Immune phenotype and functional characterization of short-term T-cell activation in patients with ankylosing spondylitis and rheumatoid arthritis

Ph.D. thesis

Dr. Balázs Szalay

Semmelweis University
Doctoral School of Clinical Medicine

Supervisor:	Dr. Attila Balog, Ph.D.
Opponents:	Dr. György Nagy, Ph.D. Dr. Klára Kitajka, Ph.D.
Chairman of the Examination Committee:	Dr. Edit Buzás, D.Sc.
Members of the Examination Committee:	Dr. Éva Pállinger, Ph.D. Dr. Viktor Sebestyén Varga, Ph.D.

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1. INTRODUCTION

Inflammation plays a prominent role in the pathogenesis of rheumatologic diseases. A number of immunological changes have been observed in ankylosing spondylitis (AS) and in rheumatoid arthritis (RA), including the variations of the adaptive immune response.

CD4+ and CD8+ T-lymphocytes are key regulators of the adaptive immune system. The function and activation of these cells determine the direction and the intensity of the adaptive immune response. It has been demonstrated that the prevalence of CD4+ and CD8+ cells and their cellular environment can be affected in AS and in RA. However, besides a few cross-sectional examinations, no prospective follow-up studies have been carried out that monitor the changes in CD4+ and CD8+ T cell subset distribution during the course of the disease in conjunction with the therapy. Moreover, no data are available as to whether the effects on T-cell subset distribution differ between individual anti-TNF-α agents.

Along the alterations observed in cell prevalence values, one can assume that T-cell activation properties may also be altered in AS and in RA. Because of technical difficulties the number of studies investigating intracellular processes are rather limited. Only a few studies have been performed in rheumatologic diseases.

As a PhD student I have been involved in the research of potential biomarkers that could be affected in patients with AS and RA. We investigated the adaptive immune phenotype in different stages of AS and RA before and after therapy changes. Using a special flow cytometric technique, I also evaluated the changes in intracellular processes during short-term T-cell activation characteristics.

We included a number of patient groups and identified alterations in the adaptive immune response that could be specific to a disease stage. Our observations could help to better understand the patogenesis of AS and RA, and it may help to identify diagnostic and therapeutic decision-making markers.

2. AIMS

In the first phase of our comprehensive study our aim was to assess the prevalence of major regulatory cells of adaptive immunity (CD4+ and CD8+ lymphocytes, Th1, Th2, Th17 and Treg cells) in AS with a flow cytometer. We also evaluated the activation status of AS by determining the expression of activation markers (CD69 and HLA-DR) and the prevalence of CD4+ and CD8+ naive and memory/effector cells. Besides baseline characteristics we also investigated the changes in immune phenotype in AS patients during IFX therapy.

We aimed to define the above mentioned representatives of the adaptive immune system in treatment naive (early) RA patients and in RA patients unresponsive to standard DMARD (disease-modifying antirheumatic drugs) therapy (DMARD nonresponders). We also determined cell prevalence values and activation markers after 4 and 8 weeks of DMARD combination therapy in early RA and during 8 weeks of therapy with three different anti-TNF-α agents (infliximab: IFX, etanercept: ETA and adalimumab: ADA) in DMARD nonresponders.

In the second phase of our study we aimed to characterize intracellular processes during short-term T-cell activation in CD4+ and CD8+ lymphocytes in both rheumatic diseases. During our study using a flow cytometer we monitored the changes in cytoplasmic Ca^{2+} level, mitochondrial Ca^{2+} level, superoxide generation and nitric oxide production for 10 minutes after the addition of phytohaemagglutinin.

Besides baseline characteristics we determined the effect of IFX in AS, DMARD combination therapy in early RA and anti-TNF-α agents in DMARD nonresponders on intracellular processes.

3. METHODS

3.1. Patients

3.1.1. Ankylosing spondylitis

Thirteen active AS patients with high Bath ankylosing spondylitis disease activity index (BASDAI) were enrolled to the study. Because of the active disease patients are given IFX at a dose of 5 mg/kg bw intravenously on week 0, 2, and 6 then on every 8th week. Blood samples were taken at 3 distinct time points: just before starting IFX (when each of them was on NSAID therapy alone), then on week 2 and 6 after initiation of IFX therapy.

3.1.2. Rheumatoid arthritis

Nineteen patients with newly diagnosed, treatment naive (early) RA and 32 active RA patients unresponding to standard DMARD combination therapy (DMARD nonresponders) were enrolled to the study. **Early RA** patients had not received any anti-RA treatment prior to our study. After establishment of the diagnosis, DMARD therapy was initiated: medium-dose oral glucocorticosteroid (GCS, 16 mg/day methylprednisolone) alone for 4 weeks; GCS was subsequently tapered to 8 mg/day, and on week 4, methotrexate (MTX) was started at 10 mg/week. Blood samples were taken before the initiation of DMARD therapy (baseline), then after 4 and 8 weeks of treatment. **DMARD nonresponders** were all on long-term MTX (at 15 mg/week) and leflunomide (LF, 20 mg/day) combination treatment, and none of them received glucocorticosteroids or other immunosuppressive agents. LF was discontinued, while MTX was given simultaneously. At this time anti-TNF-α therapy was initiated: ADA at 40 mg/2 weeks sc (n=12); ETA at 50 mg/weeks sc (n=12) or IFX on week 0, 2, and 6 at 3 mg/kg iv (n=8). Blood samples were taken before the initiation of each anti-TNF-α agent, and on week 4 and 8 of therapy.

3.1.3. Healthy patients

Nine age and gender matched healthy volunteers served as controls in AS and ten in RA. The control patients had a negative history of rheumatic symptoms and a negative status upon detailed physical and laboratory examination. Written informed consent was obtained in advance from all participants.

3.2. Sample preparation

24 mL of lithium-heparin anticoagulated blood was taken from all participants (BD Vacutainer, Beckton Dickinson & Co, Plymouth, UK) and were processed within 4 hours. Peripheral blood mononuclear cells (PBMCs) were separated with gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA). 20% of the PBMCs (approximately 5×10^6 cells) were preserved at -80ºC for cell surface staining, while 80% (approximately 2×10^7 cells) was resuspended in modified RPMI medium (Sigma-Aldrich, St. Louis,Mo, USA) for intracellular measurements (the Ca^{2+} concentration was set to 2mM).

3.3. Cell surface staining

To characterize T-cell subset prevalence values, PBMCs were stained with fluorescent antibodies (Becton Dickinson, San Diego, California, USA) against cell surface markers according to the manufacturer's instructions. Samples were measured within 1 hour after staining. At least 300,000 events were recorded for each acquisition. Cell types were defined as: helper T-cells (CD4+), cytotoxic T-cells (CD8+), Th1 cells (CD4+CXCR3+), Th2 cells (CD4+CCR4+), Th17 cells (CD4+CCR4+CCR6+), regulatory T-cells (Tregs; CD4+CD25+CD127−), naive T-cells (CD4+CD45RA+ and CD8+CD45RA+), and memory/effector T-cells (CD4+CD45RO+ and CD8+CD45RO+). The prevalence of CD4+ and CD8+ cells expressing early and late activation markers (i.e. CD69, and HLA-DR, respectively) was also determined.

3.4. Intracellular staining

In order to characterize intracellular processes associated with a specific T-cell stimulation, each specimen marked with CD4+ and CD8+ surface markers was loaded with Fluo3AM, Rhod2-AM, Dihydroethidium and DAF-FM diacetate (Molecular Probes, Carlsbad, California, USA) sensitive to cytoplasmic Ca^{2+} levels (cyt-Ca^{2+}), mitochondrial Ca^{2+} (mit-Ca^{2+}) levels, superoxide (ROS) generation and nitric oxide (NO) production, respectively. The changes in fluorescent signals were monitored up to 10 minutes after the addition of 20 μg/mL in final concentration of phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, Mo, USA), a specific activator of T-cells.

3.5. Measurements and evaluation

All measurements were performed on a BD FACSAria flow cytometer (Becton Dickinson, San Jose, California, USA). **Cell prevalence** values were determined with conventional gating using FACSDiVa software (Becton Dickinson, San Jose, California, USA). The kinetic parameters of **intracellular processes** were determined using R (R Foundation for Statistical Computing, Vienna, Austria) as follows. Measurement timeframe was divided into 100 time intervals and the medians of fluorescent values were calculated. Lowess smoothing method was applied to the median values, and each value was related to that measured at the beginning of the experiment (the resulting values became relative parameter values, rpv). The following parameters were calculated from the rpv values: area under the curve (AUC), maximum value (max) and time to reach maximum (t_{max}). One unit of the AUC value is defined as one rpv in one second. Further statistical analysis was based on the values of these parameters.

3.6. Statistical analysis

Statistical analysis was performed with the Statistica 7 software package (Statsoft, Tulsa, OK, USA). The Mann-Whitney test was applied for the comparison of the data of the controls and the patients, while the paired data in each related patient group were compared by the Friedman test. When the Friedman test demonstrated significant differences, the post hoc Dunn test was used to identify which pairs were significantly different. Levels of $p<0.05$ were taken as statistically significant.

4. RESULTS

4.1. Ankylosing spondylitis

At the beginning, BASDAI was ≥5 in each AS patient (median [interquartile range]: 6.88 [6.07–7.6]. After 6 weeks of IFX therapy, it decreased significantly: 1.79 [0.60–3.83], $P < 0.0001$.

4.1.1. Cell prevalence values in ankylosing spondylitis

The overall prevalence of **CD4+** cells within lymphocytes increased in AS. Th1 prevalence values increased by approximately 30 per cent, while Th2 prevalence was double, resulting in a skewness of Th1/Th2 ratio to a Th2 direction. Th17 prevalence increased by 70 per cent, while Treg numbers were comparable to that in controls. The prevalence values of CD4+ naive (CD45RA) T-cells, CD4+ memory/effector (CD45RO) T-cells and the expression of activation markers (CD69, HLA-DR) in CD4+ cells were comparable in patients and controls.

During IFX-treatment, the observed abnormalities did not change. Instead, the prevalence of CD4+ naive cells on week 2 and 6 decreased, while the prevalence of CD4+ memory/effector cells increased on week 6.

In general, all **CD8+** prevalence values were comparable in patients and controls irrespectively of IFX therapy.

4.1.2. Functional characteristics in ankylosing spondylitis

Before IFX therapy CD4+ and CD8+ cells presented with a delayed increase in cyt-Ca^{2+} levels after activation in AS compared to controls. Mit-Ca^{2+} kinetics also changed in a similar manner in CD8+ cells. The amount of NO generation, max and time to reach max NO levels were significantly higher in CD4+ and CD8+ cells of AS patients. Superoxide generation of T-cells did not differ between AS and controls.

With IFX therapy the delay in CD4+ cyt-Ca^{2+} levels did not normalize in AS. For CD8+ cells, cytoplasmic and mitochondrial Ca^{2+} kinetics during activation

normalized by week 6 on IFX (but not on week 2). NO kinetics became comparable in AS patients to that in controls even after 2 weeks of IFX therapy and remained unaltered by week 6.

4.2. Rheumatoid arthritis

4.2.1. Cell prevalence values in rheumatoid arthritis

Treatment naive, early RA patients exhibited higher than normal Th1, Th2, and Th17 cell and lower than normal Treg and CD8+ cell prevalence values. The CD4+ cell prevalence value was comparable to controls, so the overall ratio of CD4/CD8 was increased. The prevalence of CD4+ cells expressing early or late activation markers (CD69 and HLA-DR, respectively) and CD4+ memory/effector cells were higher, while CD4+ naive cell were lower than normal. CD8+ naive and memory/effector cells along with the expression of HLA-DR activation marker was were comparable to controls, while the expression of CD69 on CD8+ cells increased.

On week 4 of GCS treatment, the Th2 prevalence decreased significantly (while Th1 remained unaltered), therefore Th1/Th2 ratio shifted to the Th1 direction. The prevalence of activated T cells expressing CD69 normalized in CD4+ and CD8+ cells. While the expression of HLA-DR on CD4+ cells also normalized, on CD8+ cells it increased. At this point, Th1, Th17, Tregs, CD4+ naive and CD4+ memory/effector T-cell prevalence values were comparable to the baseline. However, the ratio of CD8+ naive/memory cells decreased on week 4.

By week 8 (4 weeks after the initiation of MTX therapy and start of the tapering of GCS), Th17 prevalence decreased but was still higher than normal. It was noteworthy that the Th2 cell prevalence returned to that on week 4. Compared to the controls, the prevalence of CD4+ cells and the expression of CD69 on CD8+ cells increased, while HLA-DR expression on CD8+ cells and the prevalence of CD8+ naive and memory cells normalized. Other cell prevalence values remained unaltered.

In **DMARD non-responders** Th1, Th2, and Th17 prevalence values were higher, while Treg and cytotoxic CD8+ cell prevalence was lower than normal at baseline (i.e., before the initiation of anti-TNF-α therapy). CD4+ helper cells and

activated (i.e. CD69 and HLA-DR positive) CD4+ and CD8+ cell prevalence was normal. Par hazard, baseline naive/memory cell ratios markedly differed in patient subgroups to be treated with the different anti-TNF-α agents.

T cell subset distribution markedly altered under anti-TNF-α therapy with some differences between ETA, ADA, and IFX subpopulations. By weeks 4 and 8, Th1 prevalence increased further in ETA and IFX patients, while it was constant in ADA patients. Th2 prevalence was constantly higher than normal under ADA or IFX therapy, while normalized in patients treated with ETA. Anti-TNF-α therapy did not affect Th17 prevalence, while it increased (but still not normalized) Treg prevalence, irrespectively of the anti-TNF-α agent used. (However, ADA patients exhibited a further increase in Tregs by week 8, while Treg values were comparable to those at week 4 in ETA and IFX patients).

Activated CD4+ prevalence values showed great variation according to anti-TNF-α agent used. Compared to the baseline, CD4+ HLA-DR+ prevalence increased in ADA patients on week 4 and on week 8 in ETA patients, while remaining unaltered in IFX patients. The expression of CD69 on CD4+ cells remained unaltered upon anti-TNF-α therapy. In CD8+ cells the expression of HLA-DR increased by week 8 in ETA and ADA treated patients, while under IFX remained unaltered. The expression of CD69 on CD8+ cells increased upon IFX treatment by week 4 and normalized by week 8, while in ETA treated patients CD69 decreased by week 4 and increased by week 8 compared to controls. Upon ADA therapy CD69 remained unaltered on CD8+ cells. Of note, CD4+ naive/memory cell ratio (also indicating immunoactivation) also increased by week 4 in ADA and by week 8 in ETA treated patients, but remained stable in IFX patients. CD8+ naive and memory cells were not altered and remained comparable to the controls.

4.2.2. Functional characteristics in rheumatoid arthritis

In **naive, early RA patients** at baseline, the investigated intracellular parameters (cyt-Ca^{2+}, mit-Ca^{2+}, ROS generation and NO production) of the T-cells during short-term activation were similar to those of the controls. On week 4 of GCS therapy, the cyt-Ca^{2+} response in CD4+ and CD8+ cells, also the level of ROS production in CD4+

cells during T-cell activation had decreased significantly and become lower compared to the controls and the baseline values. At week 8, cyt-Ca^{2+} response had become comparable to the baseline and control levels in CD4+ and CD8+ cells, while the ROS production was further impaired in CD4+ cells. Simultaneously, the ROS production in CD8+ cells also increased by week 8. Mit-Ca^{2+} levels and NO production remained unaltered upon DMARD combination therapy.

In **DMARD nonresponders** at baseline, the cyt-Ca^{2+} response in CD4+ and CD8+ cells was higher compared to the controls. The other parameters investigated, including the mit-Ca^{2+} level, ROS generation and NO production were comparable to those observed in the controls with the exception of the subgroup treated later with ETA. (These patients exhibited an increased ROS generation capacity in CD4+ cells at baseline). On week 4 of anti-TNF-α therapy, the CD4+ and CD8+ cyt-Ca^{2+} level became comparable to controls in all patient groups, while ROS generation was decreased significantly to below the baseline, irrespective of the nature of the anti-TNF-α agent administered (including ETA). Only the ROS generation in CD8+ cells upon ADA treatment remained unaltered. At this time point, mit-Ca^{2+} was increased in CD8+ cells after IFX therapy, while in the other groups it remained unaltered. Also, the production of NO was still comparable to that at the baseline in each patient group. At week 8, no significant alterations were detected relative to week 4 in any of the parameters tested.

5. CONCLUSIONS

1) The adaptive immune phenotype is characterized by a proinflammatory dominance in AS. At baseline (before therapy) this is demonstrated by increased prevalence of Th1 and Th2 cells (with a shift to Th2 cells), along with an increased Th17/Treg cell ratio.
2) Although the overall clinical conditions of AS patients improved upon IFX therapy, the investigated cell populations were only partially altered during 6 weeks of treatment. One can assume that the positive clinical effects of IFX in the induction phase of therapy are independent of the adaptive immune phenotype.
3) Besides changes in cell prevalence values at baseline, some intracellular processes that are integral parts of lymphocyte activation are also altered in AS. We observed delayed cit-Ca^{2+} and increased NO production in CD4+ and CD8 lymphocytes and delayed mit-Ca^{2+} handling in CD8+ cells.
4) IFX therapy in AS had different effects on cit-Ca^{2+}, mit-Ca^{2+} and NO production. In CD4+ lymphocytes IFX had no effect on cit-Ca^{2+} response, while in CD8+ cells cit-Ca^{2+} and mit-Ca^{2+} responses were normalized by week 6. The production of NO became comparable to normal after just 2 weeks of IFX therapy in both lymphocytes. Since the clinical condition of patients improved by week 2, NO production could be of more importance than intracellular Ca^{2+} handling in AS.
5) Only functional changes in CD8+ cells could contribute to the pathogenesis of AS, since a number of changes are observed in intracellular processes, while the prevalence of CD8+ cell types an the expression of activation markers of CD8+ cells remained unaltered through our study. In CD4+ cells, besides functional changes, some cell prevalence values are also altered and therefore could contribute to the pathogenesis of the disease.
6) In early RA (similarly to AS) the immune response is shifted to a proinflammatory direction, as the prevalence of Th1, Th2, and Th17 cells are increased, while that of Tregs and CD8+ lymphocytes are decreased. Immune activation is also supported by increased expression of activation markers in CD4+ and CD8+ lymphocytes along with the differentiation of CD4+ naive cells to memory/effector cells.

7) Since alterations of cell prevalence values in peripheral blood of early RA patients appear before the extra-articular lesions, the changes in immune phenotype are preceeded by clinical complications during the progression of the disease.
8) The DMARD combination therapy in early RA had different effects ony cell prevalence values and were only partially normalized. While GCS mainly affected CD8+ and Th2 cells, MTX had an impact on Th1, Th2, Th17 and CD8+ cells as well.
9) In the early staged of RA (before any therapy) the intracellular processes are intact. However, DMARD combination therapy had a major effect on these processes: after 4 weeks of GCS therapy cit-Ca^{2+} level and ROS generation decreased in CD4+ and CD8+ lymphocytes. Later, MTX normalized cit-Ca^{2+} levels and further decreased ROS generation. These intracellular processes could contribute to the therapeutic efficacy of DMARDs.
10) The proinflammatory immune phenotype can also be observed in active RA patients unresponding to DMARD combination therapy (DMARD nonresponders), but with a general cell anergy due to the lack of cellular activation.
11) Anti-TNF-α agents eliminate this anergy in DMARD nonresponders. To some extent they also affect cell prevalence values, although with different intensities and at different stages of the disease.
12) In active RA before anti-TNF-α therapy the cit-Ca^{2+} level is increased (in both CD4+ and CD8+ cells). This phenomenon could have a prognostic value in RA as it draws attention to the need of therapy change and to the initiation of anti-TNF-α treatment.
13) Anti-TNF-α agents in active RA normalized cit-Ca^{2+} level and decreased ROS production which may contribute to the alleviation of inflammation and to the significant clinical improvement of the patients.

6. PUBLICATIONS

6.1. Publications related to the thesis

Cumulative impact factor: 8.372

International publications:

- **Szalay B**, Mészáros G, Cseh Á, Ács L, Deák M, Kovács L, Vásárhelyi B, Balog A. Adaptive immunity in Ankylosing Spondylitis: phenotype and functional alterations of T-cells before and during infliximab therapy. Clin Dev Immunol. 2012;2012:808724. [IF: 3.064]

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6.2. Publications not related to the thesis

Cumulative impact factor: 34.420

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