Investigation of T lymphocyte activation and microRNA expression

PhD theses

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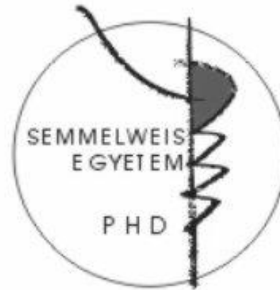

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Introduction

The physiological function of the immune system is provided by the coordinated cooperation of the signaling pathways, which regulate the response to external signals. If there is a functional alteration in one element or in more elements of this complex regulatory network that may subsequently lead to pathological processes and eventually to diseases. Decreased or increased immune functions are specific for some diseases of the immune system. Congenital and acquired immune deficiencies are characterized by decreased immune functions, while several inflammatory autoimmune diseases or the asthma are characterized by the reinforcement of some immune functions. The pathomechanism of the systemic autoimmune diseases is rather complex, in other words the alteration of a single cell type or process does not lead to autoimmune diseases. Both genetic and environmental factors play a pivotal role in these conditions. There are several essential signaling pathways, which are characteristic to some autoimmune diseases and shape the nature of the disease.

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, affecting primarily the small joints. The presence of allelic variants of HLA-DRB1*04 and the PTPN22 increases the susceptibility to the development of this disease. In addition environmental factors, e.g. smoking are also known risk factors for RA. The altered cytokine environment and the immune cell dysfunction play a key role in pathomechanism of the disease.

Tumor necrosis factor-α (TNF) is a multifunctional proinflammatory cytokine with both local and systemic effects, it plays a central role in the pathomechanism of inflammation. Soluble and membrane bound TNF binds to its receptors (TNFR1 and TNFR2) expressed on several cell types and regulate cellular functions. TNF has a central role in regulating the inflammation in RA, the increased expression of TNFR in T lymphocytes is characteristic in this disease. Increased TNF levels were reported both in the sera and in the synovial fluid of RA patients. TNF induces the release of other proinflammatory cytokines, such as IL-1, IL-6 and GM-CSF, leading to chronic inflammation of the synovium. TNF blockers belong to the most effective medications in RA, currently five commercially available anti-TNF biologicals are available, clearly showing the primary role of TNF in this disease.

During our work we examined key signal transduction pathways, essential in the regulation of immune cell functions. The CD3ζ chain carries three ITAM motifs, it has main role in regulating the T cell activation. The cross-linking of the TCR induces the phosphorylation of the protein tyrosine kinase $p56^{Lck}$, which leads to the phosphorylation of the ζ-chain, allowing the subsequent activation of additional kinases. The rapid rise of the intracellular Ca^{2+} level and the altered gene expression pattern has a central role in the cell

activation. The enhanced expression of target genes leads to cytokine production, among them IL-2 plays a major role in further cell activation events. T lymphocyte activation is regulated by the expression and the phosphorylation of the CD3ζ chains. The ζ-chain has a relatively long half-life compared to the other CD3 chains, the internalization and the recycling of the ζ-chain is regulated independently from the other CD3 subunits. Src-like adapter protein (SLAP) is a regulator of the internalization and activation-induced degradation of the ζ-chain. SLAP binds to the phosphorylated ζ-chains through its SH2 domains (both to the constitutively phosphorylated ζ-chains and to the ζ-chains phosphorylated during T cell activation). The SLAP bound ζ-chains may be degraded, or alternatively may recycle back to the plasma membrane. The CD3ζ chains can be degraded by the lysosomes, or by the proteasomes (the precise mechanism of the proteasomal degradation is not known). Decreased expression of the CD3ζ chain and the hyporesponsiveness of the T cells are characteristical in RA.

Mast cells play a central role both in the adaptive and in the innate immunity. Immature mast cells leave the bone marrow and migrate into the peripheral tissues where they complete their differentiation. It is well known that during the allergic reaction the cross linking of the high-affinity IgE-receptors lead to mast cell activation and degranulation. In addition, the high number of these cells in the inflamed synovium and their activation through Fc receptors suggest the role of mast cells in the pathomechanism of RA.

The signal transduction proteins are under the control of both transcriptional and post-transcriptional regulation; microRNAs (miRNAs) are recently described post transcriptional regulators. The mature miRNAs contain 20-26 nucleotides, which are complementary to their mRNA targets. MiRNAs, associated with special protein complexes, bind to the appropriate mRNAs and inhibit the translation or promote the degradation of the target RNAs. By evaluating the sequence complementarity, conservation and other factors, target prediction methods have a major role in exploring the potential effects of the miRNAs. The association of miRNAs with several diseases is well known.

The role of miRNA-155 in the regulation of T cell activation has been recently recognized. Altered expression of mir-155 is associated with several diseases, including RA and atopic dermatitis. MiRNA-155 knockout animals are characterized by impaired immunological memory and regulatory T cell functions, these animals are protected from collagen-induced arthritis. MiRNA-181a plays an important role in the selection and activation of T cells, miRNA-181a deficiency leads to the increase in the rate of autoreactive lymphocytes. PTPN22 and DUSP6 (both have a key role during T cell activation) are targets

of the miRNA-181a. Altered expression of miRNA-146a was also described in the synovial cells of patients with RA. The most studied targets of the miRNA-146a are some proteins of TNF signal transduction (TRAF6, IRAK1) and this miRNA targets several other T lymphocyte proteins as well. The level of miRNA-132 is decreased in the plasma samples of patients with RA, by contrast its level is increased in the synovial samples, and novel data suggest that miRNA-132 might be used as a biomarker in RA.

Aims

To study the regulation of CD3ζ chain in human T lymphocytes

1) **To investigate the effects of TNF on the expression of CD3ζ chain:**
   a) To study the protein expression of the ζ-chain.
   b) To study the ζ-chain internalization.
   c) To investigate the effects of TNF on other CD3 chains.
   d) To study the mRNA expression of the ζ-chain.
   e) To investigate the phosphorylation of ζ-chain.
2) **To study the T lymphocyte activation in the presence of TNF:**
   a) To evaluate the Ca^{2+} response.
   b) To measure the IL-2 production.
   c) To study the proliferation of T lymphocytes.
3) **To investigate the degradation of the CD3ζ chain:**
   a) To determine the place of the degradation.
   b) To investigate the expression of SLAP in the presence of TNF.
   c) To study the colocalization of the SLAP and the CD3ζ.
   d) To investigate the role of SLAP in the degradation of CD3ζ.
4) **To study the possible role of SLAP in RA:**
   a) To investigate the expression of SLAP in RA T cells.
   b) To study the effect of TNF on the SLAP expression of RA T cells.
5) **To investigate the regulation of SLAP expression.**

To study microRNAs in human T cells and in mast cells

6) **To investigate the TNF-induced expression of miR-155, miR-181a and miR-146a in T lymphocytes.**
7) **To study the expression of miRNA-132 in human mast cells.**

Methods

Cells, cell lines

Jurkat cells (Human T cell leukemia cell line, E6.1, ATCC), or PBMCs, isolated by density gradient centrifugation from whole-blood samples over Histopaque (Sigma-Aldrich), were used during our experiments. CD4+ T cells were isolated from PBMCs by using negative magnetic selection (Miltenyi Biotec). T cells were cultured in RPMI-1640 supplemented with 10% FBS and 2mM glutamine. Human mast cells were differentiated from the separated CD34+ fraction of the cord blood. To differentiate mast cells, CD34+ cells were cultured for 8 weeks in RPMI-1640, supplemented with 10% FBS, 2mM glutamine, 0.1% non essential amino acid solution, 100 ng/ml SCF, 50 ng/ml IL-6 (ImmunoTools) and 3μM lysophosphatidic acid (Sigma-Aldrich).

Patients

Samples of RA patients (n = 10) and controls (healthy donors, n = 5) were collected in the Department of Rheumatology, Semmelweis University, Budapest, Hungary. All patients were diagnosed with RA according to the classification criteria of American College of Rheumatology. Five patients were treated with anti-TNF biologicals, and five patients received non biological disease-modifying antirheumatic drugs (DMARD). Cord blood samples were obtained from the Department of Obstetrics and Gynecology, Semmelweis University, Budapest.

Cell activation, treatment

1μg/ml of PHA (Sigma-Aldrich) or anti-CD3/CD28 antibody coated beads (Invitrogen) were used to activate T lymphocytes (4-24 hours). During some experiments, cells were treated with TNF (40 ng/ml, BD Biosciences) for 2 hours. PBMCs were stimulated for 2 hours before the TNF treatment. The lysosomal and proteasomal degradation was inhibited by ammonium chloride (10 mM, Sigma-Aldrich), and by MG-132 (100 nM, Calbiochem) for a 24 hours followed by treatment with TNF. For mast cell activation, cells were presensitized by myeloma-derived IgE (Serotec) overnight and after washing were cross-linked by anti-human IgE (Dako) for 2 hours.

Western blot

Cells were lysed with ProteoJET Mammalian Cell Lysis Reagent (Fermentas; Thermo Scientific, Waltham, MA) supplemented with 1% Nonidet P-40, protease, and phosphatase inhibitor mixture (Sigma-Aldrich), and then centrifugated 14,000 x*g* at 4°C for 15 min. Samples were loaded on 12% PAGEr Gold precast gels (Lonza) and then blotted onto polyvinylidene difluoride membranes (Bio-Rad). After blocking and incubation with the

appropriate Abs, the membrane was processed by the ECL method (Amersham Biosciences), and the bands were visualized on standard X-ray film (Amersham Biosciences). OD was determined by ImageJ, plots were normalized to control.

Flow Cytometry

Cells were labeled with the appropriate antibody in 0.5% BSA / PBS buffer at 4° C. Acquisitions were performed on BD FACS Calibur instrument, and the results were evaluated by FlowJo (Tree Star) software. For the measurements of the intracellular Ca^{2+} level and the proliferation, cells were loaded with Fluo-4 AM or with CFSE dyes (both Invitrogen), respectively.

Cell transfection

In some experiments, cells were transfected with siRNAs specific for SLAP or control siRNAs (Life Technology), in other experiments GFP-tagged SLAP cDNA vectors were administered into the cells using Amaxa Nucleofector (Lonza) device for confocal microscopy studies.

Confocal microscopy

After cells were attached to an 8 well slide, they were permeabilized and labeled with the appropriate antibodies (CD3ζ, LAMP1, Golgi 58K) or dyes (Lysotracker, Draq5, cholera toxin). For image acquisitions FluoView 500 confocal laser scanning microscope equipped with four optical channels and 60x (NA: 1.45) oil immersion objective lens (Olympus) was used. For the determination of colocalization the ImageJ software GSCD plugin package was applied. Approximately 100 cells per sample were defined as region of interest (ROI) and Pearson's correlation coefficients were determined. The integrated intensity values were given as per cell averages using FluoView 5.0 software (Olympus).

Real-time RT PCR

mRNA or microRNA-fraction containing total RNA was extracted by RNeasy kit or miRNeasy kit (Quigen), respectively. For reverse transcription of miRNAs specific reverse transcription primers (Life Technologies) or in case of mRNA measurements random primers (Promega) were used. The real-time PCR was performed in on ABI 9700 (Life Technologies) instrument with specific primers. The relative expressions were correlated to the HGPRT or to the RNU6B levels.

MicroRNA target prediction

In order to increase the accuracy of the online target prediction, a cumulative list was set up from several online target prediction algorithms (e.g. PicTar, MiRBase, TargetScan), and the hierarchy was determined by ranking the different probability scores.

ELISA

IL-2 levels were measured by BD OptEIA Human IL-2 ELISA Kit (BDBiosciences), according to the manufacturer's protocol.

Statistics

Results were analyzed by Student t-test, Mann–Whitney U rank-sum test, Wilcoxon matched pairs test, or ANOVA test.

Results

To explore the possible association between the T cell hyporesponsiveness and the elevated level of TNF, which can be observed in several autoimmune diseases, we investigated the involvement of antigen receptor signal transduction.

Firstly, we studied whether the amount of the CD3 signal transducing ζ-chain is directly affected by TNF. The results of the Western blot experiments show that TNF dose-dependently (5-80 ng/ml) reduces the amount of CD3ζ chain both in Jurkat ($p < 0.05$) and in primary cells ($p < 0.05$). Our observation was also confirmed by confocal microscopy, wherein the integrated intensity of the pixels corresponding to the ζ-chain in TNF (40 ng/ml, for 24 h) treated cells was compared to the untreated cells ($p < 0.00002$). We have also shown that as long as the TNF is present in the culture medium (24-72 hours) the observed suppression of the ζ-chain is sustained, and after removal of the TNF, the ζ-chain expression returns to the basal value. Fluorescent imaging demonstrated that TNF facilitates the reduction of the cell surface ζ-chains.

Because CD3ζ is closely associated with the other TCR chains, we examined whether there is a decrease in the expression of other chains as well. Total protein levels of CD3ε- and γ-chain were analyzed by Western blot, and ε-chain expression was also measured by flow cytometry. Our results show that there is no TNF-induced change in the expression of the CD3ε and γ-chains. In further experiments we focused on the mechanism how CD3ζ is regulated. Quantitative real-time RT-PCR (4 hours to 15 days, from 2.5 to 80 ng/ml TNF) experiments showed that the mRNA expression of the CD3ζ is not affected by TNF, independently from the time or the concentration used, suggesting that CD3ζ chain is regulated post-transcriptionally.

On that basis, we assumed that TNF promotes the internalization and degradation of ζ-chain. To demonstrate this, proteasomal or lysosomal degradation was blocked before TNF treatment (24h, 40 ng/ml), then the samples were analyzed by Western blot. The selective inhibition of the lysosomal activity (NH_4Cl) did not prevent the ζ-chain downregulation, by contrast, the proteasome inhibition (MG-132) attenuated the TNF-induced CD3ζ chain downregulation ($p < 0.05$). The colocalization of the CD3ζ and the LAMP-1, a lysosome-associated protein was examined by confocal microscopy. According to our data there is no detectable TNF-induced colocalization between the two molecules.

In the next phase of our experiments we concentrated on those molecules that might be involved in the regulation of CD3ζ degradation. The src-like adapter protein (SLAP) is related to the CD3ζ chain and it is involved in the recycling and in the degradation process. Western

blot experiments showed that the level of SLAP protein is increased in response to TNF treatment ($p < 0.05$). Confocal microscopy was applied to demonstrate the association between the CD3ζ chain and the SLAP. Cells were transfected with GFP-tagged SLAP cDNA vector, then they were treated with TNF and the CD3ζ chain was labeled with specific antibody. Compared to the untreated cells, where the two proteins localize at different regions of the cell, colocalization develops upon TNF treatment ($p < 0.01$). To further study the possible role of SLAP in the regulation of CD3ζ degradation, Jurkat cells were transfected with SLAP-specific siRNAs or with control siRNAs, and then were treated with TNF. In the control siRNA-transfected samples, as it was expected, TNF reduces the amount of ζ-chain however, SLAP silencing inhibited the TNF-induced ζ-chain downregulation. SLAP can bind to the phosphorylated ζ-chain and in accordance with that, an increased proportion of the phosphorylated isoform can be seen in the presence of TNF ($p<0.05$).

Next, we investigated whether the observed decrease in the amount of ζ-chain may alter the responsiveness of the T cells. To characterize the possible role of TNF on the T cell activation, the cells were activated with anti-CD3 (for 6, 24, 72 hours), then the IL-2 levels were measured in the supernatant by ELISA method. According to our observations, in the TNF-treated group the IL-2 production was significantly lower than in the control group ($p<0.05$). Similarly, TNF treatment reduced both the amplitude and the duration of the T cell activation induced Ca^{2+} response. Interestingly, the proliferative capacity of the T cells was not affected by TNF treatment.

Recently published data suggest that SLAP deficiency leads to a significant reduction in the incidence and in the severity of zymosan-induced arthritis. On the basis of this previous observation we investigated whether there is a difference in the expression of SLAP in the T lymphocytes of RA patients. To study the SLAP expression, peripheral CD4+ T cells were isolated from patients with RA (n=10) and from age and sex-matched healthy controls (n = 6). The expression of SLAP was measured by Western blot. SLAP expression was 2.5-fold higher in the RA T cells, than in the T cells of healthy controls ($p<0.05$), which suggest the involvement of SLAP in the pathomechanism of this disease. Next, we studied if there is a difference in the TNF induced SLAP expression between the T cells of RA patients and healthy donors. The separated CD4+ T lymphocytes were activated in the absence or in the presence of TNF, and then the expression of SLAP was determined by Western blot. TNF increased the SLAP expression of the CD4 T cells isolated from healthy donors and from non-biological DMARD treated RA patients (n=5). By contrast, TNF treatment did not alter the SLAP expression of the CD4+ cells of anti-TNF therapy-treated patients (n=5).

Next we studied the TNF induced SLAP mRNA expression by real-time RT PCR method, according to our data, the TNF treatment did not alter the SLAP mRNA level. Thereafter, the post-transcriptional regulation of SLAP may be involved in this process, thus miRNAs may have a role in the regulation of SLAP expression. During the target prediction analysis for mRNA sequence of SLAP, in which outputs of 12 different algorithms were taken into consideration, mir-155 was identified as the most likely target, in addition, mir-181a is also among the predicted top targets.

We also studied which miRNAs may influence the activation of T lymphocytes. Especially those miRNAs were studied, which may have a role in the pathomechanism of RA. The expression of mir-155, which may interfere with SLAP, and may play a relevant role in RA, was determined in anti-CD3/CD28 activated human CD4+ T lymphocytes by RT-PCR method. According to our data, the expression of mir-155 was significantly upregulated during T cell activation, and this upregulation was clearly inhibited by TNF pretreatment ($p < 0.05$). Conversely, target prediction was also performed to define the possible targets of miR-155, and it was found that SLAP appears on the target list among some other proteins involved in the TCR signaling.

In accordance with earlier observations, anti-CD3/CD28 treatment downregulated mir-181a expression in T cells, and this effect was also inhibited by TNF pretreatment. TNF also regulated the expression of mir-146a. Anti-CD3/CD28 treatment induced the expression of mir-146a in T cells. The maximal expression was observed after 2 hours stimulation, and the TNF treatment shifted this peak from 2 to 6 hours. In addition, mir-146a expressed at higher levels in the TNF treated groups in most of the time points studied (2, 6, 10, 24, 72 h).

Mir-132, which can be detected in the synovial fluid of RA patients, was examined in human umbilical cord blood-derived, differentiated mast cells. Expression levels of mir-132 were measured by real-time RT-PCR. Interestingly, mir-132 was significantly upregulated ($p < 0.05$) after the IgE-induced cell activation.

Major findings

1. TNF treatment reduces the amount of CD3ζ chain selectively and reversibly in T lymphocytes, which contributes directly to the hyporesponsiveness of the cells.

2. TNF enhances the expression of SLAP, which plays a key role in the regulation of the ζ-chain, TNF promotes the colocalization between CD3ζ and SLAP. TNF-induced loss of ζ-chain can be prevented by silencing of SLAP.

3. Studies of the degradation processes of CD3ζ chain show that the activation signal induces lysosomal degradation, by contrast, TNF induces predominantly the proteasomal degradation of the CD3ζ chain.

4. SLAP expression is increased in the T lymphocytes of patients with RA. Moreover, the TNF induced SLAP upregulation was not observed in the T cells of anti-TNF biological treated RA patients.

5. Based on the results of the target prediction methods, it is likely that miRNA-mediated inhibition is involved in the regulation of the amount of SLAP.

6. TNF regulates the expression of mir-146a, mir-155 and mir-181a in T lymphocytes, and the upregulation of mir-132 during mast cell activation was also observed.

In summary, our results indicate that TNF regulates the activation of the T cells and may affect the outcome of the immune response.

List of publications

The findings of the thesis were published in the following publications:

1. **Ersek B,** Molnar V, Balogh A, Matko J, Cope AP, Buzas EI, Falus A, and Nagy G. (2012) CD3zeta-chain expression of human T lymphocytes is regulated by TNF via Src-like adaptor protein-dependent proteasomal degradation. J Immunol, 189:1602-1610.
IF: 5.788

2. Molnar V, **Ersek B,** Wiener Z, Tombol Z, Szabo PM, Igaz P, and Falus A. (2012) MicroRNA-132 targets HB-EGF upon IgE-mediated activation in murine and human mast cells. Cell Mol Life Sci, 69:793-808.
IF: 6.570

Publications not included in the thesis:

3. Nagy G, Koncz A, Telarico T, Fernandez D, **Ersek B,** Buzas E, and Perl A. (2010) Central role of nitric oxide in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. Arthritis Res Ther, 12:210.
IF: 4.689

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