Project description

The role of SIRT1 in the pathogenesis of Spondyloarthritides

Applicant

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1 Scientific background

Relevance of Spondyloarthritides

Spondyloarthritides (SpAs) are the second most frequent form of chronic inflammatory rheumatic diseases with an estimated prevalence of 0.5-1.5% in the adult population.(1,2) The disorder includes a manifold of closely related disorders characterized by axial and/or peripheral joint inflammation often associated with extra-articular inflammation of skin, eye or gut and has therefore been subdivided into subtypes. Enthesitis is a key pathologic feature of SpAs, and contributes to both axial and peripheral arthritis. Another important characteristic of SpAs is synovitis. Structural damage in SpAs is dominated by new bone formation that can result in spinal fusion and marked functional limitation. Trabecular bone loss is also present, and paradoxically, occurs in close proximity to osteoproliferation.

Relevance of the IL-17/IL-23 axis in the disease pathogenesis

T-cells are considered to be the most important players in the pathogenesis of SpAs. Genome-wide association studies linked SpAs with genes related to T-cell immune responses (HLA-B27, ERAP1, IL-23R and other Th17/23 pathway genes).(3) In parallel with these genetic advances, immunological studies have implicated Th17/23 responses in SpAs and therapeutic strategies targeting the Th17 response have proved beneficial.(4,5)

Th17 cells are a subset of T helper cells, developmentally distinct from Th1 and Th2 cells and are characterized by the production of IL-17 next to several other pro-inflammatory cytokines such as IL-6, IL-22, IL-26, IFN-γ and TNF-α.(6) Th17 cells and their effector

cytokines mediate host defensive mechanisms to various infections, especially extracellular bacteria, by recruiting neutrophils and macrophages to infected tissues.

Research into the triggers and drivers of Th17 responses has identified a major role for the cytokine IL-23 and was found to be expressed in activated monocytes, macrophages, dendritic cells, endothelial cells, T and B-cells.(7) IL-23 appears to be crucial in localized tissue inflammation, it is not required for systemic inflammatory responses.(8) Strikingly, Sherlock et al. showed that over-expression of IL-23 is sufficient to induce an inflammatory disease with enthesitis in mice, which is similar to human SpA.(9) In this study, they describe entheseal-resident T-cells that respond to IL-23 by producing IL-17 and other pro-inflammatory cytokines and thus mediate peripheral and axial enthesitis. Notably, IL-23 driven enthesitis leads to enhanced expression of TNF-α also. TNF blockade, however, had only a modest benefit in this context whereas IL-17A and IL-22 blockade were most effective.(9) In addition, other studies reported IL-23-driven hyperplastic and inflamed synovium in mice, another characteristic of SpA pathogenesis.(10)

In humans, increased levels of IL-17 as well as IL-23 were observed in serum and synovial fluid of patients with SpA.(11,12) In line with this, elevated frequencies of circulating Th17 cells and, in some cases, enhanced *in vitro* IL17-production were described.(13) An increased number of IL-17-associated cells has also been demonstrated in the facet joints of patients with ankylosing spondylitis, including both T cells and non-lymphocytic cells.(14) These data are strongly supported by therapeutic strategies targeting the IL-17/IL-23 axis. Both secukinumab (a monoclonal antibody against IL-17A) and Ustekinumab (a monoclonal antibody against the p40 subunit of IL-12 and IL-23) showed to be beneficial in the treatment of SpAs.(4,5)

The role of acetylation for the generation of Th17 cells

Th17 and regulatory T cells (Tregs) represent two reciprocally related arms of an immune response, and their uniquely plastic relationship dictates the flavor of their surrounding immune environment, allowing for shifts between pro- and anti-inflammatory states. Naive T-cells differentiate to Th17 cells under the influence of IL-1β, IL-6, IL-23, and TGF-β.(15) Their differentiation is thereby controlled by STAT3 and the “master-regulator” transcription factor RORγt, which directs a specific and heritable gene expression profile.(16, fig. 1) Moreover, on the molecular level it was demonstrated that FoxP3 (the signature transcription factor of Tregs) could bind physically to RORγt to antagonize each other’s function illustrating their reciprocal relationship.(17)

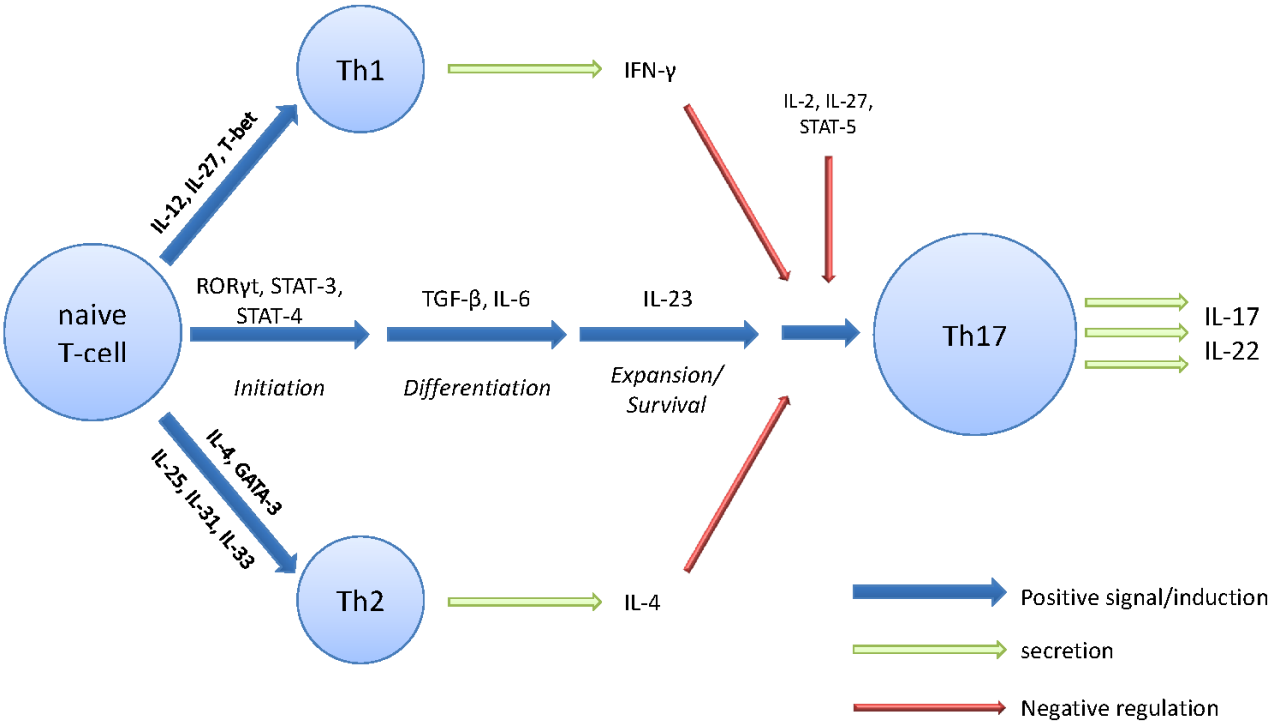

Figure 1: Overview of Th17 cell differentiation. Naive T-cells differentiate to Th17 cells in three transcriptional phases: I) Initiation phase controlled by STAT3 and the "master-regulator" transcription factor RORγt; II) Differentiation phase marked by TGF-β and IL-6 and III) Expansion/Survival Phase mediated by IL-23.

Growing evidence suggests that the epigenetic state of T-cells regulates their development, reactivity, and effector function. Sirtuins (silent mating type information regulation 2 homolog) are NAD^+-dependent protein/histone deacetylases that play critical roles in transcriptional regulation, cell cycling, replicative senescence, inflammation, and metabolism. Sirtuin1 (SIRT1) in particular acts as an epigenetic regulator that modulates the activity of several transcription factors important for immune function.(18) Recent studies identified a pivotal pro-inflammatory action of SIRT1 as a negative regulator of Treg function by deacetylation of transcription factor FoxP3.(19,20) On the other hand SIRT1 induced Th17 cells via modulating the acetylation RORγt. *In vivo*, deletion of SIRT1 in T-cells as well as chemical inhibition of SIRT1 protects mice from experimental autoimmune encephalomyelitis, another Th17-mediated disease.(21)

There is evidence that the Treg/Th17 balance is disturbed in patients with rheumatoid arthritis also.(22) In these patients, SIRT1 was found to be constitutively upregulated in synovial tissues and cells. Moreover, TNF-α stimulation resulted in enhanced expression levels of SIRT1 and protected cells from apoptosis in this study.(23) The role of protein/histone acetylation in the pathogenesis of SpA, however, is largely unknown.

Whether the deacetylase SIRT1 plays a role in the development of Th17 cells in the pathogenesis of SpA has not been investigated so far.

2 Rationale & Aims

We know that SpAs are predominantly Th17-mediated diseases and the emergence of Th17 cells in the disease pathogenesis is elusive. There is accumulating evidence that the epigenetic state of T-cells regulates their development, reactivity, and effector function. The protein/histone deacetylase SIRT1 was recently reported to induce Th17 cell generation. If SIRT1 is quantitatively or qualitatively altered in SpA T-cells has not been investigated so far.

In this project we aim to investigate the role of SIRT1 on Th17 cell differentiation in the pathogenesis of axial SpAs (aSpAs).

3 Hypotheses & Methods

- *Hypothesis 1: Naïve $CD4^+$ T-cells of aSpA patients more likely differentiate into Th17 cells*

To proof this hypothesis we will include patients with radiographic as well as non-radiographic aSpA and healthy controls (HC). Naïve $CD4^+$ T-cells will be isolated and cultured under Th0, Th17 and Treg-polarizing conditions. After 7 and 14 days IL-17 production and expression of RORγt will be analyzed by flow cytometry. All used methods are well established at the Division of Rheumatology and Immunology, Medical University Graz.

Patients

Consecutive patients with a final diagnosis of radiographic and non-radiographic aSpA fulfilling the ASAS classification criteria(24,25) of aSpA and age- and sex-matched HCs will be prospectively enrolled. A complete medical history including, age, gender, disease duration, current disease activity, HLA-B27 status and current treatments will be obtained.

Peripheral blood mononuclear cells (PBMCs)

Peripheral venous blood will be drawn and PBMCs will be isolated by Histopaque density gradient centrifugation. Cells will be washed twice with RPMI 1640 containing 10% fetal calf

serum, 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The total cell number will be determined by a Beckmann Coulter.

Separation of T-cell subsets

Naïve $CD4^+$ T-cells will be isolated by using 'MagniSort Human CD4 Naïve T-cell Enrichment Kit' (eBioscience) and autoMACSPro (Miltenyi Biotech) according to manufacturer's instructions.

Cell culture

Naïve $CD4^+$ T-cells will be cultured under Th0 (anti-CD3, anti-CD28, IL-2), Th17 (anti-CD3, anti-CD28, IL-1β, IL-6, IL-23, TGF-β and neutralizing antibodies for IL-4 and IFN-γ) and Treg-polarizing conditions (anti-CD3, anti-CD28, IL-2, TGF-β) for 14 days. On day 7 and 14, cells will be re-stimulated with PMA and ionomycin in the presence of Golgi transport inhibitor for 5 hours and analyzed by flow cytometry.

Flow cytometry

For surface and intracellular staining we will use appropriate combinations of the following antibodies: CD3, CD4, IL-17, RORγt, CCR6, CD25, CD127 and FoxP3 (all Becton Dickinson). Surface staining will be performed for 30 minutes, followed by permeabilization for 30 min and intracellular staining for 30 minutes according to a routine protocol. We will use appropriate isotype control antibodies.

Stained cells will be analyzed by a FACS Canto II (Becton Dickinson) and data will be analyzed with FlowJo.

- *Hypothesis 2: SIRT1 levels or activity are increased naive $CD4^+$ T-cell of aSpA patients*

To proof this hypothesis we will evaluate the expression of SIRT1 by flow cytometry techniques and the activity of SIRT1 of isolated naive T-cells. Alternatively, we will obtain RNA of purified $CD4^+$ T-cells. mRNA levels of SIRT1 will be measured without and following stimulation *in vitro* using RT-PCR. All used methods are well established at the Division of Rheumatology and Immunology, Medical University Graz.

Flow cytometry

For surface and intracellular staining we will use freshly isolated PBMCs and appropriate combinations of the following antibodies: CD3, CD4, CD28, CD45RA, CD45RO, RORγt, FoxP3 (all Becton Dickinson) and SIRT1 (abcam). Surface staining will be performed for 30 minutes, followed by permeabilization for 30 min and intracellular staining for 30 minutes according to a routine protocol. We will use appropriate isotype control antibodies.

Stained cells will be analyzed by a FACS Canto II (Becton Dickinson) and data will be analyzed with DIVA.

Determination of SIRT1 activity

SIRT1 activity of isolated naïve T-cells will be determined by 'SIRT1 activity assay kit' (abcam) following manufacturer's instructions.

RNA isolation & RT-PCR

Total RNA of purified naïve $CD4^+$ T-cells will be isolated using the RNeasy Mini kit (Qiagen). SIRT1 and housekeeping gene transcripts will be measured in bead-purified T-cells using a Rotor-Gene 3000 (Corbett Robotics) and the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions.

- *Hypothesis 3: Modulation of SIRT1 influences Th17 differentiation in aSpA patients*

To proof this hypothesis we will polarize naïve $CD4^+$ T-cells as described for hypothesis 1 in the presence of Ex-527 (SIRT1 inhibitor) or resveratrol (SIRT1 activator). After 7 and 14 days IL-17 production and expression of RORγt will be analyzed by flow cytometry. All used methods are well established at the Division of Rheumatology and Immunology, Medical University Graz.

Cell culture

Naïve $CD4^+$ T-cells will be cultured under Th0 (anti-CD3, anti-CD28, IL-2), Th17 (anti-CD3, anti-CD28, IL-1β, IL-6, IL-23, TGF-β and neutralizing antibodies for IL-4 and IFN-γ) and Treg-polarizing conditions (anti-CD3, anti-CD28, IL-2, TGF-β) for 14 days in the absence or presence of either Ex-527 or resveratrol. On day 7 and 14, cells will be re-stimulated with

PMA and ionomycin in the presence of Golgi transport inhibitor for 5 hours and analyzed by flow cytometry.

4 Expected impact of the study

We anticipate that the conclusions drawn from this study will link epigenetic modifications and Th17 differentiation and improve the current understanding of the involvement of environmental triggers in the pathogenesis of SpAs.

With the data obtained by this pilot study we will apply for funding by FWF or ÖNB and extend our research expertise in this field. We plan to investigate the role of SIRT1 in a curdlan-induced mouse model of SpA. Therefore, we will cross this model with $SIRT^{-/-}$ mice to evaluate if the mice are protected from SpA development. Alternatively, we will chemically inhibit SIRT1 by treatment with Ex-527. To investigate if the disease protection is depending on a reduction of Th17 cells we will test for alterations in the frequency and function of Th17 as well as Treg cells. Furthermore, we will broaden our work on human axial SpA patients and other SpA subtypes.

In addition, we plan to publish this work in a top-ranked journal in the category rheumatology or immunology.

5 Resources & cost planning

Commencement, duration and location of work:

The project will start in the beginning of 2017 at the Medical University of Graz and will last one year.

Research personnel:

Medical University of Graz:

Johannes Fessler, PhD (Applicant)

Patrizia Fasching, MSc

Christian Dejaco, MD, PhD, Associate Professor

Martin Stradner, MD, Assistant Professor

Josef Hermann, MD, Associate Professor

Winfried Graninger, MD, Professor

Requested funding:

For this study we apply for €25.000 of total funding. (See details below)

Description	in €
Master student (partial employment, one year)	7.100
Cell culture	6.900
Flow cytometry and sorting	6.000
Assay kits	2.000
Molecular biology equipment, RNA/DNA isolation, PCR	2.000
Plastics	1.000
Total	**25.000**

Available infrastructure:

All technical facilities to perform the experiments are available at the Division of Rheumatology and Immunology of the Medical University Graz. Patients are recruited from the Rheumatology out-patients clinic and healthy individuals from lab staff and hospital personnel.

6 References

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