Published in final edited form as:

Arthritis Rheumatol. 2014 October ; 66(10): 2793–2803. doi:10.1002/art.38763.

Role of PPM1A and anti-PPM1A Autoantibodies in Ankylosing Spondylitis

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Abstract

Objective—Although ankylosing spondylitis (AS) is driven by immunemediated processes, little is known about the presence and role of autoantibodies in this disease.

Methods—We performed human protein microarray analysis of sera derived from patients with AS and other autoimmune disorders to identify autoantibodies associated specifically with AS, and identified autoantibody targeting of protein phosphatase magnesium-dependent 1A (PPM1A) in AS. We performed ELISA analysis of sera from two independent AS cohorts to confirm autoantibody targeting of PPM1A, and to assess associations between levels of anti-PPM1A antibodies and AS disease severity or response (as measured by BASDAI score) to anti-TNF therapy. Levels of anti-PPM1A antibodies were also evaluated in sera from transgenic rats overexpressing HLA-B27 and human β_2 -microglobulin. The expression of PPM1A was assessed by immunohistochemistry in synovial tissues from patients with AS, rheumatoid arthritis, or osteoarthritis. The role of PPM1A on osteoblast differentiation was investigated by gene knock-down and overexpression.

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Conflicts of interest: None

Author contributions

Study conception and design. Y Kim, WH Robinson

Acquisition of data. Y Kim, D Sohn, X Zhao

Data analysis and interpretation. Y Kim, WH Robinson, D Sohn, J Sokolove, TM Lindstrom, JD Reveille, JD Taurog, B Yoo, C Lee

Results—AS was associated with autoantibody targeting of PPM1A, and levels of anti-PPM1A autoantibodies were significantly higher in patients with more advanced sacroiliitis and correlated with BASDAI score after treatment with anti-TNF agents. The levels of anti-PPM1A autoantibodies were also higher in sera of transgenic rats that are prone to develop AS than in those that are not. PPM1A was expressed in AS synovial tissue, and PPM1A overexpression promoted osteoblast differentiation, whereas PPM1A knockdown suppressed it.

Conclusions—Anti-PPM1A autoantibodies are present in AS, and our findings suggest that PPM1A may contribute to the pathogenic bone ankylosis characteristic of AS.

Introduction

Ankylosing spondylitis (AS), the prototype of a group of inter-related diseases known collectively as spondyloarthritis, is a chronic inflammatory arthritis that affects the spine, sacroiliac joints, and peripheral joints. It has a prevalence of 0.2- 0.5% in the US and frequently results in functional disability (1, 2). The diagnosis of AS is typically delayed, being made on the basis of radiographic features, such as joint erosion and subchondral-bone erosion, that are observed at late stages of the disease (3). Although the more recent use of MRI enables detection of inflammatory lesions, which may develop at early stages of the disease, the usefulness of such MRI assessment in predicting subsequent structural damage remains to be established (4). In addition, disease activity and treatment response in AS are assessed using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (5) or Assessment in Ankylosing Spondylitis (ASAS) improvement criteria (6), both of which are complex and comprise several subjective parameters. Thus, there is great need for biomarkers that can aid in early diagnosis or in assessment of disease activity in AS.

Although its pathogenesis is incompletely understood, AS is considered an immune-mediated disease: 80-90% of individuals with AS carry the human leucocyte antigen (HLA)-B27 haplotype, suggesting involvement of CD8+ T cells. Compared to other rheumatic autoimmune diseases, little is known about a possible role for autoantibodies in AS. A recent screen, however, identified the presence of several autoantibodies targeting connective, skeletal, and muscular tissue autoantigens in the blood of individuals with AS (7).

Besides aberrant activation of the immune system (8), ankylosis is a hallmark of AS. Ankylosis is the result of bony apposition occurring along periosteal sites and leading to new bone formation, a process that requires differentiation of osteoblasts. Differentiation of osteoblasts from mesenchymal cells in turn requires a series of signals, including prostaglandin E2, parathyroid hormone, bone morphogenetic proteins (BMPs), and wingless proteins (Wnt) (9). The process is regulated by activation of genes such as Runx-2, osterix, osteocalcin, and bone sialoprotein, depending on the stage of differentiation (9). Repair mechanisms activated in response to local joint destruction have been proposed to trigger the activation of osteoblasts in AS, resulting in syndesmophyte formation and in ankylosis of the affected joint (10). Limiting abnormal osteoblast activation might slow radiographic progression in AS. For example, levels of BMPs are increased in AS serum (11, 12), and systemic transfer of the BMP antagonist Noggin prevented radiographic progression in a

mouse model of AS (13). Levels of sclerostin, a natural inhibitor of Wnt, is lower in the skeleton of individuals with AS than in that of individuals with rheumatoid arthritis (RA) (14), and levels of dickkopf-1, another inhibitor of Wnt, was proposed as a predictor of radiographic progression in AS (15).

In a screen to identify autoantibodies associated with AS, we found that serum levels of autoantibodies against protein phosphatase magnesium-dependent 1A (PPM1A)—a Ser/Thr protein phosphatase that regulates BMP and Wnt signaling (16)—are higher in AS than in other autoimmune diseases. Whether PPM1A activation positively or negatively affects osteoblast differentiation is controversial. Overexpression of PPM1A dephosphorylates and thereby blocks the nuclear translocation of BMP2-induced Smad1, a transcription factor that promotes skeletal and osteogenic development (17). However, another report demonstrated that PPM1A is a positive regulator of Wnt signaling, which induces osteoblastogenesis (18).

Here, using serum samples from two independent cohorts of AS patients and from HLA-B27 transgenic rats, we show that AS is associated with the presence of anti-PPM1A autoantibodies. Moreover, we show that serum levels of anti-PPM1A autoantibodies correlate positively with the degree of sacroiliitis in AS and with the change in disease activity in response to anti-TNF therapy. In addition, we found that PPM1A protein is highly expressed in synovial tissue from AS patients and drives osteoblast differentiation, suggesting that PPM1A itself may contribute to the pathogenesis of AS.

Patients and Methods

Human samples—All biologic samples from patients with AS or other rheumatic diseases, and from healthy individuals, were obtained and studied with informed consent under Institutional Review Board (IRB)-approved protocols. Plasma and sera derived from AS and control patients came from the Multiple Autoimmune Disease Genetics Consortium (MADGC) (19), the Stanford Arthritis Center, the University of Texas Health Sciences Center, and from the rheumatology clinic of Asan Medical Center. Synovial tissues from patients with AS, rheumatoid arthritis (RA), or osteoarthritis (OA) were collected in accordance with human subjects protocols approved by the Stanford University IRB and with the patients' informed consent, as described (20). Joint fluids from patients with AS, RA, or OA were collected at Asan Medical Center and at Stanford University Medical Center. The diagnosis was limited to cases that met the modified New York criteria for AS (21), 1987 revised criteria for RA (22), and criteria for OA (23).

For serum samples collected from AS patients at Asan Medical Center, blood was drawn at the time of diagnosis, and clinical information (age, sex, BASDAI score, radiographic findings, laboratory indices, including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), etc.) were extracted from an electronic clinical database. The severity of sacroiliitis was graded according to the modified New York criteria (21) by a musculoskeletal radiologist. Disease activity of AS was determined according to the BASDAI index (5).

Rat sera—The HLA-B27/hu β ₂-microglobulin transgenic rat lines (inbred Lewis (LEW) background) used in this study have been described previously (24), and non-transgenic rats

or *Dazl*-deficient transgenic rats were used as negative controls according to previous description (25). All animal studies were performed under IACUC-approved protocols at the University of Texas Southwestern. Rats were examined for signs of arthritis at the time of blood sampling as previously described (25, 26), and serum samples from these LEW rats were analyzed.

Protein microarray—ProtoArrays V4 (Invitrogen) were used to profile the autoantibodies present in AS and control sera, and were run using the manufacturer's protocols. Arrays were scanned using the GenePix4000B Scanner (Molecular Devices Corporation, Union city, CA). Median pixel intensities of features and background were determined using GenePix Pro 5.0 software (Axon Instruments Inc., Union city, CA).

ELISA—Levels of anti-PPM1A, anti-PTPN6, and anti-influenza antibodies were measured by ELISA. ELISA plates were coated with rhPPM1A (Creative Biomart, Shirley, NY), rhPTPN6 (Creative Biomart), or influenza virus vaccine (Fluzone®, Sanofi-Pasteur; Nunc, Rochester, NY). Human PPM1A protein is 99% identical with rat PPM1A and 98.2% identical with mouse PPM1A. Secondary antibodies were HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or HRP-conjugated goat anti-rat IgG (Millipore, Billerica, MA). Optical density (OD) was measured at 450-nm absorbance. Serum concentrations of PPM1A were measured with commercially available ELISA kits (USCN Life Science Inc., Houston, TX).

Cells, Antibodies, and Plasmids—Mouse preosteoblastic MC3T3-E1 (Subclone 4 and 14) cells were purchased from ATCC and maintained in Minimum Essential Medium (MEM) α supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μ g/ml of streptomycin (Gibco, Grand Island, NY) in 5% CO₂ at 37°C. Antibodies used were against PPM1A (Abcam: p6c7 for immunoblotting, EP1684Y for immunohistochemistry), p-Smad1/5/8 (Cell Signaling Technology, Danvers, MA), active β -catenin (Millipore, Billerica, MA), and β -actin (Sigma-Aldrich, St. Louis, MO). Human cDNA for PPM1A was purchased from Open Biosystems and subcloned into p3XFLAG-CMV-10 vector (Sigma-Aldrich) for expression in mammalian cells.

RNA Interference and Plasmid Transfection—ON-TARGETplus SMARTpool small interfering RNA (siRNA) targeting mouse PPM1A (Dharmacon: L-040052-00-0005) and non-targeting control siRNA (Dharmacon: D-001810-10-05) were used for RNA interference. The siRNA sequences for mouse PPM1A are as follows: 5'-GCAAGCGGAAUGUAAUUGA-3', 5'-ACACGGCUGUGAUCGGUUU-3', 5'-UCACCAUAUACCAGGAUUU-3', and 5'-ACAAUAGACUGAACCCUUA-3'. MC3T3-E1 cells (1×10^6) were plated into 100-mm culture dishes and 24 hours later transfected with a 60-nM solution containing siRNAs by using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. For plasmid transfection, cells were transfected with 12 μ g of FLAG-PPM1A expression plasmid or p3XFLAG-CMV-10 empty vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours incubation, cells were trypsinized, plated on 24-well plates, and allowed to differentiate. To determine alkaline phosphatase (ALP) activity,

we used 1×10^5 cells/well (siRNA-transfected cells) or 2×10^5 cells/well (plasmid-transfected cells) in 1 ml of medium. For Alizarin Red S (ARS) staining, we used 2×10^5 cells/well (siRNA-transfected cells) or 3×10^5 cells/well (plasmid-transfected cells).

Differentiation of osteoblasts from MC3T3-E1 cells—Cells were incubated with osteogenic differentiation media containing 50 µg/ml of ascorbic acid (Sigma-Aldrich), 10 mM of β-glycerophosphate (Sigma-Aldrich), and 50 ng/ml of bone morphogenetic protein-2 (BMP-2) (Sigma-Aldrich) for 7 or 14 days, with differentiation media refreshed every 3 days. At day 7, ALP activity was determined. Cells were washed with PBS, fixed by air drying for 30 minutes, and stained with NBT/BCIP (Thermo Scientific, Rockford, IL) for 5 ~ 10 minutes in the dark at room temperature (RT). Cells were washed with PBS and air dried for 30 minutes, and images were taken with an Olympus BX51 microscope outfitted with an Olympus DP72 digital camera (Olympus, Tokyo, Japan). To quantify the ALP enzyme activity, we used Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam, Cambridge, MA). At day 14, we performed ARS (Sigma-Aldrich) staining to confirm calcium deposition on the cultured cells. Cells were washed with PBS and fixed with 70% ethanol for 1 hour. The fixed cells were rinsed with distilled water (DW) and stained with 40 mM of ARS solution (pH 4.2) for 10 minutes. The stained cells were briefly washed with DW four times, washed with PBS for 15 minutes, and images were taken. To quantify the degree of mineralization, we incubated cells with 400 µl of 10% acetic acid for 30 minutes at RT with rocking, detached the cells from the culture plates by scraping, and then transferred the cells and acetic acid to a 1.5-ml microcentrifuge tube. The samples were vortexed vigorously for 30 seconds, heated to 85°C for 10 minutes, and centrifuged at 20,000× g for 15 minutes. The supernatants were neutralized with 25 µl of 10% ammonium hydroxide, and the concentration of ARS was quantified by measuring the absorbance at 405 nm on a microplate reader with an ARS standard curve in the same solution.

Quantitative PCR—Total RNA was isolated from cells by using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA by using qScript cDNA synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantitative real-time PCR was performed using PerfeCTa SYBR Green SuperMix ROX (Quanta Biosciences) with the Applied Biosystems 7900HT Fast Real-Time PCR System, and mRNA levels were normalized according to levels of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (Hprt1) and β-actin (Actb). Primer sequences (Integrated DNA Technologies) were as follows: Hprt1 forward 5'-TGTTGTTGGATATGCCCTTG-3', reverse 5'-TGGCAACATCAACAGGACTC-3'; Actb forward 5'-TTCTTTGCAGCTCCTTCGTT-3', reverse 5'-ATGGAGGGGAATACAGCCC-3'; Col1a1 forward 5'-ACATGTTTCAGCTTTGTGGACC-3', reverse 5'-TAGGCCATTGTGTATGCAGC-3'; Bsp2 forward 5'-GTCTTTAAGTACCGGCCACG-3', reverse 5'-TGAAGAGTCACTGCCTCCCT-3'; OCN forward 5'-GCGCTCTGTCTCTCTGACCT-3', reverse 5'-GCCGGAGTCTGTTCCTACTACC-3'; Osx forward 5'-CAACCTGCTAGAGATCTGAG-3', reverse 5'-TGCAATAGGAGAGAGCGA-3'; Runx2 forward 5'-ACACCGTGTGTCAGCAAAGC-3', reverse 5'-GCTCACGTCGCTCATCTTG-3'.

Immunohistochemistry (IHC)—Synovial tissues embedded in optimal cutting temperature (OCT) compound were cut on a cryostat (Thermo Scientific, Kalamazoo, MI) and fixed in 4% paraformaldehyde for 10 minutes at 4°C. The tissue sections were then washed twice (5 minutes each) with PBS, permeabilized with 0.1% Triton X-100 for 3 minutes at RT, and washed again with PBS. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ solution. The tissue sections were blocked with 5% normal goat serum for 20 minutes and then incubated with 3 µg/ml of PPM1A antibodies (Abcam) or control rabbit IgG (Cell Signaling Technology, Danvers, MA) for 1 hour at RT. They were washed twice with PBS and incubated with biotinylated secondary antibody (Vectastain EliteABC Kit; Vector Laboratories, Orton Southgate, Peterborough, UK) at a 1:250 dilution for 30 minutes. The tissue sections were washed with PBS, incubated with complexed avidin-biotin horseradish peroxidase ABC reagent (Vector Laboratories) for 30 minutes, and exposed to 3,3-diaminobenzidine (DAB) substrate according to the manufacturer's instructions (Vector Laboratories). The nuclei were subsequently counterstained with hematoxylin (Vector Laboratories). Tissues were examined and photographed with an Olympus BX51 microscope outfitted with an Olympus DP72 digital camera.

Immunoblotting—Cells were lysed with cell lysis buffer (M-PER Mammalian Protein Extraction Reagent; Thermo Scientific, Logan, UT) containing a Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were separated on 4-12% Bis-Tris gels (Bio-Rad), transferred to PVDF membranes (Millipore), blocked with 5% (w/v) milk, and probed with primary and secondary antibodies in milk. Signal was detected with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Statistical analysis—Differences between two groups were calculated by using the Mann-Whitney U test or unpaired Student's *t*-test, and differences between three or more groups were analyzed by one-way ANOVA and Bartlett's test for equal variances. Relationships between parameters were tested by using Spearman's rank correlation coefficient. Statistical analyses were considered significant when $P < 0.05$.

Results

Detection of anti-PPM1A antibodies in AS sera from a U.S.-based cohort

We used high-density protein microarrays, containing 8,087 human proteins, to test antibody reactivity in sera derived from individuals with pulmonary artery hypertension ($n=23$), RA ($n=21$), juvenile idiopathic arthritis ($n=15$), psoriatic arthritis (PsA; $n=34$), psoriasis ($n=6$), or AS ($n=16$). We found that reactivity to PPM1A was higher in sera from patients with AS than in that from patients with other autoimmune diseases in the U.S.-based Multiple Autoimmune Disease Genetics Consortium (MADGC) cohort (Figure 1A) as well as in a second U.S.-based cohort at the University of Texas Health Science Center at Houston (data not shown). Because TGF- β causes sacroiliitis at the time of bone proliferation in advanced AS (27), we focused our investigation on antibodies against PPM1A, known as an inhibitor of TGF- β signaling (28). ELISA analysis confirmed that serum levels of anti-PPM1A autoantibodies are significantly higher in patients with AS compared to healthy individuals

or individuals with anti-CCP-negative RA or PsA in the Stanford Arthritis Center (Supplementary Figure 1).

Level of anti-PPM1A autoantibodies and PPM1A in AS sera from a Korean cohort

To confirm our findings in an independent cohort of treatment-naïve AS patients (n=45), RA patients (n=20) and healthy individuals (n=30), we evaluated levels of anti-PPM1A autoantibodies in a cohort of Korean patients. Levels of anti-PPM1A autoantibodies were significantly higher in the AS patients than in RA patients or the healthy controls (Figure 1B), whereas levels of anti-PTPN6 antibodies (anti-tyrosine phosphatase antibodies), used as a negative control, were not significantly different between groups (Supplementary Figures 2). When levels of anti-PPM1A antibodies more than 2 SDs above control is considered positive, the sensitivity and specificity was 66.67% and 73.33% for AS. Interestingly, levels of anti-PPM1A autoantibodies were higher in AS patients with high grade radiographic sacroiliitis (grade 3 or 4) than in those with low grade radiographic sacroiliitis (grade 2) (Figure 1C). Clinical parameters including age, sex, disease duration, HLA B27 allele, BASDAI score, and levels of inflammatory markers (ESR and CRP) were not found to be associated with the level of anti-PPM1A antibodies (Supplementary Table 1). Given that PPM1A is an intracellular protein, we performed ELISAs on sera to determine whether it might be released into extracellular compartments or into the blood. Indeed, we found that serum concentrations of PPM1A protein were significantly higher in sera derived from AS patients as compared to sera from RA patients or healthy controls (Figure 1D).

Serum levels of anti-PPM1A autoantibodies decrease after anti-TNF therapy

To determine whether levels of anti-PPM1A autoantibodies change in response to anti-TNF treatment of AS, we measured anti-PPM1A autoantibodies in 6 AS patients (4 treated with adalimumab and 2 with infliximab) before and 3 months after initiation of treatment with a TNF inhibitor. Levels of anti-PPM1A autoantibodies decreased significantly after treatment (Figure 2A), whereas levels of anti-influenza antibodies did not (Figures 2B). BASDAI score, an indicator of disease activity and clinical response (5), also decreased significantly after treatment (Supplementary Figure 3). Notably, a drop in BASDAI score was positively correlated with a drop in level of anti-PPM1A autoantibodies ($r^2 = 0.703$, $P < 0.05$), suggesting that serum levels of anti-PPM1A autoantibodies might be able to serve as a pharmacodynamic biomarker of response to anti-TNF therapy in AS (Figure 2C).

Levels of anti-PPM1A autoantibodies in AS-prone transgenic rats

Transgenic rats that overexpress HLA-B27 and human β_2 -microglobulin developed AS phenotypes dependent on the transgene copy number (24, 25). Among 41 double transgenic rats, at the time of the blood draw twenty one (age at blood drawing ranged from 122 to 349 days) exhibited peripheral arthritis and twenty (age range 93-218 days) did not. Levels of anti-PPM1A autoantibodies were significantly higher in the AS-prone transgenic rats irrespective of clinically evident arthritis as compared to the controls (non-transgenic rats or hypospermatogenic *Dazl*-deficient transgenic rats) ($P < 0.001$) (Figure 3).

PPM1A is highly expressed in AS synovium

Because we found that levels of anti-PPM1A autoantibodies are higher in AS synovial fluids (Figure 4A) and sera (Figure 1B) than in fluids from other diseases, we sought to determine whether expression of PPM1A protein is also higher in synovial tissue derived from AS patients than in synovial tissue from patients with other diseases. Immunohistochemical (IHC) analysis revealed that PPM1A was higher in AS synovial tissues than in RA or OA synovial tissues (Figure 4B). As shown in Figure 4B (box inset), PPM1A localized to the nuclei of AS synoviocytes.

PPM1A promotes osteoblast differentiation in vitro

To evaluate the role of PPM1A on osteoblastogenesis, we modulated the expression of PPM1A by knock-down or overexpression in the mouse preosteoblast cell line MC3T3-E1 and assessed differentiation of these cells into osteoblasts after 1-2 week of incubation in osteogenic media. Knock-down of PPM1A significantly decreased ALP activity and nodule formation (assessed by ARS; Figures 5A, C, and E); conversely, overexpression of PPM1A increased ALP activity and nodule formation (Figures 5B, D, and F).

Furthermore, PPM1A knock-down significantly decreased, and PPM1A overexpression increased, expression of collagen type 1, bone sialoprotein 2, and osteocalcin during differentiation (Figure 6A and B). Osteocalcin expression was the most prominently affected. We found that PPM1A overexpression did not activate Smad1/5/8 or β -catenin, suggesting that PPM1A promotes osteoblast differentiation independently of BMP and Wnt signaling (Figure 6C).

Discussion

We demonstrate that serum levels of anti-PPM1A autoantibodies are higher in sera derived from individuals with AS than from individuals with other autoimmune diseases. Further, HLA-B27 transgenic rats that spontaneously develop AS-like arthritis also had anti-PPM1A autoantibodies. Anti-PPM1A autoantibodies were higher in AS patients with high-grade sacroiliitis as compared to those with low-grade sacroiliitis. Moreover, serum levels of anti-PPM1A autoantibodies decreased in AS patients treated with anti-TNF agents, and the change in levels of anti-PPM1A autoantibodies correlated with the change in disease activity. Finally, we found that PPM1A protein is highly expressed in synovial tissue from AS patients and drives osteoblast differentiation, suggesting that PPM1A itself contributes to the pathogenesis of AS.

Although autoantibodies are an immunologic hallmark of many autoimmune diseases (29), AS has been largely regarded a seronegative disease to date because of a lack of reliable autoantibody biomarkers. Nevertheless, some groups have reported the presence of autoantibodies to collagen in the blood of individuals with AS (30), and others reported the presence of circulating plasma cells and an increase in immunoglobulin levels, comparable to that observed in patients with RA (31, 32). Levels of autoantibodies to mutated citrullinated vimentin, which are considered a specific serologic biomarker of RA (33), were also shown to be elevated in AS sera (34), although anti-citrullinated protein antibodies are

not generally detected in patients with AS. Recently, autoantibodies to multiple autoantigens were detected in patients with AS (compared to patients with RA or healthy controls) by using nucleic acid programmable protein arrays (7), demonstrating that 44% of AS patients possess autoantibodies to multiple autoantigens and that 60% of these autoantibodies are present in AS patients but not RA patients. Here we profiled the autoantibodies present in the blood of AS patients by using a protein array containing more than eight thousand human proteins and sera acquired from patients with AS and other autoimmune diseases. We detected the presence of antibody reactivity to PPM1A in AS serum samples, and validated this finding in an independent cohort of AS patients.

Interestingly, anti-PPM1A antibodies were detected irrespective of evident arthritis in the AS-prone rats. Males exhibit increased susceptibility for the disease, and male AS-prone rats developed epididymoorchitis around 1-2 months of age prior to develop arthritis. Hypospermatogenic *Dazl*-deficient rats (used as a negative control in this study) were free from epididymoorchitis as well as subsequent arthritis, and persistent testicular inflammation and/or antigenic stimulation are essential prerequisites for the subsequent development of AS (25). Furthermore, we cannot exclude the possibility of subclinical arthritis or spondylitis in the rats without gross evidence of arthritis.

Levels of anti-PPM1A antibodies were higher in the transgenic rats overexpressing HLA-B27 that are prone to develop AS as compared to *Dazl*-deficient mice overexpressing HLA-B27, suggesting that PPM1A activity is independent of HLA-B27 expression. However, TNF as well as TGF- β activation is characteristic of AS (27), and PPM1A overexpression might be influenced by these conditions. Reuter S. *et al.* reported that PPM1A, induced by TNF stimulation in K562 cells, was suppressed by pretreatment with an NF- κ B inhibitor (35), suggesting that PPM1A is a downstream gene activated by TNF-NF- κ B signaling. Furthermore, PPM1A is a known inhibitor of TGF- β signaling, and ectopic expression of PPM1A in C2C12 cells abolished TGF- β -induced antiproliferative response (28). Taken together, we suggest that PPM1A might be induced by TNF and/or TGF- β .

In AS patients initiated on anti-TNF therapy, there was a positive correlation between the change in anti-PPM1A antibody levels and the change in BASDAI scores. It is possible that this observation arises from suppression of TNF-induced PPM1A activation (35) by anti-TNF therapy. Moreover, considering the efficacy of anti-TNF therapy in controlling disease activity in AS (36, 37), it is possible that the increase in baseline levels of anti-PPM1A autoantibodies is caused by an increase in exposure of (normally intracellular) PPM1A to the immune system, with a drop in the levels of anti-PPM1A autoantibodies after anti-TNF therapy reflecting a decrease in exposure of PPM1A following the control of synovial and/or enthesial inflammation. Although these findings remain to be validated in an independent cohort, they suggest that baseline levels of anti-PPM1A antibodies may serve as a serologic biomarker of AS, and that the decrease in anti-PPM1A autoantibody levels after anti-TNF therapy may serve as a pharmacodynamic biomarker. Whether anti-PPM1A autoantibodies contribute to the pathogenesis of AS, or are simply secondary to an underlying disease processes in AS, remains to be determined. Further studies on the role of anti-PPM1A autoantibodies and/or PPM1A-containing immune complexes in AS and its rat model are

needed to further define the biological role of anti-PPM1A autoantibodies in the pathogenesis of AS.

In addition to the extracellular release of PPM1A and its targeting by autoantibodies in AS, we identified a role for endogenous PPM1A in osteoblast differentiation, a process that contributes to the AS phenotype (9). That AS is dominated by anabolic processes after the onset of chronic inflammation and is characterized by bony overgrowth and ankylosis (10) further supports a role for osteoblasts, and hence PPM1A, in the pathogenesis of AS. The molecular mechanisms of bone formation in AS involve BMP and Wnt signaling (38), and PPM1A can inhibit BMP signaling by decreasing protein levels of Smad1, Smad5, and Smad8 in C2C12 mouse myoblasts (39). However, we show that PPM1A enhances osteoblast differentiation independently of BMP and Wnt in an *in vitro* setting. Our experiments were designed to identify the direct role of PPM1A in osteoblast differentiation, by using two different strains of mouse MC3T3-E1, a monopotent cell line that can only differentiate into osteoblasts when cultured in osteogenic media (40, 41). We demonstrate that preosteoblasts overexpressing PPM1A differentiated into mature osteoblasts, and that this differentiation was accompanied by an increase in expression of markers of osteoblast formation, but not by an increase in activation of Smad1/5/8 and β -catenin, key molecules of BMPs and Wnt signal pathway. We propose that overexpression of PPM1A in AS promotes the differentiation and activation of osteoblasts by activating several target genes, especially osteocalcin.

In conclusion, we show that high levels of anti-PPM1A autoantibodies are present in AS patients and are associated with greater radiographic severity of AS and with greater disease activity after anti-TNF therapy. We also demonstrate increased expression of PPM1A in AS synovium and identify intracellular PPM1A as a potential enhancer of osteoblastogenesis. Thus, our data suggest that levels of anti-PPM1A autoantibodies in the blood of AS patients may serve as a diagnostic biomarker, as a biomarker of disease severity, and as a biomarker of response to anti-TNF therapy. These findings could not only give rise to an actionable mechanistic biomarker (42) for the management of patients with AS, but also identify PPM1A as a novel therapeutic target in attenuating bony ankylosis and thus radiographic progression in AS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge Drs. Peter Gregersen and Annette Lee for provision of samples from the MADGC cohort.

Financial support: This research was supported by NIH NHLBI Proteomics Center N01-HV-00242, NIH NIAMS R01 AR063676, and Department of Veterans Affairs funding to W.H.R. Yong-Gil Kim was supported by the National Research Foundation of Korea (NRF-2013R1A1A1009271) and Dong Hyun Sohn was supported by Arthritis Foundation Postdoctoral Fellowship (Award # 5119).

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Figure 1. AS is associated with an increase in serum levels of anti-PPM1A autoantibodies

A, Protein microarray analysis of autoreactivity to PPM1A in sera from patients with toxin-induced pulmonary artery hypertension (toxin PAH; $n = 8$), PAH associated with connective tissue disease (PAH CTD; $n = 8$), idiopathic PAH ($n = 7$), anti-cyclic citrullinated peptide-negative (anti-CCP-) rheumatoid arthritis (RA) ($n = 11$), anti-CCP+ RA ($n = 10$), psoriasis ($n = 6$), juvenile idiopathic arthritis (JIA; $n = 15$), psoriatic arthritis (PsA; $n = 34$), or ankylosing spondylitis (AS; $n = 16$) in the Multiple Autoimmune Disease Genetics Consortium (MADGC) cohort. **B**, ELISA analysis of anti-PPM1A autoantibodies level in sera from healthy individuals and patients with AS or RA in a Korean cohort. **C**, Levels of anti-PPM1A autoantibodies in sera from Korean AS patients with high-grade (3 or 4) or low-grade (2) radiographic sacroiliitis. **D**, ELISA analysis of levels of PPM1A protein in sera from healthy individuals and individuals with AS or RA in a Korean cohort. The mean $\pm$ SEM of values for each group is shown. * $P < 0.05$; ** $P < 0.01$ by one-way ANOVA or Student's t -test, comparing AS samples to all other samples.

OD, optical density

Figure 2. Serum levels of anti-PPM1A autoantibodies decrease after anti-TNF therapy and correlate with change in disease activity in AS

Serum levels of (A) anti-PPM1A autoantibodies and (B) anti-influenza antibodies in AS patients at baseline and after 3 months' treatment with anti-TNF agents, and C, correlation between the change in serum levels of anti-PPM1A autoantibodies (... Anti-PPM1A Ab) and the change in BASDAI score (... BASDAI) after 3 months' treatment with anti-TNF agents. Points represent values for individual patients. ** $P < 0.01$ by Mann-Whitney U test. BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; OD, optical density; ns, not significant; A, Adalimumab; I, Infliximab.

Figure 3. Serum levels of anti-PPM1A autoantibodies are elevated in AS-prone rats irrespective of evident arthritis

ELISA analysis of levels of anti-PPM1A autoantibodies in sera from HLA-B27/human β 2-microglobulin transgenic (TG) rats (n=41), *Dazl*-deficient TG rats (n=9) and non-TG rats (n=26). *Dazl*-deficient TG rats were also used as negative controls because deficiency of the *Dazl* gene in these rats prevents both epididymoorchitis and arthritis (25). The mean $\pm$ SEM of values for each group is shown. *** $P < 0.001$ by one-way ANOVA. hB2m, human β 2-microglobulin; OD, optical density

Figure 4. PPM1A is strongly expressed in synovial tissue from individuals with AS

A, ELISA analysis of levels of anti-PPM1A autoantibodies in synovial fluids from patients with AS ($n=14$), RA ($n=10$) and OA ($n=10$). **B**, Immunohistochemical (IHC) images of AS, RA and OA synovial tissues stained with anti-PPM1A antibodies or IgG isotype controls. All IHC images are shown at 200 $\times$ magnification, and are representative of images from 3 independent experiments. The box in the right lower corner of left upper IHC image is a magnified image of the marked area at the center of panel. The mean $\pm$ SEM of values for each group is shown. ** $P < 0.01$ by one-way ANOVA.

AS, ankylosing spondylitis; RA, rheumatoid arthritis; OA, osteoarthritis; OD, optical density

Figure 5. PPM1A drives osteoblast differentiation

Preosteoblastic MC3T3E1 cells were transiently transfected with siRNA against PPM1A (si-PPM1A) or with a non-targeting control siRNA, or with a PPM1A expression plasmid (FLAG-PPM1A) or control vector, and cultured in osteogenic media for 7 to 14 days. **A**, Knock-down of PPM1A protein in si-PPM1A-transfected cells. **B**, Overexpression of PPM1A in FLAG-PPM1A-transfected cells. **C**, ALP activity and **(E)** nodule formation in cells transfected with si-PPM1A or control siRNA. **D**, ALP activity and **(F)** nodule formation in cells transfected with FLAG-PPM1A or control vector. Bar chart values are the mean $\pm$ SEM of triplicates and representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6. Gene and protein expression associated with osteoblast differentiation

Quantitative real-time PCR analysis of gene expression associated with osteoblast differentiation in (A) si-PPM1A-transfected or (B) FLAG-PPM1A-transfected cells stimulated with osteogenic media. Col1a1, collagen type 1; Bsp2, bone sialoprotein 2; OCN, osteocalcin; Osx, osterix; Runx-2, runt-related transcription factor 2. C, Immunoblot analysis of phospho-Smad1/5/8 and active β-catenin in si-PPM1A-transfected or FLAG-PPM1A-transfected cells stimulated with osteogenic media for 15, 30, or 60 minutes. Bar chart values are the mean ± SEM of triplicates and representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.