodominant epitopes were identified. The relevance of this finding for the pathogenesis of AS and RA remains to be established.

KEY WORDS: Ankylosing spondylitis, Rheumatoid arthritis, G1 immunity, T-cell epitope, Aggrecan.

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846
The spondyloarthropathies are frequently occurring inflammatory rheumatic diseases [1], in part leading to a significant burden of disease with pain and disability probably not so much different from rheumatoid arthritis (RA) [2]. Ankylosing spondylitis (AS) and undifferentiated spondyloarthropathies are the most frequent subtypes [1, 3, 4]. Although the strong association with HLA-B27 was reported more than 25 yr ago, the pathogenesis of the spondyloarthropathies has remained obscure [5, 6]. In AS, similar to reactive arthritis, there is evidence that T cells play an important role [5, 7–9]. T-cell responses to bacteria-derived antigens such as Klebsiella [10] or to autoantigens derived from cartilage such as the proteoglycan aggrecan have been demonstrated in AS [11–15]. Moreover, immunity to this molecule in BALB/c mice has been shown to induce spondylitis and inflammatory polyarthritis [14, 15]. The outcome of 20–50% of the patients with reactive arthritis and inflammatory bowel disease carrying HLA-B27 is AS [16, 17]. This association has raised the question of whether the immunopathology in AS is caused or triggered [5, 18] by an antibacterial immune response and/or perpetuated by an immune response directed against an unknown self-antigen. The fact that no bacterial DNA could be detected in biopsies from sacroiliac joints by the polymerase chain reaction technique (PCR) [19], and that in spondyloarthropathies such diverse structures as spine, enthesis, eye and aorta are involved, makes bacterial persistence at these sites rather unlikely and argues in favour of an autoimmune response. An increasing amount of studies using magnetic resonance imaging (MRI) have shown that the most severe inflammation in spondyloarthropathies is an osteitis occurring at bone/cartilage interphases [7, 20, 21]. This finding has been supplemented by histological investigations indicating that, especially in early phases of spondyloarthropathies, mononuclear cells invade and erode the cartilage at different sites [7, 22, 23]. Based on these findings it has been proposed that the cartilage is the primary target of the immune response in spondyloarthropathies [18, 24–26]. The Montreal group has provided evidence over the last few years that the cartilage proteoglycan aggrecan might be a candidate autoantigen in AS [11, 12, 14, 18, 27, 28], and that it is the G1 globular domain of this molecule where immunity is mainly targeted [27, 28]. These findings are supported by others [15, 29]. While the animal model of HLA-B27-transgenic rats [30] displays a polyarthritis, a spondylitis is less prominent. A mouse model for AS has recently gained more attention. Injection of the G1 domain of aggrecan into BALB/c mice induces not only peripheral arthritis but also spondylitis [27]. It could be shown that T cells play an important role in this model and G1-derived immunodominant T-cell epitopes have been identified [27, 28]. There are limited data in humans about the cellular immune response to the G1 protein to date: these are mainly based on lymphocyte proliferation. With this technique, a T-cell response to the aggrecan has been reported in AS and RA patients, but also in osteoarthritis patients [12, 14, 31]. Furthermore, T lymphocytes have also been reported to respond positively to human cartilage (HC) gp-39 and collagen II, two other cartilage-derived antigens, in RA [32–34]. In this study we applied the more sensitive and more specific technique of antigen-specific cyometry to investigate the T-cell response in patients with AS, RA and healthy controls to these cartilage-derived autoantigens [35]. Using induction of interferon-γ (IFNγ) by CD4+ T cells as the primary outcome parameter, we examined the antigen-specific T-cell response in peripheral blood (PB) and synovial fluid (SF) to determine whether T cells specific for the G1 domain of aggrecan and to single G1-derived peptides are detectable in AS patients and controls and compared this to the response after stimulation with two other cartilage-derived proteins, the human cartilage glycoprotein (HC gp)-39 and collagen II protein. We used patients with RA as a reference group to compare responses to the proteoglycan aggrecan.

**Patients and methods**

**Patients**

Peripheral blood from 47 and synovial fluid from seven AS patients, PB from 22 and SF from four RA patients, and PB from 20 normal controls were collected in the out-patient clinic of the University Hospital Benjamin Franklin, Berlin. All AS patients fulfilled the modified 1984 New York criteria for a diagnosis of AS [36] and all RA patients the American College of Rheumatology criteria for the diagnosis of RA [37]. The characteristics of the patients and normal controls are shown in Table 1. All AS patients were in an active state of disease with current inflammatory back pain, a joint effusion or an elevated C-reactive protein with at least two of these three parameters being positive. However, the degree of activity varied and we did not attempt to quantify disease activity in more detail. All RA patients were active as judged by a modified disease activity score (DAS) >3 despite treatment with various disease-modifying drugs [38].

**The cartilage-derived antigen G1 domain of aggrecan**

Human aggrecan G1 domain (AG1) proteins were expressed and purified in an adenovirus expression system. Briefly, a cDNA fragment encoding the N-terminal 431 amino acids of human aggrecan with a His-tag at its C-terminus was generated from a human chondrocyte RNA preparation by reverse transcriptase (RT)-PCR (5'-GGAGATCTACTATGGCCACTTTACCTCGGTTTTCG-3' and 5'-CGATCTCAATGGTTGGTGGTGGATGTTGATGATGCTCAGCGAAGCCAGTGCGC3'). This PCR fragment was cloned into a pCR2.1 vector using a TA cloning

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**Table 1. Characteristics of patients and controls**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Source</th>
<th>n</th>
<th>Age (yr)</th>
<th>Disease duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankylosing spondylitis</td>
<td>PB</td>
<td>47</td>
<td>35.4±8</td>
<td>6.7±7</td>
</tr>
<tr>
<td>Ankylosing spondylitis</td>
<td>SF</td>
<td>7</td>
<td>43.3±9</td>
<td>9.6±1</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>PB</td>
<td>22</td>
<td>56.4±5</td>
<td>6.2±8</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>SF</td>
<td>4</td>
<td>51.2±7</td>
<td>7.4±9</td>
</tr>
<tr>
<td>Normal controls</td>
<td>PB</td>
<td>20</td>
<td>42.5±7</td>
<td></td>
</tr>
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</table>

*95% of patients were positive for HLA-B27.*
kit (Invitrogen Inc., Carlsbad, CA). The construct, including the human aggrecan G1 globular domain and a partial interglobular domain (IGD) plus six histidine residues at its C-terminal, was sub-cloned into the pQBI-AdCMV5-IRES-GFP transfer vector from the ADENO-QUEST KIT (Quantum Biotechnologies Inc., Montreal, QC) at the BgIII site. After being linearized by FseI restriction enzyme digestion, 1 mg of the recombinant transfer vector plasmid was cotransfected into 293 cells with 1 mg of QBI-viral DNA from the same kit using Lipofectamine plus reagent (LifeTech Co., Burlington, ON). Screening and purification of recombinant adenovirus were performed according to the Adeno-Quest application manual included in the kit. For recombinant AG1 production, 293 cells were split on to 150-mm dishes in 1 to 10 dilution in Dulbecco’s Modified Eagle Medium (DMEM) plus 5% fetal calf serum (FCS), grown for 2 days until cells were ~90% confluent, then the media were changed into 293 serum-free media (LifeTech Co., Burlington, ON); meanwhile recombinant virus was harvested at 50 MOI. On day 3 after infection, the supernatant containing recombinant AG1 protein was collected. The supernatant was applied to a Sephadex G-25 column equilibrated with phosphate-buffered saline (PBS), pH 7.4; the protein-containing fractions were collected and applied to a Ni-NTA agarose column (Qiagen Inc., Mississauga, ON). The column was washed with 40 mM imidazole in PBS containing 0.3 M NaCl, pH 7.4. Recombinant versican G1 (VG1) and AG1 were eluted with 100 mM imidazole in PBS, pH 7.4, containing 0.3 M NaCl.

**HC gp-39 and collagen II**

HC gp-39 is present in cartilage, but also in other structures [39]. There have been several reports showing that HC gp-39 might be a possible autoantigen both in animal models of RA and in RA patients [32, 33]. HC gp-39, recombinantly produced, was kindly provided by A.M.M. Boots, at Organon, Soden, Germany. Screening and purification of recombinant adenovirus were performed according to the Adeno-Quest application manual included in the kit. For recombinant AG1 production, 293 cells were split on to 150-mm dishes in 1 to 10 dilution in Dulbecco’s Modified Eagle Medium (DMEM) plus 5% fetal calf serum (FCS), grown for 2 days until cells were ~90% confluent, then the media were changed into 293 serum-free media (LifeTech Co., Burlington, ON); meanwhile recombinant virus was harvested at 50 MOI. On day 3 after infection, the supernatant containing recombinant AG1 protein was collected. The supernatant was applied to a Sephadex G-25 column equilibrated with phosphate-buffered saline (PBS), pH 7.4; the protein-containing fractions were collected and applied to a Ni-NTA agarose column (Qiagen Inc., Mississauga, ON). The column was washed with 40 mM imidazole in PBS containing 0.3 M NaCl, pH 7.4. Recombinant versican G1 (VG1) and AG1 were eluted with 100 mM imidazole in PBS, pH 7.4, containing 0.3 M NaCl.

**Peptide synthesis**

Peptides were synthesized by a robotic multiple peptide synthesizer (SYRO, MultiSynTech, Bochum, Germany) using an Fmoc-tBu solid-phase synthesis strategy [41]. Wang resin (p-benzyloxybenzylalcohol-polystyrene) (Novabiochem, Bad Soden, Germany, was used as solid support. Side-chain-protected Fmoc-amino acids were obtained from Senn Chemicals (Dielsdorf, Switzerland) and Novabiochem (Bad Soden, Germany). Peptides were characterized by reversed-phase high-pressure liquid chromatography (HPLC) (M480 pump, UVD-320 S diode-array UV-detector, GINA 160 autosampler, Gynkotek, Germering Munich, Germany) on Nucleosil C18, 100A, 5 μm (Macherey-Nagel, Düren, Germany) and electrospray mass-spectrometry (ESI-Quattro II, Micromass Ltd, Altrincham, UK). Forty-six overlapping 18-mer peptides overlapping by 10 amino acid, which cover all 394 amino acid residues of G1 protein, were synthesized.

**In vitro stimulation of CD4+ T cells by protein antigen or peptides**

T cells were stimulated in vitro by protein antigens as described previously [42]. Briefly, 1 ml of whole heparinized peripheral blood or whole synovial fluid (1 ml containing 5 x 10⁶ cells) was stimulated in the presence of anti-CD28 (Immunotech, Marseille, France; 1 μg/ml) for 6 h with 20 mg/ml G1 protein, or 20 mg/ml HC gp-39, or 20 mg/ml collagen II, or Staphylococcus enterotoxin B (SEB, Sigma, 1 mg/ml; used as positive control). These concentrations were found to be optimal in preliminary experiments (data not shown). HC gp-39 was investigated in AS but not in RA. Brefeldin A (10 mg/ml, Sigma) was added for the last 4 h of the stimulation. The culture tubes were left at a 5° slant at 37°C in a CO₂ incubator. Afterwards cells were incubated in EDTA (2 mM final concentration) for 15 min at room temperature. Nine millilitres of FACS lysing solution (1:1) (Becton Dickinson, San Jose, CA) was added to 1 ml of blood for 10 min at room temperature to lyse erythrocytes and fix monocytes. Then the cells were washed again with PBS BSA. For SF, cells were washed with PBS after EDTA incubation and 1 ml of 2% formaldehyde was added to the pellet for 20 min at room temperature, then the cells were washed again with PBS BSA.

For stimulation with peptides, the 46 G1 peptides were put into five pools of peptides, with 8–10 in each pool. Pool 1 contained peptides 1–10, pool 2 peptides 11–19, pool 3 peptides 20–28, pool 4 peptides 29–38, and pool 5 peptides 39–46. For stimulation with peptides, mononuclear cells (MC) instead of whole blood were used. Immediately after the blood was drawn in heparinized syringes, MC of 15 AS and 10 RA patients, whose blood was available again, were obtained by Ficoll-paque density gradient centrifugation (Pharmacia, Uppsala, Sweden). The cells were then suspended in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) with 100 unit/ml penicillin, 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany), 2 mM L-glutamine (Biochrom KG), and 10% heat-inactivated fetal calf serum (Gibco BRL). At least 1 x 10⁶ cells/ml were stimulated with G1 pools of peptides (each peptide 5 mg/ml in the presence of anti-CD28, in the presence of anti-CD28 alone (negative control), or with SEB as a positive control. Finally, staining with monoclonal antibodies directed against the surface markers CD4 and CD69 and against intracellular cytokines and analysis by flow cytometry (FACS) were performed (see below). For fine epitope mapping, fresh blood was taken again from patients who responded to pools of peptides, and MC were stimulated with single peptides.

**Analysis of HLA class II restriction**

Monoclonal antibodies (mAbs) specific for the three main human class II MHC products, HLA-DR, HLA-DQ and HLA-DP were purchased from Becton Dickinson (murine Ig G1, San Jose, CA). These antibodies have been observed to block the corresponding class II mediated responses in vitro. The mAbs were thoroughly dialysed against PBS and were used at a predetermined optimal blocking concentration of 2.5 mg/ml in the culture. To determine whether the responding T cells were stimulated by peptide presented by antigen-presenting cells (APC) in the context of MHC class II molecules, blocking antibodies specific for HLA-DR, -DQ or -DP were added to cultures for 30 min at 37°C and samples without blocking antibodies were included as controls. Then the cells were washed with PBS/BSA and resuspended in RPMI 1640 medium with anti-CD28 and stimulated with the peptides of interest for 6 h as described above.

**Staining for T-cell surface markers, intracellular cytokines and analysis by flow cytometry**

T cells were stained after in vitro stimulation as described previously [42]. Briefly, cells from whole PB or SF or MC were stained after protein antigen presentation. T cells were stained with monoclonal antibodies directed against the surface markers CD4 and CD69 and against intracellular cytokines, and staining was performed with flow cytometry as described above.
washed with PBS/BSA, centrifuged (300 g, 10 min, 4 °C), and cells were quadruple stained for CD4-, CD69-surface markers and two intracellular cytokines, either IFNγ/TNFα or interleukin 4 (IL-4)/IL-10. All stainings were performed in FACS® Permeabilizing Solution (Becton Dickinson, Heidelberg, Germany). To avoid non-specific binding of antibodies to Fc-receptors, all the staining was done in the presence of BSA (3 mg/ml, Centeon Pharma, Berlin, Germany). The following antibodies were used: anti-human CD4 PerCP (clone Leu-3a), anti-CD69 FITC and anti-CD69 phycoerythrin (PE) (Leu-23) obtained from Becton Dickinson. The antibodies against TNFα (Hölzel Diagnostika, Köln, Germany) were coupled to FITC (Sigma), antibodies to IFNγ were coupled to Cy5 (Amersham Pharmacia Biotech, Freiburg, Germany), antibodies to IL-4 (4D9) were coupled to PE (Becton Dickinson), antibodies to IL-10 were labelled to APC (Pharmingen, San Jose, CA). Positive cells were subsequently quantified by flow cytometry using a FACS Calibur from Becton Dickinson (San Jose, CA) with Cellquest-software. After gating on CD4+ T cells, only cytokine-positive T cells which were also positive for the early activation surface antigen CD69 were counted. To analyse whether the two cytokines, which were stained simultaneously, were produced by the same or different cells, CD4+ T cells positive for two cytokines were also counted at the same time.

CD4+ T cells were regarded as positive after antigen-specific stimulation as judged by the percentage of CD69 cytokine double-positive cells if at least 30 cells and 0.02% of the gated CD4+ T cells were positive without background staining (stimulation with anti-CD28 without antigen only) [42, 43]. If the background staining was above 10 cells the percentage of CD69+ T cells positive for intracellular cytokine staining had to be at least three times higher than the background staining to be accepted as positive. CD69 is an early T-cell activation marker and is up-regulated shortly after stimulation with specific antigens [44]. Thus, specificity of intracellular cytokine staining is increased by excluding cytokine + CD69- T cells (non-specific staining of the intracellular cytokines) from analysis.

HLA typing

HLA-DR typing was done by PCR-based methods using sequence-specific primers.

Statistics

χ²-test was used to compare frequency in different groups; the unpaired version of the Wilcoxon test was used to analyse between-group differences of percentages of G1-specific positive T cells; the paired version of the Wilcoxon test was used to analyse differences of percentages of G1-specific positive T cells between PB and SF. Differences were considered to be significant if there was a two-tailed P value of less than 0.05.

Results

Antigen-specific cytokine secretion in ankylosing spondylitis and rheumatoid arthritis compared with healthy controls

As shown in Fig. 1, there is an increased frequency of IFNγ-positive T cells in PB specific for the aggrecan G1 protein in AS and RA: 61.7% (29/47) of the AS patients and 54.5% (12/22) of the RA patients compared with only 2/20 (10%) of the controls had increased percentages of IFNγ-positive T cells in response to G1.

TNFα-positive CD4 T cells responding to G1 stimulation were detected at an even higher percentage in AS and RA patients, but also in controls: 91.5% (43/47), 81.8% (18/22), 50% (10/20), respectively (Fig. 1). This difference between AS and RA compared with controls is significant for both cytokines (P < 0.05), but the small difference between AS and RA was not significant for both cytokines. In most of the AS patients whose T cells responded to G1 domain (26 out of 29), the IFNγ-positive CD4+ T cells were also positive for TNFα (not shown). An example of IFNγ and TNFα secretion of PB CD4+ T cells in response to stimulation with these antigens is shown for one AS patient in Fig. 2 and for one RA patient in Fig. 3.

There was also a significant difference between the groups when the medians of the responding CD4+ T-cell subsets were compared. The percentage of G1-specific IFNγ-positive CD4 cells was [median (25–75th percentile)] 0.04% (0–0.08%), 0.035% (0–0.08%) and 0% (0–0%) in AS, RA and controls, respectively; the difference between AS and controls (P = 0.001) and between RA and controls was significant (P = 0.009). The percentage of G1-specific TNFα-positive CD4 T cells was 0.05% (0.02–0.09%), 0.04% (0.02–0.15%), 0.015% (0.0025–0.04%) in AS, RA and controls, respectively (P = 0.005 for the comparison of AS with controls and P = 0.017 for the comparison of RA with controls). Again, no significant difference was observed between AS and RA.

In SF, 71.5% (5/7) of the AS patients responded to in vitro stimulation with G1 by IFNγ production and 57.2% (4/7) by TNFα production (Fig. 4). In SF from RA patients, a response to G1 was detectable in all four patients (100%) as judged by IFNγ production and in 50% of the patients by TNFα production (Fig. 4). Interestingly, in SF the percentage of patients responding by IFNγ production was higher than the percentage responding by TNFα synthesis, while it was the other
way around in PB (Figs 1 and 4). An example of IFNγ and TNFα production of synovial and PB CD4+ T cells in response to these antigens is shown for one AS patient in Fig. 5. The G1-specific T-cell response in SF [0.12% (0.07–0.23%) for IFNγ; 0.14% (0.01–0.23%) for TNFα] was significantly (P=0.005 for IFNγ; P=0.008 for TNFα) higher than that in PB [0.04% (0.02–0.12%) for IFNγ; 0.04% (0.01–0.06%) for TNFα] (Fig. 4).

Fig. 2. Example of an antigen-specific response to the G1 domain of the proteoglycan aggrecan compared with stimulation without antigen (Ag) or with the human cartilage-derived antigen glycoprotein (gp)-39 or collagen II in a patient with AS. After staining for T-cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T-cell subpopulation is indicated.

Fig. 3. Example of an antigen-specific response to the G1 domain of the proteoglycan aggrecan compared with stimulation without antigen (Ag) or with the human cartilage-derived antigen collagen II in a patient with RA. After staining for T-cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T-cell subpopulation is indicated.

Fig. 4. Percentage of patients with AS and with RA responding to the in vitro stimulation with the G1 domain of the proteoglycan aggrecan. Response was measured either by IFNγ or TNFα production of CD4+ T cells after antigen-specific stimulation in comparison with stimulation without antigen. For more details see Patients and methods section. Analysis was done with whole synovial fluid.

None of the AS patients showed a T-cell response to HC gp-39 or to collagen II (an example is shown in Fig. 2) and none of the RA patients showed a T-cell response to collagen II (an example is shown in Fig. 3; HC gp-39 was not tested in RA). No increased percentages of IL-4- or IL-10-positive CD4+ T cells were observed after stimulation with G1, HC gp-39 or collagen II in any of the three groups (data not shown).

Characterization of immunodominant G1 epitopes
A positive response of CD4+ T cells derived from PB to pools of G1 peptides was observed in 15 AS and in 10 RA patients, who also showed a positive response to the whole protein (Tables 2 and 3). Some of the AS patients recognized only one peptide while others showed a T-cell response to two or more peptides. In all of the RA patients a T-cell response to two or more peptides was detectable. Restimulation of PB T cells from the responding patients with single peptides (all out of the positive pools) indicated that peptides 13, 17 and 35 were stimulatory both in AS and RA patients, while peptide 30 was stimulatory only in some AS but not in RA patients, and peptide 9 was stimulatory only in some RA but not in AS patients (Tables 2 and 3). The amino acid sequence and its position in the G1 protein is shown for the single peptides in Tables 2 and 3.

HLA typing
In the majority of the AS patients a full HLA class II typing was performed. The results for the HLA-DR typing are shown in Table 2. None of the peptides was confined to a single HLA-DR type indicating that they can be presented by different -DR types. However, not all of the -DR types seem to be associated with these peptides. There is a negative association of peptides 13
and 17 with HLA-DR3 and -DR5, of peptide 30 with HLA-DR4 and -DR7 and of peptide 35 with HLA-DR7. This association was statistically not significant, possibly because of a small number. The T-cell response to peptide 13 and 35 could be blocked by anti-class HLA-DR antibodies by about 50% in five patients investigated: a reduction of the percentage of IFN$\gamma$-positive CD4$^+$ T cells from a median of 0.020 (range 0.015–0.035) to 0.01 (0–0.02) ($P=0.038$ for this difference) was found for peptide 13 and a reduction from 0.046 (0.025–0.06) to 0.024 (0.01–0.035) ($P=0.041$ for the difference) for peptide 35. No inhibition of the T-cell response was observed after adding anti-DQ or anti-DP antibodies (data not shown). Thus, these data indicate that the immune response to G1-derived peptides is HLA class II restricted, although several G1-derived peptides can be presented by several HLA class II types.

### Discussion

The main aim of this study was to determine by new sensitive cytokine cytometric analysis whether response to cartilage-derived putative autoantigens G1, HC gp-39 or collagen II are observed in AS and RA. Our results showed, judged by the frequency of induction of IFN$\gamma$-positive cells among CD4$^+$ T cells, that there is a response to the G1 domain of aggrecan in almost two-thirds of patients with AS (61.7%) and in half of the patients with RA (54.5%). In contrast, normal healthy individuals showed response reactivity only in a few cases (10%). This is even more striking since we did not

![Fig. 5. Example of an antigen-specific response to the G1 domain of the proteoglycan aggrecan compared with stimulation without antigen (Ag) in a patient with AS. The T-cell response in synovial fluid (SF) is higher than peripheral blood (PB). After staining for T-cell surface markers and intracellular cytokines a gate for CD4$^+$ T cells was set. The percentage of IFN$\gamma$/CD69- or TNF$\alpha$/CD69-positive cells of the CD4$^+$ T-cell subpopulation is indicated.](https://academic.oup.com/rheumatology/article-abstract/42/7/846/1784448/8411860)
Fig. 6. Example of an AS patient with an antigen-specific response to one of the pools of peptides (pool 4), but not to pool 1, derived from the G1 domain of the proteoglycan aggrecan compared with stimulation without antigen (Ag). Out of pool 4, containing peptides 29–38, only the single peptide 35 but not peptide 37 was recognized by this patient’s CD4+ T cells. After staining for T-cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T-cell subpopulation is indicated.

find a T-cell response to two other cartilage-derived antigens, the HC gp-39 and collagen II. T-cell responses to HC gp-39 [33] and collagen II [34] have been reported before in RA, but have not been investigated in AS previously. The recombinant collagen II used in this study has not been tested before in human or animal studies. Thus, we can not absolutely exclude that the negative T-cell response is due to this antigen presentation. Importantly, in this study, the response of synovial fluid (SF) CD4+ T cells to the G1 domain was examined in AS and RA patients for the first time. The data clearly show that a significantly higher number of antigen-specific T cells is present in SF than PB. Taken together, all these results indicate that the G1 domain of aggrecan might play a pathogenetically relevant role in the cellular autoimmune response in AS and RA.

In the context of the pathogenesis of spondylarthropathies it is of interest to stress that aggrecan is present in fibrocartilaginous enthesal regions of the tendons which insert at the bone, but not in the human mid-tendon [45, 46]. Furthermore, the G1 domain of the aggrecan molecule is the major degradation product of intervertebral discs [18, 47]. These are all sites that are primarily affected in spondylarthropathies, but not in RA. Aggrecan is the large aggregating proteoglycan from cartilage containing chondroitin sulphate and keratan sulphate, which are attached to a multidomain protein core. It aggregates by binding via the G1 domain to hyaluronic acid and this is further stabilized by a separate globular link protein. A major site of aggrecan cleavage within the interglobular domain lies between the G1 and G2 domains [48, 49] releasing the G1 domain from the rest of the molecule. The G1 domain accumulates in articular cartilage with ageing through its binding to hyaluronan [50]. G1-containing fragments are abundant in synovial fluid [31].

The results presented here do not prove that the G1-specific T-cell response plays a primary role in causing immunopathology in AS and/or RA. It may be a secondary event after cartilage destruction caused by other mechanisms. The fact that the T-cell response to this autoantigen does not seem to be specific for one rheumatic disease is compatible with: (i) aggrecan being a major component of human cartilage which is affected by various rheumatic diseases such as AS, RA and also osteoarthritis; (ii) the physiological role, cleavage and breakdown of aggrecan; and (iii) previous results describing immune reactivity to the G1 domain at both the cellular and the humoral level in different rheumatic diseases [14, 31, 51]. None the less, the demonstration of such a cellular response in both PB and SF of AS and RA patients is encouraging enough to pursue this question in future experiments, particularly since experimental immunity to G1 causes the induction of an inflammatory erosive polyarthritis (as in RA) and a spondylitis (as in AS) [27, 28].

Furthermore, the T-cell response to the aggrecan G1 domain is so far the clearest and strongest autoimmune reaction to cartilage-derived autoantigens both in AS and RA. The T-cell response to both the whole G1 protein and to G1-derived pools of peptides and to single peptides, as shown in our study, confirms the presence of a G1-specific immune response and, importantly, it also excludes a false-positive response due to contamination in the protein/peptide preparations. In comparison, it has been difficult to show T-cell responses to other potential autoantigens such as collagen II and gp-39 in RA [33, 34] and no such investigations have been performed in AS. Thus, independently of its potential role as a causative autoantigen, the aggrecan G1 domain could be a candidate for antigen-specific tolerance induction through bystander suppression, both for AS and RA, similarly as it has been tried with oral collagen II treatment in RA [52].

The examination, quantification and visualization of cellular immune responses have recently become more sophisticated by using flow cytometry [42–44], which allows determination not only of the cellular subtypes under investigation but also, at the same time, the measurement of the intracellular cytokines secreted by the same cells after non-specific or antigen-specific stimulation of T cells in vitro [42]. The technique is capable of detecting antigen-specific T-cell frequencies as low as $1 \times 10^{-5}$ [35, 43, 53] Such an improvement in sensitivity is essential if T-cell responses to autoantigens are sought, which are normally difficult to detect because of low frequencies [43]. In our study, we concentrated on CD4+ T-cell responses because we started by investigating whole recombinant proteins, which are normally processed via the pathway II of antigen presentation. The epitopes created are normally only presented to CD4+ T cells.
In parallel to the IFN-γ response, we also observed a high G1-specific TNF-α response. However, it remains to be determined whether T-cell responses to candidate antigens including autoantigens can be assessed by TNF-α secretion. Our data indicate that antigen-triggered TNF-α secretion by T cells could be more sensitive but less specific than IFN-γ secretion. The investigation of the TNF-α response is of special interest in consideration of: (i) the high amount of TNF-α present in inflamed sacroiliac joints of AS patients [22], (ii) the reportedly lower amount of TNF-α secreted in peripheral blood of AS patients [54] and (iii) the efficacy of anti-TNF-α treatment in AS and other spondylarthropathy patients [55].

An antigen-specific T-cell secretion of IL-4 or IL-10 could not be detected in this study. The production of the T₃₁ cytokines IFN-γ and TNF-α upon antigen contact but not of T₄₂ (IL-4) or T₄₅ regulatory (IL-10) cytokines might indicate that: (i) the G1 response might play a role in the immunopathology of AS and possibly in RA and (ii) that this does not appear to be counteracted by suppressive cytokines.

The identification of T-cell epitopes is crucial for the understanding of the host response in autoimmune diseases. MHC molecules on the surface of antigen-presenting cells present peptide fragments derived from proteins to T lymphocytes. Once a target protein is defined for a T-cell response, the antigenic epitope can be mapped with synthetic peptides [56]. In our study, four T-cell epitopes within the G1 protein (amino acid residues 116–133, 148–165, 252–269 and 292–309) were identified in AS patients and also in RA patients (amino acid residues 84–101, 116–133, 148–165 and 292–309). Previous work on G1 induction of an erosive polyarthritis and spondylitis in BALB/c mice has revealed that the G1 domain of the proteoglycan aggrecan contains an immunodominant arthritogenic region identified by two distinct T-cell epitopes [28]. Adoptive transfer of T cells specific for these peptides also induced arthritis [28]. The identified immunodominant epitopes found in mice and in our study were not identical, a finding which is not surprising if a different MHC background is considered.

We showed in this study that not a single but several peptides out of the G1 domain are recognized by T cells, that the immunodominant peptides identified in AS and RA patients are overlapping, and that the immunodominant peptides might, at least partly, be determined by the HLA-DR type. All this indicates that there does not seem to be a single causative antigen, at least not on the CD4 T-cell level, but rather a broad T-cell response to the G1 domain with the implications discussed above. However, in context of the strong HLA-B27 association in AS, the identification of epitopes presented by HLA-B27 to CD8+ T cells [5] would certainly be of great interest. Thus, while the CD4+ T-cell response might be rather non-specific, this does not exclude a CD8 T-cell response to one or few arthritogenic peptides derived from the G1 domain. The experiments to address this question are currently in progress.

Final proof for a critical role of the G1 molecule in the pathogenesis of AS will come from the detection of antigen-specific T cells in cartilage [7, 22], possibly through tetramer technology [57], or, both for AS and RA, by induction of G1-specific T-cell tolerance, possibly through mucosal tolerance [52]. The latter approach could be used for both AS and RA even if the G1 molecule is not pathogenetic. Owing to the strong HLA-B27 association in AS, it will also be important to investigate the G1-specific response of CD8+ T cells. Furthermore, it will be very interesting to look for G1-directed immune responses in other spondylarthropathies such as reactive arthritis and psoriatic arthritis in which, clinically, the same anatomical structures are involved.

Taken together, a new piece has been added to the puzzle of the immunopathology of AS: this study clearly suggests that CD4+ T cells of the majority of AS and RA patients recognize the G1 domain of aggrecan—a molecule that is present in clinically relevant anatomical structures involved in AS and other spondylarthropathies.

Conflict of interest

The authors have declared no conflicts of interest.

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