PhD Thesis

The role of the tyrosines of the ZAP-70 kinase in the T-cell activation and in the development of non-genomic glucocorticoid effects

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Pécs
2012.

1. Introduction

1.1 Characteristics of T lymphocytes

T lymphocytes belong to the cellular components of the adaptive immune system, they set out approximately 75-80% of the lymphocytes in the peripheral blood of a healthy human individual. They have a pivotal role in the recognition of foreign antigens (e.g. bacteria, viruses, tumor cells), in the development of defense mechanisms against these antigens as well as in the regulation of the immune system. Moreover, T lymphocytes participate in the tolerance mechanisms by protecting the self structures.

1.1.1 T-cell activation signaling cascade

Close TcR-ligand binding initiates many signaling steps in the T-cells which lead to their activation/differentiation and engagement of effector mechanisms. TcRs recognize only peptides - digested fragments of an antigen - by an MHC dependent manner. This mechanism is strictly regulated: nucleated cells and thrombocytes present intracellular peptides through MHCI to the CD8+ Tc cells. Extracellular peptides are expressed by professional antigen presenting cells - B-cells, macrophages, dendritic cells - by MHCII to the helper T cells. This biological process is called as MHC-restriction. TcR-MHC-peptide engagement alone is not sufficient to initiate full T-cell activation. Co-receptors such as CD4/8 need to bind to the constant part of the MHC molecule. Moreover, co-stimulation between CD28 and CD80/86, CD40-CD40L and other adhesion molecule pairs are also a prerequisite of the activation. Intracellular domains of the TcR are not long enough to be able to mediate signals inside of the cells. Therefore, TcR associated CD3 complex plays an important role in the initiation of intracellular signals. CD3 complex consists of γ, δ and ε chains. In addition, a ζζ homodimer or a ζη heterodimer associates to these chains. In the chains of CD3 molecule ITAM (Immunoreceptor Tyrosine-based Activation Motif) sequences (YxxLx (6-8) YxxL) are present. Tyrosines residues of the ITAM sequences become phosphorylated in the early steps of the T-cell activation and play and set off the signal transduction. Upon close TcR – peptide – MHC binding early phosphorylation steps are initiated. First, the Src non-receptor tyrosine kinase family member, Lck (CD4/8 associated) is primed by the phosphatase CD45 through the removal of an inhibitory phosphate group from the tyrosine (Y)505. Next, Lck is activated by the phosphorylation of Y394 by the activated TcR complex. Activated Lck, in turn, phosphorylates ITAMS of the CD3 complex. The phosphorylated CD3ζ chain provides a docking site for the Syk family member ZAP-70 kinase (zeta-chain-associated protein kinase of 70kDa). ZAP-70 is phosphorylated by Lck and activated to become a key organizer of downstream TcR signaling steps. LAT and SLP-76 adapter proteins are the main target molecules of the ZAP-70. Phosphorylation of these molecules, leads to the formation of a multimolecular complex involving GRB2, Itk, GADS and Vav that results in activation of PLCγ1. PLCγ1, in turn, cleaves PIP_2 producing two second messengers: IP_3 and DAG. DAG initiates two major pathways the Ras and PKCθ signaling. Ras triggers the MAP-kinase cascade that results in the translocation of transcription factors (eg. AP-1). Activation of PKCθ leads also to transcriptional regulation mediated by the NFκB pathway. IP_3 releases Ca^{2+} from the endoplasmatic reticulum (intracellular Ca^{2+}-store) that is followed by the opening of plasma membrane Ca^{2+} channels as well (capacitative influx). Then, elevated intracellular Ca^{2+} level activates calcineurin, calmodulin and finally the transcription factor NFAT. As a consequence of the above mentioned signaling cascades a number of transcription factors

are activated (AP-1, NFAT, NFkB) leading to complex gene expression in the activated T cells. Two important gene products of the T-cell activation are the IL-2 cytokine and the α chain of IL-2 receptor. On activated T-cells IL-2 binds to its high affitity receptor composed by α, β and γ chains. IL-2 regulates the T-cell activation through autocrine mechanisms and induces T-cell proliferation.

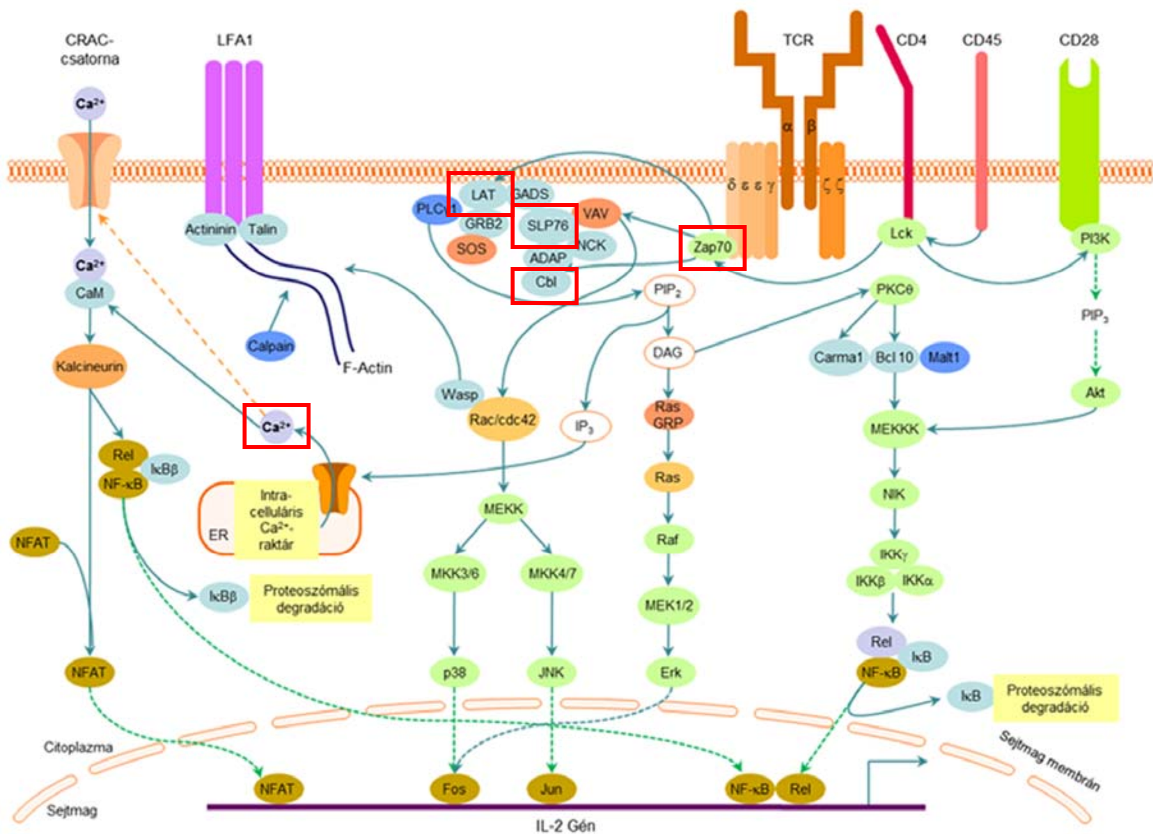

Fig.1. Signaling pathway of the T-cell activation. *Detailed in chapter 1.1.1. Original:http://www.cellsignal.com/pathways/lymphocyte.jsp- Cell Signaling Technology. Enframed those molecules and* Ca^{2+} *that we observed in our experiments.*

1.1.2 The ZAP-70 kinase

The ZAP-70 kinase is member of the Syk non-receptor tyrosine kinase family and is expressed in T cells, NK cells and basophile granulocytes. ZAP-70 consists of two N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain separated by interdomains A and B, respectively. ZAP-70 contains 31 Y residues in total, 11 of which have been identified as phosphorylation sites by mass spectroscopy; however, the physiological function of some is still unknown. For example, little is known about Y069, Y126, Y178 and Y238, located in the two SH2 domains and interdomain A, although Y126 is considered to be involved in the autophosphorylation of the kinase. Interdomain B contains three regulatory Y residues at 292, 315 and 319. Y292 plays an inhibitory role in TCRmediated signaling and serves as docking site for casitas B-lineage lymphoma. Y315 binds Vav and sarcoma virus CT10 oncogene homolog II and bears both positive and negative regulatory functions in T-cell activation. Y319 plays a positive regulatory function and is important in PLC-γ1 and Ras-mediated signaling. Y residues found in interdomain B also influence T-cell development. Three tyrosines of the kinase domain are considered to take part in T-cell activation. Y474 is the docking site for Shc; Y492 has inhibitory, while Y493 has an activatory role in T-cell activation. Y597 and Y598 are negative regulatory sites.

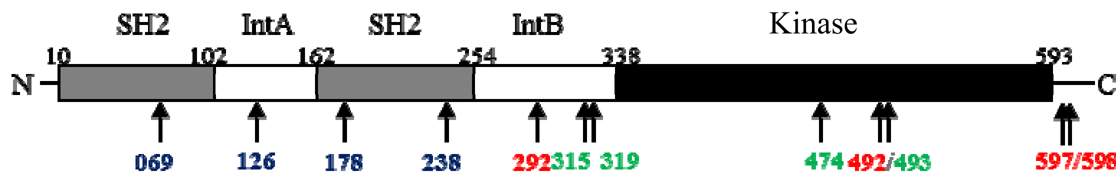

Fig. 2. Structure of the ZAP-70 kinase and its tyrosine reidues that take part in the T-cell activation. *In: Szabo M, Czompoly T, Kvell K, Talaber G, Bartis D, Nemeth P, Berki T, Boldizsar F. Fine-tuning of proximal T cell receptor (TcR) signaling by ZAP-70 tyrosine-residues in Jurkat cells. Int. Immunol. 2012 Feb;24(2):79-87.*

The lack of ZAP-70 leads to severe combined immunedeficiency, SCID. The Tc-cells of these patients are almost completely missing, the Th-cell number is normal or elevated but the T-cell signalling is impaired in these cells as well.

Missense mutation of the gene encoding the ZAP-70 can be observed in the SKG mice. The mutation is a W163C switch in the C-terminal SH2 domain of the molecule. This mouse model draws the attention to the role of the ZAP-70 in the development of autoimmune diseases.

The ZAP-70 is expressed in B-cell leukaemia cells as well (normally B-cells express Syk molecule). The B-CLL is characterized by the clonal proliferation of CD19+, CD5+, CD23+ B-cells. The procession of the disease is heterogeneous, therefore numerous investigations have been done to find factors, that can prognostize the course of B-CLL. Besides the CD38 expression and the mutation of the variable region of the Ig heavy chain, ZAP-70 positivity refers also a worse prognosis.

1.2 Glucocorticoid hormones (GC)

1.2.1 Synthesis of glucocorticoid hormones and their physiological effects

GCs are cholesterol derived steroid hormones synthetized in the zona fasciculata of the adrenal cortex. They act through glucocorticoid receptors (GR) which are members of the nuclear receptor family. GCs influence several metabolic effects: elevation of blood sugar level, stimulation of gluconeogenesis in the liver, production of amino and fatty acids, and blocking the secretion of CRH and ACTH by a negative feedback. In addition to the regulation of metabolism GCs also influence the function of the immune system. GCs decrease the number of basophile, neutrophil granulocytes and mast cells, induce apoptosis in Th1-, Th2- and B-cells. GCs inhibit the T-cell signalling, resulting in the decrease of cytokine especially IL-2 synthesis. However they increase the number of regulatory T-cells and Th17 cells.

1.2.2 Glucocorticoid effects

1.2.2.1 Genomic glucocorticoid effects

According to the classical genomic pathway GC-s diffuse across the plasma membrane and bind to their cytoplasmic receptor. Upon ligand binding comformation changes of the GR occure, it dissociates from heat shock proteins and dimerises. The ligand binded, dimerized receptor ligand complex translocates to the nucleus. It associates with glucocorticoid responsive elements (GRE) in the promoter region of several genes, leading to transcription. Binding to negative GREs promotes transrepression, while binding to positive GREs initiates transactivation that result in immunosuppressive effects. The GR can also directly inhibit the pro-inflammatory transcripton factors. This process above needs hours or even days to develop. However, not all GC effects can be explained by the genomic mechanism. Recently, accumulating evidence is available about non-genomic glucocorticoid effects.

1.2.2.2 Non-genomic glucocorticoid effects

Non-genomic glucocorticoid effects develop by high dose glucocorticoid administration (30 mg–1g/day) within a short period of time (within minutes). This dosage is used in the treatment of asthma, anaphylactic schock, in transplantation and at the relapses of autoimmune diseases.

Non-genomic GC effects include:

i) signals mediated through the membrane GR;
ii) direct effects in the plasma membrane;
iii) mitochondrial effects;
iv) interactions between the activated GR and other cytoplasmic signaling molecules

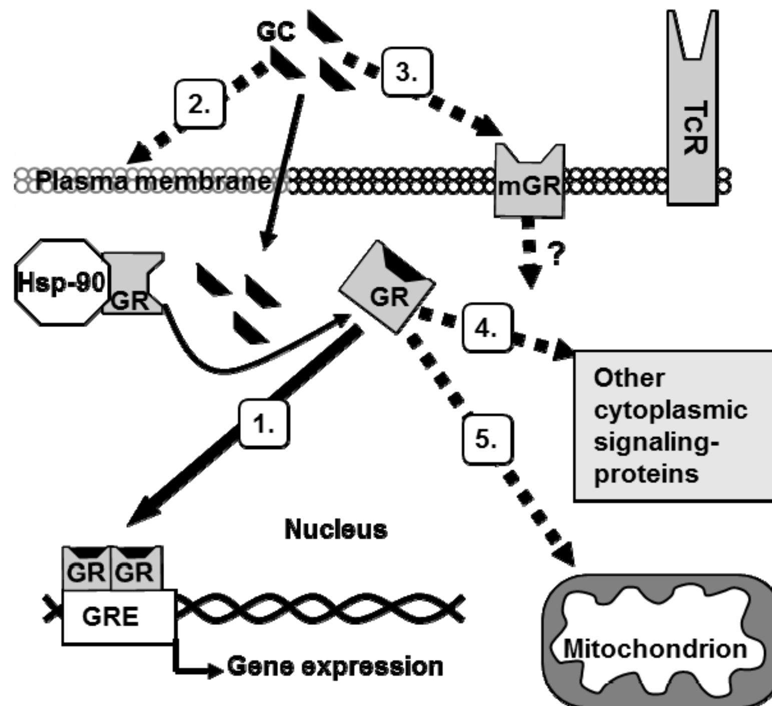

Fig. 3. Genomic (solid arrow) and non-genomic (dashed arrows) glucocorticoid effects. *In: Boldizsar F, Talaber G, Szabo M, Bartis D, Palinkas L, Nemeth P, Berki T. Emerging pathways of non-genomic glucocorticoid (GC) signalling in T cells. Immunobiology 2010 Jul; 215:521-6.*

1.2.3 The role of ZAP-70 kinase in the development of non-genomic glucocorticoid effects

Previously, we observed the non-genomic glucocorticoid effects in the Jurkat human T-cell leukemia cell line. Short time, high dose Dexamethasone (henceforth DX - a widely used synthetic glucocorticoid analogue) treatment alone results increased phosphorylation of Jurkat cells, while DX pre-treatment inhibits the anti-CD3 induced phosphorylation.

We chose the ZAP-70 kinase as a central molecule in the T-cell activation for further analysis. Short time, high-dose DX treatment led to tyrosine-phosphorylation of the ZAP-70 kinase, moreover DX additionally increased the anti-CD3 induced tyrosine phosphorylation. To further investigate the function of ZAP-70 kinase in non-genomic glucocorticoid effects we proposed a novel model. In the lack of ligands, GR, Lck and ZAP-70 form a multimolecular complex with the HSP-90 near to the plasma membrane. After few minutes of GC administration the GR dissociates from the HSP-90 and associates with ZAP-70 leading to its phosphorylation by p56-Lck.

Upon anti-CD3 and DX treatment the ZAP-70 kinase is phosphorylated. ZAP-70 consists of both activatory and inhibitory tyrosine residues. This phenomenon raises the question that phosphorylation of various tyrosines mediates different signals to downstream molecules.

2. Objectives

Our aim was to observe the function of various tyrosine residues in the ZAP-70 kinase during T-cell activation and non-genomic glucocorticoid signaling.

1. We aimed to establish a model sytem in which the different tyrosine residues of ZAP-70 separately can be analyzed. For this purpose P116 cells (ZAP-70-deficient Jurkat subclone) were lentivirally-transfected with wild type or point-mutated ZAP-70 constructs. In those tyrosine (Y) residues at 069, 126, 178, 238, 292, 315, 492 or 493 positions were replaced with phenylalanine (F).

2. In this system we clarify the role of different tyrosine residues of the ZAP-70 kinase in the T-cell activation emphasizing those with unknown function (Y069, Y126, Y178 and Y238).

- We define the function of the ZAP-70's tyrosines in the development of the activation induced Ca^{2+} signal.
- We observe that how the various tyrosine residues mediates the autoregulation of ZAP-70 kinase.
- SLP-76 and LAT are substrates of the ZAP-70 kinase and play an important role in the development of the intracellular Ca^{2+} signal. Therefore we aimed to analyze how the point mutations of the ZAP-70 influence the anti-CD3 induced SLP-76 and LAT phosphorylation.

3. According to our previous results ZAP-70 kinase participates in the non-genomic glucocorticoid effects. Short time, high dose GC analogue treatment induces phosphorylation and GR association of the ZAP-70. Anti-CD3 caused ZAP-70 phosphorylation is further increased by GC treatment.

- In our recent work we aimed to characterize which tyrosine residues of the ZAP-70 take part in the development of non-genomic glucocorticoid effects. We also clarify if the Y-F amino acid switch changes the ZAP-70-GR association.
- We wanted to analyze the role of the SLP-76, LAT and Cbl molecules - substrates of the ZAP-70 kinase – in the non-genomic glucocorticoid signaling.
- We aimed to ascertain, if those tyrosine residues of the ZAP-70, that mediate non-genomic glucocorticoid effects, will influence the GC induced phosphorylation of SLP-76, LAT or Cbl.
- We also elucidated how the GC pre-treatment affects the anti-CD3 induced T-cell signaling pathways: the phosphorylation of the SLP-76, LAT and Cbl, and the development of the intracellular Ca^{2+} signal.

3. Matherials and methods

3.1 Chemicals and buffers

All fine chemicals were obtained from Sigma unless otherwise stated.

3.2 Cloning and site-directed mutagenesis of the human ZAP-70

The full-length human ZAP-70 coding sequence (henceforth WT-ZAP-70) was amplified using the primers P1 (forward) and P2 (reverse) (annealing temperature: 57°C; product length: 1881 bp) containing BamH1 and Sal1 restriciton sites, respectively. We used a cDNA library transcribed from human peripheral T-cell total RNA as template for the cloning PCR. Next, the PCR product was purified and cloned into a TA vector using the InsTAclone PCR cloning kit (Fermentas) according to the manufacturer's instructions; thereafter, the plasmid with the insert was sequenced. Site-directed mutagenesis of the human ZAP-70 was done in two steps using the TA-cloned WT sequence as a template. First, PCRs were done using primers containing the Y-F mutations at different amino acid (AA) positions; the following forward and reverse primer combinations were used: P1-P069, P1-P126, P1-P178, P1-P238, P1-292, P315-P2, P492-P2 and P493-P2. PCR products were purified and used as megaprimers in the second step PCRs in pair with P1 or P2 to amplify the full-length ZAP-70 constructs containing the Y-F mutations. The final products were TA cloned and sequenced which verified the targeted mutations. All PCRs were done with a high fidelity Proof Start DNA Polymerase (Qiagen) according to the manufacturer's instructions.

3.3 Stable transfection of the ZAP-70 constructs into ZAP-70-deficient P116 cell line using lentiviral vectors

P116 cells were transfected with the lentiviral vectors containing the mutated or the full-length WT-ZAP-70 cDNA. Point-mutated or WT-ZAP-70 cDNA has been inserted into the pWPTS lentiviral transfer plasmid under control of elongation factor-1 (EF1) promoter. This late second-generation lentiviral construct contains central polypurine tract and WPREs that increase transgene integration and expression. For lentivirus production, an envelope construct (pMD.G), a packaging plasmid (R8.91), and the transfer plasmid (pWPTS with EF1-ZAP-70) were transiently co-transfected by calcium–phosphate method into 293T cells pre-treated with chloroquine (1 mM final concentration). Following an overnight incubation and medium change, the supernatant of the virus producer cells was harvested after 24 h, centrifuged (2000 r.p.m., 10 min, 4°C) and filtered (0.45-μm pore size polyvinylidene fluoride-coated filters) to eliminate rough cellular debris. P116 cells were transfected by spinoculation at MOI = 10 (MOI: multiplicity of infection or virus/cell ratio).

3.4 Cell lines

The Jurkat, P116 (ZAP-70-deficient Jurkat subclone, kindly provided by E. Monostori, Biological Research Centre, Szeged, Hungary), or transgenic P116 cells transfected with the wild-type (WT) or point-mutated ZAP-70 were cultured under appropriate conditions (37°C, humidified atmosphere, containing 5% CO2) in RPMI supplemented with 10% FCS (Gibco), sodium pyruvate (1 mM) and glucose (4.5 g/1), penicillin and streptomycin.

3.5 Antbodies

The following antibodies were used for western bloting: mouse monoclonal anti-phosphotyrosine (clone PY20, 1:5000) and anti-ZAP-70 (clone 29/ZAP-70 Kinase, 1:5000) antibodies were from BD Pharmingen (San Jose, CA, USA); mouse monoclonal anti-b-actin (clone AC-74, 1:50000) was from Sigma, rabbit polyclonal anti-SLP-76 (1:1000), anti-LAT (1:1000) and anti-Cbl (1:1000) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-GR (clone 5E4 from hybridoma cell line prouced by our research group).

As secondary antibodies, HRP-conjugated goat anti-mouse IgG (1:1000; Hunnavix, Hungary) or anti-rabbit IgG HRPO (1:1000; Pierce) were used.

For immunoprecipitation (IP), we used mouse monoclonal anti-SLP-76 (clone F-7; 2 lg per sample), anti-LAT (clone 11B.12, 2 µg / sample), anti-Cbl (clone A-9, 2 µg / sample) antibodies from Santa Cruz Biotechnology and rabbit polyclonal ZAP-70 (a kind gift from E. Monostori, Biological Research Centre, Szeged, Hungary).

For flow cytometry, we used mouse monoclonal FITCconjugated anti-ZAP-70 antibody (clone 2F3.2; Upstate Biotechnology) recognizing AAs 1–254 of the ZAP-70 kinase and mouse monoclonal PE-conjugated ZAP-70 antibody (clone 1E7.2; eBioscience) recognizing AAs 282–307.

Phospho-specific PE-conjugated mouse anti-SLP-76 pY128(clone J141-668.36.58) and mouse anti-LAT pY171 (clone I58-1169)antibody was purchased from BD Biosciences.

3.6 Intracellular staining and flow cytometry for the detection of intracellular ZAP-70 expression

Cells (10^6) per sample were fixed in PBS containing 4% PFA for 20 min and then permeabilized in saponine buffer (PBS containing 0.1% NaN3, 0.1% BSA and 0.1% saponine). Cells were labeled with two different antibodies recognizing two distinct epitopes of ZAP-70 in permeabilization buffer for 45 min on ice. Next, samples were washed twice with saponine buffer and once with PBS containing 0.1% BSA and 0.1% NaN3. Flow cytometric acquisition and analysis were done with the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CellQuest software. Cells were gated based on their forward scatter/side scatter parameters, 10 000 events were acquired in each sample. Mean fluorescence intensity values (MFI) were calculated based on histograms. As negative controls, unlabeled autofluorescent samples and isotype-matched control antibodies for each color were used.

3.7 Treatment of the cells

Before treatment cells were resuspended in RPMI medium. To examine the TcR/CD3 pathway cells were activated with anti-human-CD3 (clone OKT-3; ATCC CRL-8001, 1 mg/ml stock solution) in 5µl/10^6 cell concentration for 2 minutes.

To exert non-genomic glucocorticid effects cells were incubated with 10^{-5}M dexamethasone (DX- synthetic glucocorticoid analogue, dissolved in DMSO) for 2 minutes. This concentration corresponds with the high dose steroid amount used in medical treatment.

In case of combined treatment 2 minutes DX pre-treatment was followed by 2 minutes anti-CD3 activation. Treatments were performed at 37°C, under continuous shaking in a

Thermo Mixer (Eppendorf). For Western blot experiments the reaction was stopped in liquid nitrogene, for Phospho-Flow measurements in 4% PFA. For the intracellular Ca^{2+} measurement, during the combined treatment cells were pre-treated with 10^{-5} M DX for 10 minutes.

3.8 Immunoprecipitation, Western blot

Resting or treated cells were lysed in Triton X lysis buffer (50 mM HEPES, 10 mM Na-pyrophosphate, 10 mM EDTA, 100 mM NaF, 10% glycerol and 1% Triton X-100, pH 7.3, freshly completed with protease inhibitor and Na-orthovanadate) for 30 min on ice and then centrifuged for 10 min at 13 000 rpm. The supernatant was either boiled in SDS sample buffer (125 mM Tris, 4% SDS, 10% glycerol, 0.006% bromophenol blue and 10% mercaptoethanol) for 10 min or further used for IP. For IP, cell lysates were incubated in blocking buffer (Tris buffered saline containing 10% BSA and 0.1% NaN3) with the appropriate amount of antibody (see in Antibodies) for 2 h and then incubated with Protein-G (GE Healthcare, UK) for 2 h. After washing three times, immunocomplexes were removed from the Protein-G by 10 min boiling in SDS sample buffer. Samples were separated on 7.5 or 10% SDS–polyacrylamide gel using a MiniProtean system (Bio-Rad, Hercules, CA, USA) and blotted onto nitrocellulose membranes with Trans-Blot equipment (Bio-Rad) overnight. Membranes were soaked in blocking buffer [2% BSA or 5% non-fatty dry milk (Bio-Rad), 10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature (RT) and then incubated with the appropriate dilution of primary antibodies (see in Antibodies) in 10-times diluted blocking buffer for an additional 2 h at RT. Washing of the membranes was performed in wash buffer (10 mM Tris, 100 mM NaCl and 0.1% Tween-20, pH 7.4). After washing, blots were developed with HRPO conjugated secondary antibodies. Following additional washing, blots were visualized using Super Signal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Signals were detected with the Fuji LAS4000 imaging system (Fuji, Japan). Antibodies were removed using Restore Western Blot Stripping Buffer (Pierce) and after blocking (see above), blots were re-probed with the second primary antibody. Densitometry of the blots was performed with the Scion-Image software (Scion Corporation, Frederick, MD, USA).

3.9 Flow cytometric measurement of the intracelular Ca^{2+}-signal

To detect the changes in the free intracellular Ca^{2+} level, cells were loaded with the Ca^{2+} selective indicator dye Fluo-3-AM (Invitrogen, Carlsbad, CA, USA). Flow cytometric measurements were performed and analyzed with a FACS Calibur flow cytometer using the CellQuest software. Baseline Ca^{2+} level of resting or DX pre-treated cells was measured for 50 s and then OKT-3 was added to the samples and the measurements lasted for a total of 5 min. To analyze the dynamics of the Ca^{2+} signal, the changes of FL1 intensity, being proportional to the intracellular Ca^{2+} level, were plotted against time. Briefly, gates were created along the time axis of the activation dot plots at definite time points and the mean FL1 fluorescence intensities were calculated from every gate. These values were divided with the basal fluorescence intensities measured in the same sample before the addition of the activating agent and represented as ‘FL-1 change’ (y-axis).

3.10 Measurement of the anti-CD3 induced phosphorylation changes by Phospho-Flow method

Resting or OKT-3-activated cells were fixed with 4% PFA at 37°C for 10 min under continuous shaking and then permeabilized in Phosflow Perm Buffer III (BD Pharmingen) for 30 min on ice. Samples were then washed in PBS containing 0.1% BSA and 0.1% NaN_3 and incubated with anti-SLP-76 pY128 or anti-LAT pY171 antibodies for 45 min at RT. Samples were washed in PBS containing 0.1% BSA and 0.1% NaN3 and finally resuspended in PBS. Flow cytometric acquisition and analysis were performed with a FACS Calibur flow cytometer using the CellQuest software (BD Biosciences).

3.11 Statistics

During oue work descriptive statistics was used to determine group means and the standard errors of the means (mean $\pm$SEM). Differences between two groups were tested for statistical significance using Student's t-test. A value of $P \leq 0.05$ was considered statistically significant.

1. Results

4.1 ZAP-70 expression in the tyrosine (Y)-phenylalenine (F) point-mutated cell lines

To elucidate the role of different Y residues in TCR signaling, we have generated Jurkat cell lines stably expressing Y-F point mutants of the ZAP-70 protein. To this end, we transfected P116 cells with WT or point-mutated ZAP-70 construct. In the mutated cells Y residues at 069, 126,178, 238, 292, 315, 492 and 493 were changed to F. The expression of the ZAP-70 molecule was confirmed with WB and flow cytometry.

All transgenic cell lines expressed comparable levels of the ZAP-70 except the F238-ZAP-70 cell line, despite three repeated transfections, we could not establish the F238-ZAP-70-expressing cell line although the construct was complete as assessed by repeated sequence and restriction analysis. Although we have no direct evidence why the expression of the F238-ZAP-70 was not successful, we might speculate that this mutation would affect post-translational modifications of the molecule leading to defective protein folding and subsequent breakdown. To examine Y238 would be of special interest because based on an own preliminary sequence analysis is found in a putative ITIM sequence (236VEYLKL241).

The antibodies used for flow cytometry recognized two different parts of ZAP-70. Importantly, the cell line expressing F292-ZAP-70 could not be stained with anti- ZAP-70-PE clone 1E7.2 because the epitope of this antibody lies between AAs 284 and 307. Therefore, targeted Y-F mutation at position 292 disrupted the epitope necessary for antigen recognition.

4.2 Point mutations of the ZAP-70 kinase influence the TcR/CD3 signaling pathways

4.2.1 Changes in the anti-CD3 induced intracellular Ca^{2+} signal in the ZAP-70 point mutated cell lines

Next we examined how point mutations at different tyrosine residues of the ZAP-70 kinase influence the development of the activation induced Ca^{2+} signal. In the absence of ZAP-70, the TCR signaling is blocked at an early stage leading to impaired T-cell activation including the lack of the intracellular Ca^{2+} signal. A previous study showed, that introduction of the WT-ZAP-70 into P116 cells led to the restoration of the impaired anti-CD3-induced Ca^{2+} signal. Targeted Y-F mutations had diverse effects on the anti-CD3-elicited Ca^{2+} signal. As expected, mutation of the known inhibitory Y residues 292 and 492 led to an increased Ca^{2+} signal when compared with the WT-ZAP-70-expressing cells, while the mutation of the activator Y493 in the kinase domain completely abolished the anti-CD3-induced Ca^{2+} signal. Importantly, the opposing regulatory function of the neighboring Y492 and Y493 was mirrored in the Ca^{2+} signaling results. Mutation of the regulatory/activator Y315 led to a slightly decreased Ca^{2+} signal. The amplitude of the Ca^{2+} signal was significantly higher in the F069-ZAP-70-expressing cell line than in the WT-ZAP-70-expressing cell line, similar to what was found in the F292 and F492 mutant cell lines. Y-F mutation at the 178 AA position did not affect the anti-CD3-induced Ca^{2+} signaling.

4.2.2 Point mutations at tyrosine residues of the ZAP-70 kinase change the anti-CD3 induced tyrosine phosphorylation pattern, and the autoregulation of the kinase

Using Western blot we observed how point mutations of the ZAP-70 influence the anti-CD3-induced tyrosine phosphorylation patterns of the different cell lines. Phospho-protein bands of 35, 55, 70 (most likely ZAP-70) and 115 kDa showed differences in the mutants compared with the WT-ZAP-70-expressing cells. A general hyperphosphorylation could be observed in the F292-ZAP-70-expressing cell. As expected, P116 ZAP-70-deficient cells did not activate upon anti-CD3 treatment. To confirm that the 70-kDa band observed on the whole cell lysate phospho-blot was indeed ZAP-70, we performed immunoprecipitation with anti-ZAP-70 antibody. Increased phosphorylation of the ZAP-70 could be observed in the F292- and F493-ZAP-70 mutant cells. Y-F mutation at residue 315 led to slightly decreased ZAP-70 phosphorylation while other mutations did not alter the anti-CD3-induced ZAP-70 phosphorylation. Based the results we can establish that these three tyrosine residues of the ZAP-70 take part in the autophosphorylation (autoregulation) of the kinase.

4.2.3 Phosphorylation of SLP-76 and LAT is regulated by specific tyrosine residues of the ZAP-70

SLP-76 and LAT are substrates of the ZAP-70 kinase and also participate in PLCγ activation and regulation of the intracellular Ca^{2+} signal. Therefore, by means of immunoprecipitation and Western blot we examined the phosphorylation of these two molecules upon anti-CD3 activation in the point mutated cell lines.

Activation evoked SLP-76 hyperphosphorylation in the F069-, F178-, F292-, F315- and F492-ZAP-70-expressing cell lines compared with the control WT-ZAP-70-expressing cells. SLP-76 phosphorylation was impaired in the F493-ZAP-70 cells.

In case of LAT mutations at Y residues 292 and 315 lead to increased, at Y residue 493 to decreased anti-CD3 induced phosphorylation compared to the WT control.

With the Phospho-Flow technique, phosphorylation events can be analyzed in a Y-specific manner. So next, we analyzed with the help of phospho-specific antibodies the phosphorylation of SLP-76 Y128 and LAT Y171 in both resting and anti-CD3 activated point mutant cells. We found a significantly decreased phosphorylation of SLP-76 Y128 in the anti-CD3-stimulated F493-ZAP-70-expressing cell line when compared with the WT-ZAP-70-transfected P116 cells. This was in line with the above IP experiment, indicating that phosphorylation of Y178 in the SLP-76 depends on Y493 activation in the ZAP-70. A marked decrease could be observed of anti-CD3-induced Y128 phosphorylation in the F126-, F315- and F492-ZAP-70 cell lines (MFI change: 2.1 ± 0.1, 2.5 ± 0.6 and 1.3 ± 0.1 respectively) compared with the WT-ZAP-70 expressing cells (MFI change: 4.5 ± 1.31). Interestingly, Y-F mutation at residues 126, 178 and 492 led to the hyperphosphorylation of Y128 of SLP-76 in unstimulated cells, indicating that these Y residues in ZAP-70 might possess autoinhibitory function.

Y-F amino acid switch at Y492 led to hyperphosphorylation of LAT Y171 in resting cells. Similar to the results of the immunoprecipitation point mutation at Y493 of the ZAP-70 inhibits the anti-CD3 induced LAT Y171 phosphorylation.

4.3 Non-genomic glucocorticoid effects in the Y-F point mutated ZAP-70 kinase expressing cell lines

4.3.1 Point mutations at different tyrosine residues of the ZAP-70 kinase change the DX induced phosphorylation pattern

According to previous results short time, high dose DX treatment leads to phosphorylation of many signaling molecules. In our recent experiments we checked on Western blot how the Y-F amino acid switch of the ZAP-70 influences the phosphorylation pattern of the different cell lines upon 2 minutes DX treatment. F069-, F126-, F178- and F238-ZAP-70 expressing cells showed moderate increased phosphorylation upon DX treatment and the phosphorylation pattern was distinct according to the molecular weight in the various cell lines. Mutations at Y315 and 492 led to general hyperphosphorylation compared to the WT-ZAP-70 expressing DX treated samples. Protein bands at 20, 36, 48, 56 and 70 kDa (most likely the ZAP-70) showed marked phosphorylation patterns.

4.3.2 Tyrosine residues at 315 and 492 positions mediate non-genomic glucocorticoid signals

Previously our research group observed the increased phosphorylation of ZAP-70 kinase upon short time high-dose GC treatment. To clarify which Y residues of the kinase take part in this process, immunoprecipitation of the ZAP-70 was performed on the ZAP-70 point-mutated cells after 2 minutes, high dose DX treatment. Decreased phosphorylation could be observed in the cell lines expressing F315- or F492-ZAP-70 compared to the WT DX-treated sample indicating that these two Y residues might be involved in non-genomic GC actions.

Since the DX-induced ZAP-70 phosphorylation was coupled with ZAP-70-GR association we checked, if the Y-F point mutations influence this physical interaction. ZAP-70 and GR co-precipitation was examined on Western blot. Two minutes DX treatment caused increased association of the GR to the WT ZAP-70 kinase. Despite of the phosphorylation changes observed in the F315- and F492-ZAP-70 expressing cells, we found no difference in the ZAP-70-GR association among the point-mutant ZAP-70 expressing cell lines showing that Y residues are not directly involved in the physical contact between these molecules.

4.3.3 Impacts of short term high dose DX treatment on the TcR/CD3 signaling pathways

Our WB data indicates that the ZAP-70 kinase is not the only target protein of non-genomic GC actions in DX-treated cells. In T-cell signaling downstream ligands of the ZAP-70 kinase are the SLP-76, LAT and Cbl molecules. Therefore, next, we elucidated if 2 minutes high-dose DX treatment affect the phosphorylation of these 3 molecules in the Jurkat cells. After 2 minutes DX administration immunoprecipitation of SLP-76, LAT and Cbl was performed, that showed increased phosphorylation in SLP-76, LAT and Cbl.

From the clinical point of view, cross talk between the TcR/CD3- and non-genomic GC signaling pathways is of special importance, because it is suspected to be in the

background of some immunosuppressive effects. To model this condition we combined the DX and anti-CD3 treatments. According to our previous experiments co-stimulation with DX and anti-CD3 caused ZAP-70 hyperphosphorylation in Jurkat cells when compared with single treatments. Hereby, DX pretreatment partially inhibited the anti-CD3 induced phosphorylation of SLP-76. In the case of LAT, the combined DX, anti-CD3 treated cells showed additional increase of phosphorylation when compared with the DX or anti-CD3 treated samples. Finally, Cbl was phosphorylated upon anti-CD3 or combined DX+anti-CD3 treatments, but these did not reach the phosphorylation level after DX treatment alone.
Elevation of intracellular Ca^{2+} level is a key event in T-cell activation. We tested if short term DX pre-treatment had an effect on the anti-CD3 induced Ca^{2+} signal. 10^{-5} M DX treatment of WT-ZAP-70 expressing cells led to a significant decrease of the Ca^{2+} signal compared to the anti-CD3 activated sample.

4.3.4 Y315 and 492 point mutations in the ZAP-70 inhibit the short time, high dose DX induced SLP-76 and Cbl phosphorylation

As described in chapter 4.3.2 tyrosines 315 and 492 of the ZAP-70 mediate non genomic glucocorticoid effects. The SLP-76, LAT and Cbl molecules could be targets of the ZAP-70 kinase in non-genomic glucocorticoid signaling. To see if the phosphorylation of Y315 and Y492 in ZAP-70 is indeed required for the transmission of non-genomic GC signal to the downstream target molecules, we tested the short-term high-dose DX treatment-induced SLP-76, LAT and Cbl phosphorylation in the F315- and F492-ZAP-70 expressing cell line. After immunoprecipitation Western blot was performed to detect phosphorylation changes. Mutation at Y315 and 492 inhibited the DX induced phosphorylation of SLP-76 and Cbl In case of LAT the point mutations did not influence the DX induced increased phosphorylaton.

5. Summary of the new results

1. We established human T-cell leukemia based, stable, transgenic cell lines, in which we could separately observe the function of 8 (069, 126, 178, 238, 292, 315, 492, 493) ZAP-70 tyrosine residues, especially targeting those with unknown function (069,126, 178 and 238).

2. Y069 of the ZAP-70 plays inhibitory, Y126 activatory, while Y178 partly inhibitory role in the T-cell activation.

3. According to our data ZAP-70 Y292 and Y492 residues participate in negative autoregulation, while Y315 is a positive autoregulatory site.

4. Y-F amino acid switch of ZAP-70 Y126 and Y178 residues leads to SLP-76 Y128; at position Y492 to both SLP-76 Y128 and LAT Y171 hyperphosphorylation in resting cells.

5. Based on our results ZAP-70 regulates the development of the intracellular Ca^{2+} signal rather through the SLP-76 and not LAT.

6. Y315 and Y492 of the ZAP-70 mediate non-genomic glucocorticoid effects.

7. LAT, SLP-76 and Cbl take part in non-genomic glucocorticoid signaling, but the ZAP-70 mediated non-genomic glucocorticoid effects are transmitted by SLP-76 and Cbl, not LAT.

8. Similar as observed in the ZAP-70 kinase combined DX-anti-CD3 treatment further increased the tyrosine phosphorylation of LAT and Cbl. The anti-CD3 induced SLP-76 phosphorylation, and the development of the intracellular Ca^{2+} signal were partly inhibited by DX.

6. Summary, discussion

6.1 The role of tyrosines of the ZAP-70 kinase in the regulaton of the TcR/CD3 signaling

The TcR/CD3 signaling pathway has been extensively characterized in the last 20 years. After the identification of the most important signaling molecules of the TcR/CD3 signaling cascade, several mutant T-cell lines became available to analyze the fine details of signaling procedures.

ZAP-70 is essential for a complete TcR/CD3 signaling. In ZAP-70-deficient P116 cell the signal transduction, in ZAP-70 deficient mice both the signaling and the T-cell development is impaired. In humans, defective ZAP-70 function leads to a SCID phenotype. The function of kinases is regulated through phosphorylation events, therefore, studying the role of their potential phosphorylation sites in point mutated cell lines provides new details about fine regulation of signaling pathways.

Hereby, we established 8 ZAP-70 point mutated cell lines and evaluated the role of the mutated tyrosines in the T-cell activation and in non-genomic glucocorticoid signaling. We especially focused on tyrosine residues with yet unknown function.

Mutations at positions 069 and 126 had opposing effects on the Ca^{2+} signal: the former increased while the latter decreased the amplitude compared to the WT-ZAP-70 expressing cells. Point mutation at Y178 did not influence the anti-CD3 induced Ca^{2+} signal. Y069 is found within the N-terminal SH2 domain of ZAP-70, therefore, its effect on TcR/CD3 signaling might be mediated either through altered binding capacity of the activated ZAP-70 to the CD3 ζ chains (structural explanation) or, alternatively, this residue might impact the kinase activity of the molecule (autoregulatory/ functional explanation). To clarify this question further experiments need to be done. Y126 led to a decreased Ca^{2+} signal, which was very similar to what was observed in case of Y-F mutation at residue Y315. Based on this analogy, we propose that Y126 in interdomain A could have a similar positive regulatory role in ZAP-70-mediated signaling to Y315 in interdomain B. Interestingly, mutation of Y126 in the ZAP-70 led to basal hyperphosphorylation of Y128 in SLP-76, so this residue might be in volved in the regulation of a decreased Ca^{2+} signal upon anti-CD3 treatment.

We also clarified the role of the ZAP-70 tyrosines in the regulation of the SLP-76 and LAT mediated Ca^{2+} signaling. Activation of the PLCγ ia essential in the development of the intercellular Ca^{2+} signal in T cells. LAT and SLP-76 are two main activators of PLCγ, they build a connection between ZAP-70 and PLCγ. Previous data claims that activation of PLCγ depends both on LAT and SLP-76.

We observed that point mutations of the ZAP-70 tyrosines influence the LAT phosphorylation less, than the phosphorylation of SLP-76. We concluded that ZAP-70 regulates the development of the intracellular Ca^{2+} signal through SLP-76 and not LAT.

Mutation of Y493 to F led to a serious block in all early signaling steps, in a similar extent to what was observed in the ZAP-70-deficient P116 cells. Phosphorylation of Y493 is critical in downstream steps of T-cell activation. Its loss leads to a functional block of the TCR/CD3 signaling pathway. Hyperphosphorylation of ZAP-70 could be observed in F493-ZAP-70-expressing cells upon anti-CD3 treatment, most likely due to the hyperphosphorylation of other Y residue(s). This indicates that Y493 phosphorylation has important negative autoregulatory function. According to our results Y493 is not the only

tyrosine residue that takes part in the autoregulation of the kinase. Besides Y493, Y292 has also negative autoregulatory role, in their absence ZAP-70 becomes hyperphosphorylated. Decreased ZAP-70 phosphorylation was found in cells expressing F315-ZAP-70, that indicates its positive autoregulatory function.

***Table 1**. Summary of the results performed on the ZAP-70 point mutated resting or anti-CD3 activated cell lines*

Y-F position	**Ca^{2+}**	**ZAP-70***	**SLP-76***	**SLP-76^{Y128}**		**LAT***	**LAT^{Y171}**		**Function**	
a-CD3	+	+	+	**-**	+	+	**-**	+	**TcR/CD3**	**Autoregulation**
069	↑	-	↑	-	-	-	-	-	inhibitory	-
126	↓	-	-	↑	↓	-	-	-	activatory	-
178	-	-	↑	↑	-	-	-	-	partly inhibitory	-
238	NE	NE	NE	NE	NE	NE	NE	NE	?	?
292	↑	↑	↑	-	-	↑	-	-	inhibitory	negative
315	↓	↓	↑	-	↓	↑	-	-	activatory	pozitve
492	↑	-	↑	↑	↓	-	↑	-	inhibitory	-
493	↓	↑	↓	-	↓	↓	-	↓	activatory	negative

*All results were compared with the results of the WT-ZAP-70 expressing cells. Changes are indicated with arrows:↑ increase, ↓ decrease, - no change, NE: not examined, *immunoprecipitation.*

6.2 Molecular targets of non-genomic glucocorticoid effects in Jurkat cells

The ZAP-70 kinase is not only a key molecule of the T-cell activation, but plays an important role in non-genomic glucocorticoid signaling. With the aid of established ZAP-70 point mutated cell lines we elucidated the non-genomic glucocorticoid effects.

Short term, high dose DX treatment leads to decreased phosphorylation in F315- and F492-ZAP-70 expressing cell lines. These two tyrosine residue mediate the non-genomic GC signals. Interestingly the two tyrosines have opposing role in the TcR signaling: Y315 has activatory, Y492 has inhibitory function. These results suggest that fine phosphorylation differences can be observed in the phosphorylation pattern of the ZAP-70 kinase upon non-genomic glucocorticoid signaling and TcR/CD3 signaling. GC-induced association of the ZAP-70 and the GR has been described earlier. In this work now, we checked if the Y-F amino acid switch influences the ZAP-70-GR association. We could not find any changes in the ZAP-70-GR association due to ZAP-70 point mutation, which turns the attention towards alternative mechanisms.

We also analyzed the role of the substrates of the ZAP-70 in non-genomic glucocorticoid signaling. Besides the two adapters LAT and SLP-76 we also focused on the role of the negative regulator Cbl. All three molecules showed increased tyrosine phosphorylation, so all three molecules mediate non-genomic glucocorticoid effects. Furthermore, we checked that the F315 and F492-ZAP-70 expressing cells could influence the DX increased phosphorylation of LAT, SLP-76 and Cbl. In the case of Cbl and SLP-76 the mutation inhibited the DX induced tyrosine phosphorylation, but did not influence the phosphorylation of LAT. According to these findings the ZAP-70 mediated non-genomic glucocorticoid effects are further transmitted by SLP-76 and Cbl. LAT becomes phosphorylated by Itk and Lck as well, so non-genomic GC effects observed in LAT might be induced by these molecules.

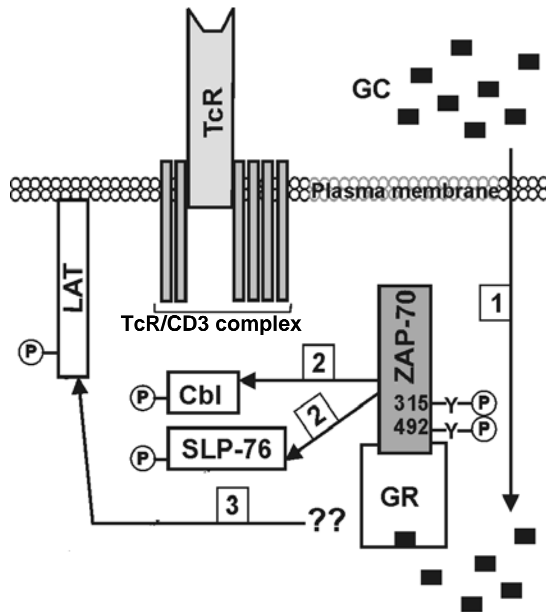

Fig. 4.: Putative mechanisms of non-genomic GC effects on the TcR signaling molecules examined in our experiments. *After diffusion through the plasma membrane GCs bind to their cytoplasmic receptors (1st arrow) The ligand activated GR associates with the ZAP-70 kinase. Y315 and Y492 of the ZAP-70 becomes phosphorylated. The ZAP-70 phosphorylates SLP-76 and Cbl (2nd arrow), while LAT becomes phosphorylated in a ZAP-70 independent (until now unknown) manner (3rd arrow).*

6.3 Non-genomic glucocorticoid effects on T-cell activation: cross-talk between TcR/CD3 and glucocorticoid receptor pathways

Next we clarified how DX treatment influences the T-cell activation and exerts its immunosuppressive effects. Therefore, we performed combined (DX + anti-CD3) treatments and observed the three substrates of the kinase: SLP-76, LAT and Cbl. Moreover, we followed the effect of DX on the anti-CD3 induced Ca^{2+} signal. Similar to phosphorylation changes observed in the ZAP-70, phosphorylation of LAT and Cbl increased further upon the combined treatment, whereas DX partly inhibited the anti-CD3-induced phosphorylation of SLP-76. Besides the phosphorylation changes, increase in the intracellular Ca^{2+} level is a key event in T-cell activation. The anti-CD3 induced Ca^{2+} signal could be partly inhibited with short time, high dose DX pre-treatment similar to the phosphorylation of SLP-76. These data are concordant with our earlier observation, that Ca^{2+} signal in Jurkat T cells is regulated through SLP-76.

Table 2. Summary of DX and anti-CD3+DX effects in Jurkat and ZAP-70 point mutated cells.

A

	ZAP-70	LAT	LAT*	SLP-76	SLP-76*	Cbl	Cbl*	Ca^{2+}
DX	+	+	+	+	+	+	+	+
a-CD3	-	-	+	-	+	-	+	+
Jurkat/ WT	↑	↑	↑	↑	↓	↑	↑	↓

B

Y-F position	**ZAP-70**	**LAT**	**SLP-76**	**Cbl**
DX	+	+	+	+
315	↓	-	↓	↓
492	↓	-	↓	↓

A: Result of experiments performed on *Jurkat/WT cells upon DX or combined (anti-CD3+DX) treatment. Changes were compared to Jurkat/WT untreated or Jurkat/WT anti-CD3 treated (*) samples.* ***B:*** *Phosphorylation changes of F315- or F492-ZAP-70 expressing cells upon DX treatment. Phosphorylaton changes were compared to the WT DX treated samples. Changes are indicated with arrows:↑ increase, ↓ decrease, - no change.*

In conclusion we can assess that the TcR/CD3 signaling is regulated by fine internships of signaling molecules. Tyrosine residues of the ZAP-70 play an important regulatory role in the early steps of T-cell activation. Besides, these tyrosine residues also take part in the (auto)regulation of the kinase.

Tyrosine residues of the ZAP-70 as well as substrates of the kinase are key mediators of non-genomic glucocorticoid effects. Due to fine internship and complex regulation of the proximal signaling molecules the GC and the combined (anti-CD3+DX) effects are transmitted differently.

Clarifying the exact function of tyrosines in the ZAP-70 kinase during T-cell activation and non-genomic glucocorticoid signaling can give further insights into the pathomechanisms of leukemias or autoimmune diseases. It provides information to understand better the immunosuppressive mechanisms, that can be important in the future drug targeting as well. However, our knowledge is far not complete about the complex regulation of the T-cell activation and non-genomic glucocorticoid signaling pathways.

7. Publications (cumulative IF: 21,217)

Publications related to the thesis (IF: 12,989)

1. **M. Szabo,** T. Czompoly, K. Kvell, G. Talaber, D. Bartis, P. Nemeth, T. Berki, F. Boldizsar: *Fine-tuning of proximal T cell receptor (TcR) signaling by ZAP-70 tyrosine-residues in Jurkat cells.*
   International Immunology 2012, 24(2):79-87.
   IF:3,301*
2. Boldizsar F.[1], **Szabo M.** [1], Kvell K. , Czompoly T. , Talaber G. , Bjorkan J. , Bartis D., Nemeth P., Berki T. : *ZAP-70 tyrosines 315 and 492 transmit non-genomic glucocorticoid (GC) effects in Jurkat cells*
   Molecular Immunology (közlésre beküldve)
3. F. Boldizsár, G. Talabér**, M. Szabó**, D. Bartis L. Pálinkás, P. Németh and T. Berki: *Emerging pathways of non-genomic glucocorticoid signaling in T cells*
   Immunobiology 2010, 215(7):521-6.
   IF: 4,114
4. D. Bartis, F. Boldizsar, K. Kvell, **M. Szabó**, L. Pálinkás, P. Németh, É. Monostori, T. Berki: *Intermolecular relations between the glucocorticoid receptor, ZAP-70 kinase, and Hsp-90.*
   Biochemical Biophysical Research Communications 2007, 354(1): 253-8
   IF:2,749
5. D. Bartis, F. Boldizsár, **M. Szabó**, L. Pálinkás, P. Németh and T. Berki: *Dexamethasone induces rapid tyrosine-phosphorilation of ZAP-70 in Jurkat cells.*
   Journal of Steroid Biochemistry and Molecular Biology 2006, 98: 147-154
   IF:2,825

Other publications (IF: 8,228)

6. **Szabó M**.: *Sokoldalú hormonterápia, Hatóanyag a mellékvesekéregből*
   Élet és Tudomány 2011, LXVI. évfolyam, 47. szám 1491-1493. oldal IF: -
7. G. Talabér, F. Boldizsár, D. Bartis, L. Pálinkás, **M. Szabó**, G. Berta, G. Sétáló Jr, P. Németh and T. Berki: *Mitochondrial translocation of the glucocorticoid receptor in double-positive thymocytes correlates with their sensitivity to glucocorticoid-induced apoptosis.*
   International Immunology 2009, 21(11):1269-76
   IF: 3,403
8. Y. Hayashi, P. Engelmann, R. Foldbjerg, **M. Szabó**, I. Somogyi, E. Pollák, L. Molnár, H. Autrup, D. S. Sutherland, J. Scott-Fordsmand, L.-H. Heckmann: *Earthworms and Humans in Vitro: Characterizing Evolutionarily Conserved Stress and Immune Responses to Silver Nanoparticles.*
   Environmental Science & Technology 2012, 46(7): 4166-4173.
   IF:4,825*

*according to the latest (2010) impact factors.
[1]shared first author

Citable abstracts (first authored): 4 (1)
Lectures on international conferences (first authored): 4 (1)
Lectures on hungarian conferences (first authored): 11 (4)
Posters on international conferences (first authored): 15 (6)
Posters on hungarian conferences (first authored): 16 (5)

8. Acknowledgements

I would like to thank to my project leaders, to **Ferenc Boldizsár MD, PhD** and to **Prof. Tímea Berki MD, PhD**, to the opportunity to join their research group and to the support to achieve my workplans.
Thanks to **Prof. Péter Németh MD, PhD,** head of the Institute to the opportunity to join to his PhD programe.
I would like to thank to **Domokos Bartis MD, PhD,** to his advices, to **Krisztián Kvell MD, PhD** and **Tamás Czömpöly MD, PhD** to their help to establish the ZAP-70 point mutated cell lines.
Thanks to **Gergely Talabér MD, PhD** and **Árpád Lábadi MD** to their help in my laboratory work and to their friendship.
Many thanks go to **Péter Engelmann PhD,** to the critical perusal of my thesis and to his support in all fields of my life.
Thanks to **Lászlóné Pápa** to her help in the cell culturing and to **Anikó Katona Pálné** to the organisations and to her help in the everyday life.
I would like to thank to all of the peole working in the **Department of Immunology and Biotechnology** to their support during my work.
Last, but not least I would like to thank to my **family** to their patience, encouragement and support.