CD74 is a T cell antigen in spondyloarthritis

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Abstract

Objective

Spondyloarthritis (SpA) is a chronic inflammatory disease of unknown aetiology. Previously, we identified autoantibodies against CD74 in sera of SpA patients. The aim of this study was to evaluate CD74 as a T cell antigen in SpA.

Methods

Recombinant CD74 protein and a panel of selected peptides representing its amino acid residues were examined for their capability to stimulate peripheral blood mononuclear cells from patients with SpA. In particular, cytokine production by CD4+ T cells was evaluated with flow cytometric detection of intracellular TNF-α, IFNγ, TGFβ and IL-17A. Patients' sera were tested for antibodies against CD74 using ELISA. Samples from patients with rheumatoid arthritis and healthy blood donors were similarly tested as controls.

Results

Significantly more CD4+ T cells from SpA patients produced TNF-α, IFNγ and IL-17A in response to recombinant CD74 than patients with rheumatoid arthritis or healthy blood donors. Among evaluated epitopes, the most promiscuous one lies within the peptide of the amino acid residues 142-185, which appeared more immunogenic. Further, the proportion of cytokine producing CD4+ T cells was significantly higher among SpA patients with autoantibodies against CD74.

Conclusion

CD74 is a T cell antigen in SpA, eliciting Th1 and Th17 responses, which may be relevant in disease pathogenesis. Recognition of the highly immunogenic amino acid residues of CD74 may contribute to our understanding of autoimmune responses of T helper cells in SpA.

Key words

spondyloarthritis, arthritis, invariant chain, epitopes, T-lymphocyte, macrophage migration inhibitory factor receptor

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Introduction

Spondyloarthritis (SpA) is a group of chronic inflammatory diseases characterised by inflammation of axial and/or peripheral joints, enthesitis and dactylitis (1, 2). Ankylosing spondylitis, non-radiographic axial spondyloarthritis, psoriatic arthritis, inflammatory bowel disease-associated arthritis, reactive arthritis and undifferentiated arthritis are diagnoses falling under SpA. Depending on pattern of joint involvement, SpA can be divided into an axial and a peripheral form (3-5). The former affects mainly the axial skeleton and the latter typically involves asymmetric peripheral arthritis, enthesitis and dactylitis. The prevalence of axial spondyloarthritis (axSpA) ranges from 0.36–0.7% in Europe (6). AxSpA starts most frequently at an age between 15 and 45 years and affects equally females and males (5).

The pathogenesis of axSpA is not entirely clear. The pathogenic involvement of HLA-B27 in SpA may stem from its interaction with killer cell immunoglobulin receptors (KIR) and leukocyte immunoglobulin-like receptors (LILR) or alternatively from its propensity to misfold and induce the unfolded protein response (UPR) (7, 8). Recently, in a mouse model of axSpA IL-17 producing, RORγt expressing T cells have been identified at the sites of enthesitis (9). Several lines of evidence, including GWAS, animal studies and the high effectivity of inhibiting TNF in reducing signs and symptoms of SpA support the pathogenic role of TNF (10). B cells were so far not thought to be implicated in the pathogenesis of axSpA. However, we have recently described antibodies against CD74 which were present in the majority of the patients with axSpA (11, 12). That raised the question whether CD74 is T cell antigen. The aim of our study was to characterise the T cell response to CD74 in SpA.

Material and methods

Study subjects

Peripheral venous fully heparinised blood and serum from SpA patients (n=52) and controls was collected in Hannover, Germany. 46 patients suffered from axial spondyloarthritis (20 non radiographic axial spondyloarthritis (nraxSpA) and 26 radiographic axial spondyloarthritis (raxSpA)), 6 had a predominantly peripheral involvement, according to the Assessment of SpondyloArthritis international society (ASAS) classification criteria (3, 4). 25 patients with rheumatoid arthritis (RA) and 15 healthy blood donors were recruited as controls. Laboratory data and disease activity scores were obtained in the Department of Clinical Immunology and Rheumatology of the Medical University of Hannover. BASDAI and DAS28 as markers for disease activity were obtained at the same time as the blood samples. Sera were used to measure autoantibodies against CD74 by ELISA, as described below.

The mean age of the 52 SpA patients was 46.7±13 years, 22 (42.3%) of them were male. The mean BASDAI of the axSpA patients was 4.4±4. 21/52 (40.4%) SpA patients had a disease duration of less than 5 years, 18/52 (34.6%) between 5–20 years and 13/52 (25%) for more than 20 years. 38/52 (73.1%) were HLA-B27 positive, 9/52 (17.3%) were negative and in 5 patients the HLA-B27 status was unknown. 38/52 (73.1%) SpA patients were treated with a TNF inhibitor-based therapy. 20 of those were treated with a TNF inhibitor exclusively and the rest (*i.e.* 18/38) were receiving a TNF inhibitor in combination with a disease modifying anti-rheumatic drug (DMARD) (methotrexate (16/18) or sulfasalazine (2/18)). 11/52 (21.2%) were treated with non-steroidal anti-inflammatory drugs (NSAIDs) and 4/52 (7.7%) were receiving a DMARD-based therapy (methotrexate (1/4) or sulfasalazine (3/4).

The 25 patients with RA fulfilled the American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) criteria of 2010 (13). Their mean age was 54±11 years, 8/25 (32%) of them were male. The mean DAS28 score was 2.51±3.1. 10/25 (40%) RA patients were treated with a TNF inhibitor (5/10 were treated with a TNF inhibitor exclusively and the rest (*i.e.* 5/10) were receiving a TNF inhibitor in combination with a DMARD). 1/25 was treated with rituximab, 2/25 with abatacept and all other RA patients were receiving DMARDs.

All studied RA patients had antibodies against citrullinated proteins.

We also studied 15 blood donors with a mean age of 41±18 years, 7/15 (46.7%) of whom were male.

The study was performed in compliance with national policies and the 1964 Helsinki Declaration and its later amendments. Approval was also obtained from the Ethics committee of the Medical University of Hannover (approval number: 5582). A written consent form has been obtained from all blood donors for publication of all presented data.

Cell isolation and intracellular flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated with Biocoll cell separating solution (Biochrom AG Berlin, Germany), washed three times with PBS (Biochrom AG Berlin, Germany) and suspended at $2x10^6$ cells/ml in RPMI medium containing 10 % fetal calf serum, 1% penicillin and streptomycin, 1% L-glutamine and 1% sodium pyruvate (Biochrom AG Berlin, Germany). Cells were cultured in 24 well plates (Greiner bio- one, Frickenhausen, Germany) at a density of $1x10^6$ cells/well and were incubated with 0.2 μg/ml of the recombinant CD74 protein (Sino Biological Inc., Beijing, China) or the transmembrane protein 199 (TMEN 199; Biomatic, Canada), which was used as control antigen, or with a synthetic peptide from a panel of six peptides representing amino acid residues of CD74 protein (Sino Biological Inc., Beijing, China) for 16 hours. Peptide sequences are depicted in Figure 4A. Incubation with same amount of carrier (*i.e.* 2 μl PBS) was included as negative control and incubation with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml; Sigma Aldrich, Steinheim, Germany) as positive control. One hour after beginning of incubation of cells with peptides, brefeldin A was added in each well at a dose of 10 μg/ml in order to inhibit cytokine secretion. After completion of 16-hour incubation, cells were collected, transferred into tubes for flow cytometric analysis (BD Biosciences Franklin Lakes, USA), washed twice in PBS and resuspended in PBS BSA 0.1% (Biochrom AG Berlin Germany). Surface staining was performed with anti-human CD3 (PerCP-Cyanine5.5, Ebioscience, San Diego, USA) and anti-human CD4 (BV421, BD Biosciences, Franklin Lakes, USA). To exclude dead cells we added at that stage eFluor 520 viability dye (Ebioscience, San Diego, USA). Cells were incubated with staining antibodies for 30 minutes at 4°C in the dark and were then washed twice with 300 μl PBS BSA 0.1%. Subsequently, cells were fixed with 100 μl paraformaldehyde 4% (Merck Darmstadt, Germany). After 10 minutes they were washed three times with 300 μl PBS BSA 0.1%. For permeabilisation of cell membranes 50 μl 0.1% saponine (Riedel- de Haën, Seelze, Germany) were added in each sample, which was followed by intracellular staining with anti-human TNF-α (APC-Vio770, Miltenyibiotec, San Diago, USA), anti-human IFNγ (V500, BD Biosciences, Franklin Lakes, USA), anti-human TGFβ (PE, BD Biosciences, Franklin Lakes, USA) and anti-human IL-17A (PE/Cy7, Bio Legend, San Diego, USA) or matched isotype control antibodies. Last, cells were resuspended in 200 μl PBS BSA 0.1% and analysed with FACS Canto II (BD Biosciences Franklin Lakes, USA). Flow cytometry data were evaluated with FlowJo software version 8 (Tree Star Inc., Ashland, USA). After selecting singlet lymphocytes we gated on $CD3^+$ cells and then on $CD3^+CD4^+$ and $CD3^+CD4^+$ cytokine expressing cells as shown in Supplementary Figure 1.

Detection of autoantibodies against CD74

IgA antibodies against CD74 were measured using an ELISA kit produced by Aesku Diagnostics, Wendelsheim, Germany (SpA Detect kit, RAF 3190). The assay procedure was performed according to the manufacturer`s instructions as previously described (11). Briefly, 10 μl of the serum from each tested subject were diluted in 1 ml of sample buffer (Aesku Diagnostics). 100 μl of the diluted serum were subsequently incubated in a measurement well for 30 minutes at room temperature. Samples were then skipped and wells were washed 3 times with washing solution (Aesku Diagnostics). Thereafter, horseradish peroxidase–conjugated anti-human IgA (100 μl) was added to each well for 30 minutes and was subsequently skipped. Wells were washed again 3 times with washing solution and tetramethylbenzidine solution (50 μl) was added for 30 minutes. After addition of stop solution (50 μl), optical density was measured with an ELISA reader.

Statistical analysis

Differences between the two groups were evaluated with Mann-Whitney, whereas for comparison of more than two groups Kruskal-Wallis test was performed. The data on immunodominance of different peptides were evaluated with Wilcoxon matched pairs signed rank test. For statistical calculation and diagrams we used GraphPad prism 5.00 (GraphPad, La Jolla, USA).

Results

Cytokine production by $CD4^+$ T cells after stimulation with CD74

Given the association of CD74 autoantibodies with SpA (11, 12), we evaluated CD74 as a T cell antigen, focusing on Th1 and Th17 responses which may be relevant in disease pathogenesis (8, 20). Therefore, we incubated PBMC with recombinant CD74 protein and evaluated cytokine production by $CD4^+$ T cells. We detected a significantly higher proportion of TNF-α-producing $CD4^+$ T cells in samples from patients with SpA as compared to samples from patients with RA or healthy donors (HD) (Fig. 1A). Similarly, significantly higher numbers of IFNγ-producing $CD4^+$ T cells and IL-17A-producing $CD4^+$ T cells were found in samples from patients with SpA (Fig. 1B and 1C, respectively). With respect to the proportion of TGFβ-producing $CD4^+$ T cells, differences between SpA and RA patients or HD remained non-significant (Fig. 1D). Stimulation of PBMC with the control antigen, TMEN 199, yielded no measurable cytokine expression in all groups of patients and controls (not shown). Separate analysis of SpA patients with different disease duration, revealed no significant differences in CD74-induced cytokine production (Fig. 2A-D). Furthermore,

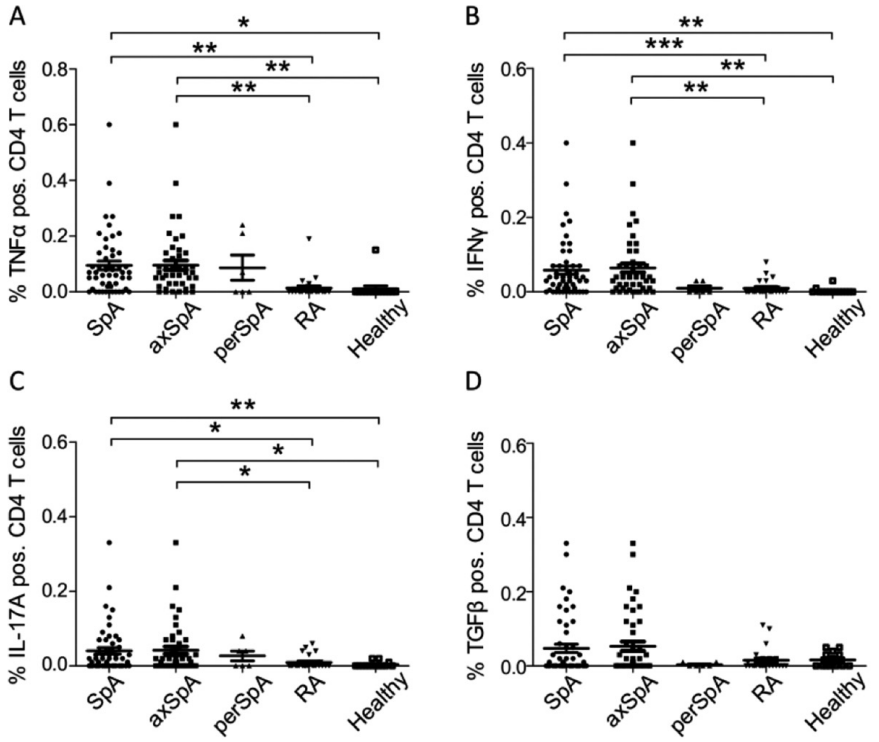

Fig. 1. CD74-induced cytokine production by CD4+ T cells from patients with SpA (n=52) (axSpA: n=46 and perSpA: n=6) as compared to patients with RA (n=25) and HD (n=15). Percentages of TNF-α (**A**), IFNγ (**B**), IL-17A (**C**) or TGFβ-producing CD4+ T cells (**D**) after incubation of PBMC from the indicated group of patients or HD with recombinant CD74 (*p<0.05; **p<0.01, ***p<0.001).

Table I. Cross table showing anti-CD74 status of all studied subjects.

	SpA	Non-SpA (RA + HD)	Total
Anti-CD74 pos.	31	4 + 0	35
Anti-CD74 neg.	21	21 + 15	57
Total	52	25 + 15	

evaluating the effect of different treatments, we found no significant differences, with the exception of increased TGFβ-producing CD4+ T cells in TNF-inhibitor treated patients as compared to the ones receiving NSAID (Fig. 2E-H). Overall, detection of higher percentages of TNF-α-, IFNγ- or IL-17A-producing CD4+ T cells from patients with SpA in response to CD74, suggests that CD74 is an antigen promoting Th1 and Th17 differentiation.

Anti-CD74 antibody status is associated with cytokine production by CD4+ T cells

Several previous studies have described the association between SpA and autoantibodies against CD74 (11, 12, 14). Here we detected anti-CD74 autoantibodies in 31/52 (59.6%) patients with SpA. Based on this cohort (Table I), the sensitivity of anti-CD74 autoantibodies for diagnosing SpA was calculated 59.6% and the specificity amounted to 90%, which is in line with the findings of the International Spondyloarthritis Autoantibody (InterSpA) trial, which evaluated the same assay (sensitivity 47% and specificity 95%) (11). The positive likelihood ratio was 5.96, while the negative likelihood ratio was 0.45. Treatment with TNF-inhibitors did not influence the detection of anti-CD74 autoantibodies; 22/38 (57.9%) SpA patients with TNF-inhibitors and 8/14 (57.1%) of patients who received no TNF-inhibitor were were detected positive.

Antigen-specific T helper cell responses can drive the development of antibody responses against cognate antigens (15). We therefore went on testing whether CD4+ T cell responses to CD74 associate with the detection of autoantibodies against CD74. The proportion of CD4+ T cells producing TNFα, IFNγ or IL-17A in response to recombinant CD74 were significantly higher among SpA patients with autoantibodies against CD74 compared to those without (Fig. 3).

Immunodominance of selected peptides located within the extracellular domain of CD74

We next performed a more detailed analysis evaluating the immunogenicity of different epitopes within CD74, using a panel of 6 synthetic overlapping peptides, spanning the complete isoform of CD74 which was used as an antigen in the ELISA (Fig. 4A). CD4+ T cell cytokine responses after cell incubation with CD74 or each of the 6 peptides were evaluated in 15 patients with axSpA. The two most frequently recognised peptides were peptide 1 (a.a. 17-51) and 4 (a.a. 142-185), with peptide 4 eliciting a higher proportion of TNF-α- or IFNγ-producing CD4+ T cells (Fig. 3B-E and Suppl. Fig. 2). Each of these two peptides could elicit a cytokine response in 11 out of 15 patients. Further, 11 out of 15 tested patients were responding to recombinant CD74 (Suppl. Fig. 2). Antibodies against CD74 were detected in 8 out of the 15 tested patients. The proportion of CD4+ T cells producing TNF-α in response to peptide 4 was significantly higher among axSpA patients with autoantibodies against CD74 compared to those without (Fig. 5). For the remainder of tested cytokine responses (*i.e.* IFNγ, IL-17A and TGFβ) we detected no significant differences. As was the case with all 52 studied SpA patients, the proportion of CD4+ T cells producing TNF-α in response to recombinant CD74 was significantly higher among patients with autoantibodies against CD74 compared to those without these autoantibodies (Suppl. Fig. 3). No significant differences were detected between anti-CD74 positive and negative SpA patients exhibiting CD4+ T cell responses against peptide 1 or the rest of peptides.

Discussion

We have previously identified autoantibodies against CD74 in sera from

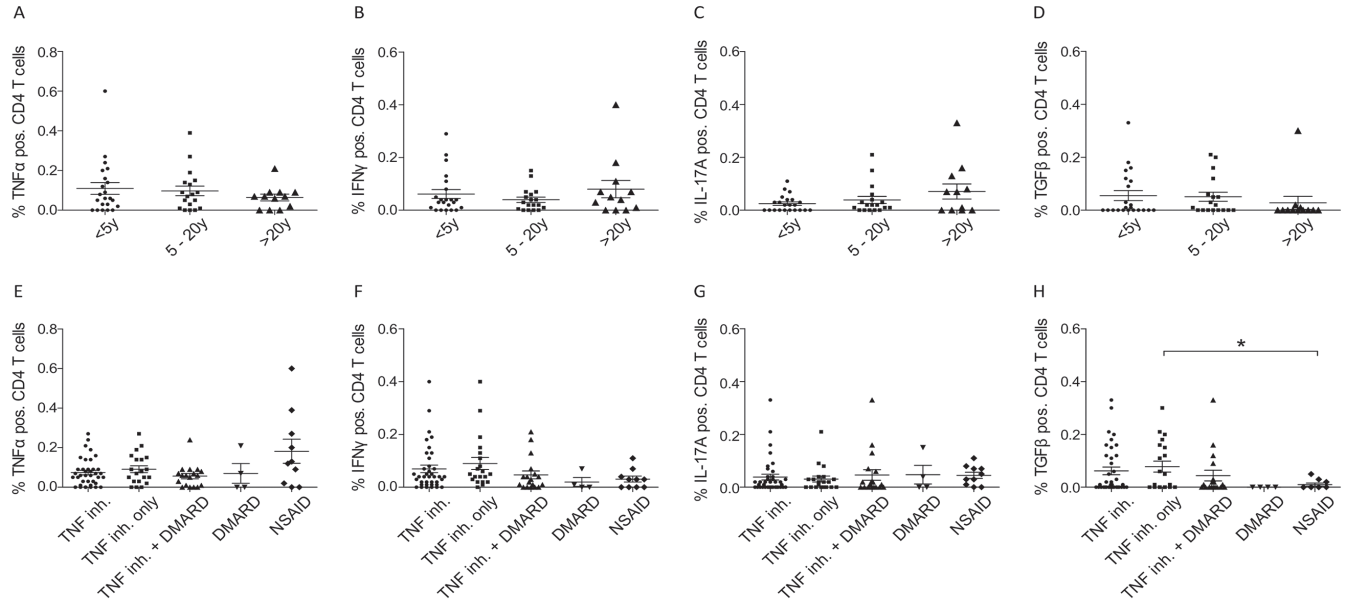

Fig. 2. Effect of disease duration and treatment on CD74-induced cytokine production by CD4+ T cells from patients with SpA.
A-**D**: effect of disease duration (<5 years(y): n=22, 5-20 y: n=18, >20 y: n=12), percentages of TNF-α- (**A**), IFNγ- (**B**), IL-17A- (**C**) or TGFβ-producing CD4+ T cells (**D**) after incubation of PBMC from the indicated group of patients or HD with recombinant CD74 protein.
E-**H**: effect of therapy (TNF inhibitor (inh.)-therapy: n=38, of whom n=20 were receiving only TNF-inhibitor and n=18 a combination of a TNF-inhibitor with a DMARD, DMARD: n=4 and NSAID: n=11), percentages of TNF-α (**E**), IFNγ (**F**), IL-17A (**G**) or TGFβ-producing CD4+ T cells (**H**) after incubation of PBMC from the indicated group of patients or HD with recombinant CD74 protein (*p<0.05).

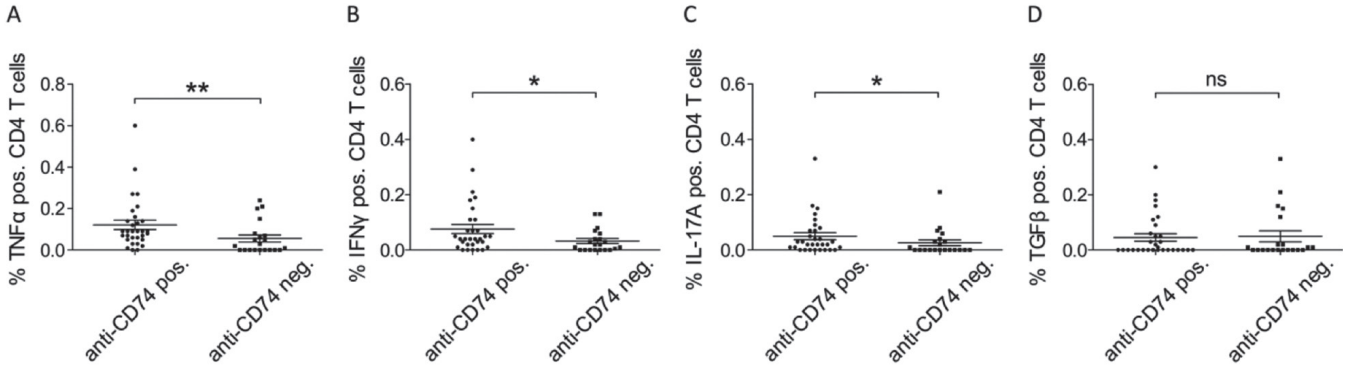

Fig. 3. Higher percentages of cytokine producing CD4+ T cells among anti-CD74 positive SpA patients (n=31) in comparison to anti-CD74 negative SpA patients (n=21). Intracellular production of TNF-α (**A**), IFNγ (**B**), IL-17A (**C**) or TGFβ by CD4+ T cells after incubation with recombinant CD74 in correlation to detection of CD74 autoantibodies in all studied patients with SpA (*p<0.05; **p<0.01).

patients with SpA (11, 12). The current study provides first evidence that CD74 is a T cell antigen in SpA. Further, CD74 responding CD4+ T cells were producing TNF-α, IFNγ or IL-17A, and appear therefore to differentiate into Th1 and Th17 cells.

CD74 is a type 2 transmembrane protein (16, 17). Human CD74 consists of an extracytoplasmic domain of 160 amino acids, a 26-amino acid transmembrane region and an intracytoplasmic domain of approximately 28 amino acids (16, 18). It is predominantly localised intracellularly (17). However, MHC class II-expressing cells, such as dendritic cells, monocytes, macrophages and B cells express CD74 also on cell surface (16, 19). The most well-known function of CD74 is its role in chaperoning MHC class II molecules and sorting them to intracellular compartments (20). Besides the involvement of CD74 in antigen presentation, the fact that it can be expressed independently from MHC class II molecules suggests additional functions (21-23). CD74 is a receptor for the proinflammatory cytokine MIF (macrophage migration inhibition factor) (23, 24). MIF binding to CD74 induces signal cascades including NF-κB and MAP kinases leading to secretion of proinflammatory cytokines (19, 24). With respect to SpA, higher levels of MIF have been described in patients' sera and synovial fluid, correlating with spinal progression in ankylosing spondylitis, which suggests the pathogenicity of MIF (25). Interestingly, these MIF-CD74 triggered proinflammatory signalling cascades can be activated through an anti-CD74 antibody (18), suggesting that activating anti-CD74 antibodies could assume a pathogenic role in SpA.

Autoreactive T cells are not necessarily pathogenic, as they can be found in healthy individuals (26). In this case

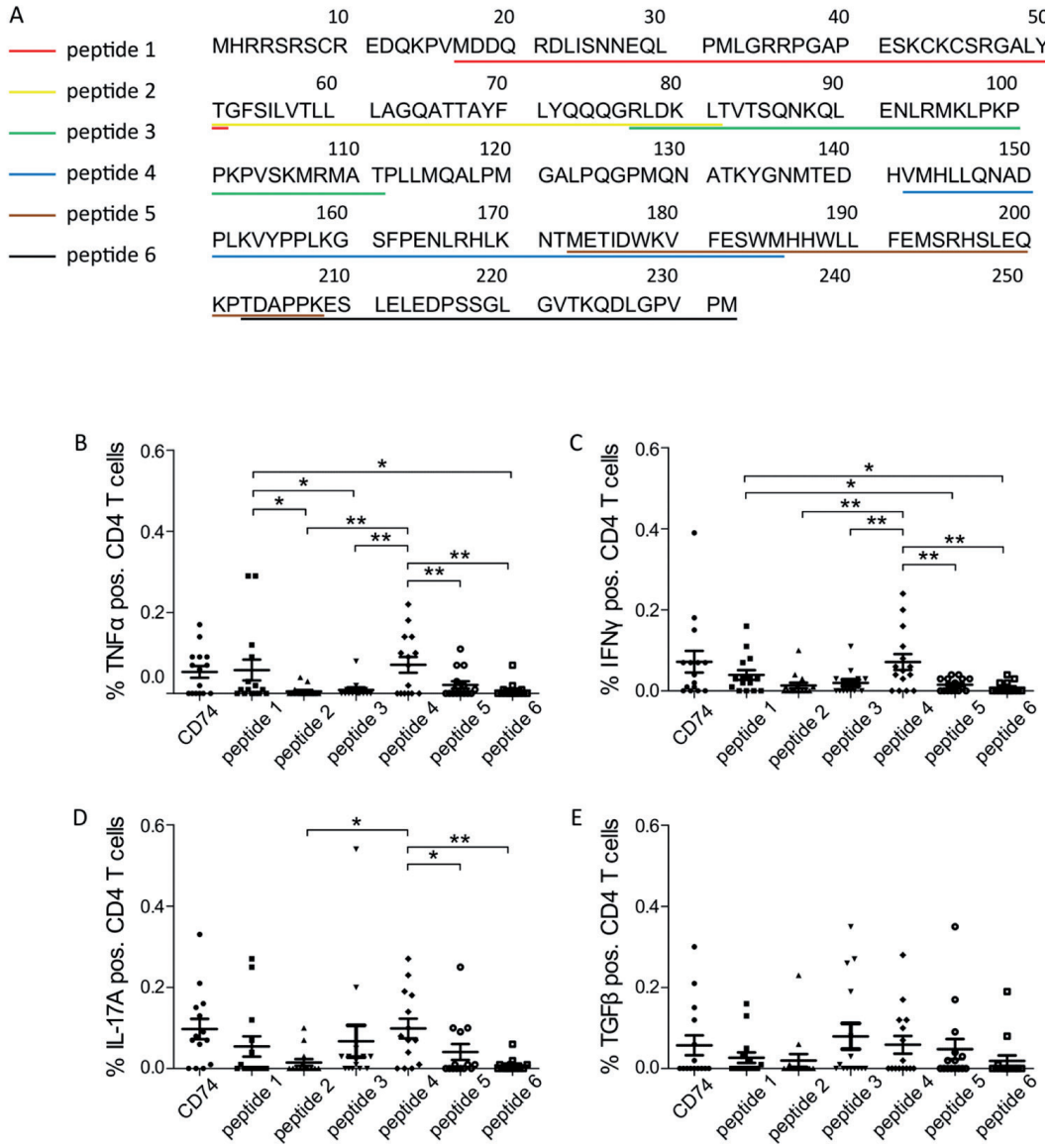

Fig. 4. Immunodominace of 6 selected peptides representing different epitopes of CD74 (**A**) were evaluated for their immunogenicity. Percentages of TNFα (**B**), IFNγ (**C**), IL-17A (**D**) or TGFβ-producing CD4⁺ T cells (**E**) after incubation of PBMC from patients with axSpA (n=15) with the indicated peptide or recombinant CD74 protein (*p<0.05; **p<0.01).

they appear to be balanced by regulatory T cells. In our study, very few T cells of blood donors produced TNF-α, IFNγ or IL-17A in response to recombinant CD74. However, production of TGFβ was more abundant, suggesting an adequate regulatory activity, which may be suppressing CD74-reactive T cells in healthy blood donors. In contrast to healthy donors, in patients with SpA, we found abundant numbers of CD4⁺ T cells, responding to CD74 by

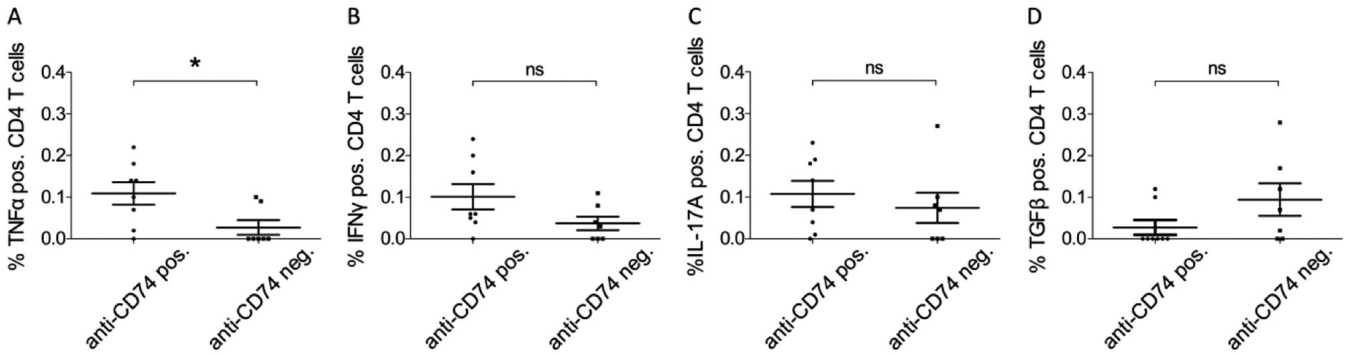

Fig. 5. Higher percentage of TNFα-producing CD4⁺ T cells among anti-CD74 positive SpA patients (n=8), after PBMC stimulation with peptide 4, as compared to anti-CD74 negative SpA patients (n=7). Intracellular production of TNF-α (**A**), IFNγ (**B**), IL-17A (**C**) or TGFβ by CD4⁺ T cells after incubation with peptide 4 in correlation to detection of CD74 autoantibodies in all studied patients with SpA (*p<0.05).

producing TNF-α, IFNγ or IL-17A. These data suggest that CD74 induces Th1 and Th17 differentiation. Several lines of evidence suggest the pathogenic role of Th1 and Th17 responses in chronic inflammatory arthropathies, including SpA (27-29). Treatment with TNF-inhibitors did not affect CD74-induced cytokine production, with exception of enhanced TGFβ production in patients receiving TNF-inhibitors (30, 31). The induction of TGFβ by TNF-inhibitors has been previously described in human T cells and macrophages. TNF-inhibitor-induced expression of TGFβ has been suggested to assume an anti-inflammatory effect in several inflammatory diseases such as in RA and Crohn's disease and needs to be further investigated in SpA.

Detection of pathogenic cellular or antibody immune responses may provide new approaches to treat autoimmune diseases (32). One therapeutic approach could be the induction of tolerogenic B and T cell responses against the antigen triggering pathogenic autoimmune responses. A prerequisite for such a therapeutic approach would be the identification of dominant epitopes. Dominant epitopes could be exploited to design diverse tolerogenic strategies, such as peptide vaccines, dendritic cell vaccines, delivery of altered peptide ligands and induction of oral tolerance (33). Here we have identified a highly immunogenic epitope of CD74, which could be helpful in designing therapeutic tolerogenic approaches. Of course evidence on the pathogenicity of immune responses against CD74, would be a prerequisite before designing such therapeutic approaches.

Several reports have implicated alterations in gastrointestinal tract microbiota and/or bacterial gut infection with the pathogenesis of joint inflammation in SpA (34). Identification of antigen and epitope-specific immune responses may shed light on the mechanisms underlying inflammation in SpA, especially in view of its suggested bacterial-related origins. With respect to the immune responses described in the present work, it is noteworthy that CD74 and its highly immunogenic amino acid residues (peptide 1 and 4), appear to highly homologous to a *K. pneumoniae* protein (CEO54-29830). Given the long ago described association between SpA and *K. pneumoniae* (35-37), homology between CD74 and *K. pneumoniae* might be explaining the mounting of humoral and cellular autoimmune responses against CD74 in SpA.

In summary, this study provides the first evidence that the humoral immune response against CD74 is associated with a T cell response in patients with spondyloarthritis. Moreover, our data suggest that CD74 induces Th1- and Th17-skewed T cell responses. These findings strengthen the view, that CD74 is a relevant autoantigen in the pathogenesis of SpA. Identification of autoreactive T cells could open the road to new therapeutic targets, for example to therapies directed against T cell activation or aiming at antigen specific tolerisation.

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