INVESTIGATION OF THE EFFECTS OF PARTIAL ZAP-70 DEFICIENCY ON *IN VIVO* T CELL ACTIVATION, DIFFERENTIATION AND ON THE DEVELOPMENT OF AUTOIMMUNE ARTHRITIS

PhD Thesis

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1. Introduction

1.1 Characteristics of the ZAP-70 molecule

The zeta-chain associated protein of 70 kDa (ZAP-70) was first described by Chan and colleagues in Jurkat cells stimulated through the T cell receptor. Under physiological conditions ZAP-70 is expressed by T cells and NK cells, however in chronic lymphocytic leukemia (CLL) and B cell acute lymphoblastic leukemia (B-ALL) it was found in a subset of B cells, as well. According to latest results, ZAP-70 is expressed in immature and mature B cells under normal conditions as well, however the expression level is significantly lower than that observed in T cells and the lack of ZAP-70 causes no disruption in the development or activation of B cells.

ZAP-70 is a member of the Syk (spleen tyrosine kinase) protein kinase family, and it is a key player in the maturation and activation of T cells. Loss-of function mutations or lack of ZAP-70 expression leads to a rare form of severe combined immunodeficiency (SCID) in both humans and mice, as in its absence the development of T cells is arrested in the double positive stage (CD4+CD8+), resulting in virtually no mature T cells in the peripheral lymphoid organs.

1.2 T cell development

The maturation and and selection of T cells is a dynamic process taking place in the thymus. The development of T cells starts in the bone marrow, the cells that later colonize the thymus arise from the hematopoietic stem cells (HSC) of the bone marrow.

In the pro-T cells phase the main markers of thymocytes are c-kit, CD44 and CD25. Early thymic progenitor cells (ETP) (c-kit^{hi} CD44^{+}CD25^{-}) reach DN2a (c-kit^{hi} CD44^{+} CD25^{+}) and later DN2b (c-kit^{lo} CD44^{+} CD25^{+}) stages. Thymocytes are at that stage CD4^{-} and CD8^{-} double negative (DN), their development is independent of the T cell receptor. The DN thymocytes migrate tot he cortex, where signals originating from cortical epithelial cells (cTEC) promote proliferation and commitment tot he T cell lineage, and paralelly, thymocytes also contribute to the maturation of cTEC precursors into mature cTECs. Rearrangement of T cell receptor (TcR) genes occur in the DN3 (CD4^{-}CD8^{-}CD25^{+}CD44^{-}) stage. This is when the β-selection takes place: if the rearrangement of TcRβ gene is successful, the TcR β protein is expressed ont he surface of the cells, and after combination with the pre-TcR α-chain forms the pre-TcR complex. This is when the decision between αβ or γδ T cell lineage differention occurs. The CD4 and CD8 co-receptor appears on t he surface of thymocytes expressing the αβ TcR. The resulting CD4^{+} CD8^{+} double positive (DP) thymocytes start to migrate towards the central part of the thymus, the medulla.

The DP thymocytes go through strict selection steps. During the positive selection those DP thymocytes, whose αβ TcR recognizes the own antigen presented on the major histocompatibility complex (MHC) of cTEC recieve survival signals through their TcR and continue their differentiation. As the result of positive selection single positive (SP) thymocytes develop, the thymocytes recognizing MHC I differentiate into CD8 SP, the ones recognizing MHC II differentiate into CD4 SP cells. Those

DP thymocytes, whose TcR does not recognize the own peptide-MHC complex does not survive selection and die via apoptosis. The negative selection of autoreactive thymocytes takes place in the medulla, where the medullary epithelial cells (mTEC) play a key role. With the help of the AIRE and Fzf2 transcription factors the mTEC cells express and present a great variety of own tissue antigens through their MHC to the developing thymocytes. To avoid autoimmunity, the thymocytes recognizing own antigens with high affinity are deleted with clonal deletion. However, during the so-called agonist selection some cell populations survive negative selection even though they recognize own antigens, an example is natural regulatory T cells (nTreg). Mature thymocytes that survived selection enter the circulation, where the naive $CD4^+$ T cells become activated and proliferate, and differentiate into various helper T cell (Th) subgroups after encounter with a presented antigen.

1.3 The role of ZAP-70 in T cell development

ZAP-70 and Syk, members of the Syk protein kinase family play a key role in the development of T cells. The expression of the two proteins shows a different pattern during T cell differentiation, Syk is expressed in the early, DN stages, while ZAP-70 is expressed later, in the DP thymocytes. The expression of ZAP-70 is upregulated during the positive selection. The signaling through the TcR during positive selection contributes the the upregulationm creating a positive feedback loop. It was shown that the static expression of ZAP-70 (lack of upregulation) leads to the lack of positive feedback loop, resulting in the inhibited differentiation of $CD8^+$ SP from DP cells. Later it was also shown that the differentiation of $CD4^+$ SP thymcytes was disturbed, as well after inadequate signaling through the TcR. To sum up, ZAP-70 plays an important role in the development of both $CD4^+$ and $CD8^+$ SP thymocytes, supposedly through the TcR-ZAP-70 positive feedback circuit, that is essential for the positive selection of thymocytes.

1.4 ZAP-70 deficient mouse strains

The first mouse strain deficient for ZAP-70 was first produced by Negishi and colleagues. In contrast to SCID patients, ZAP-$70^{-/-}$ mice have no $CD4^+$ or $CD8^+$ T cells in their peripheral lymphoid organs, and only $CD4^-CD8^-$ double negative and double positive thymocytes can be found in their thymus. In the spleen there are no αβ T cells, however the number of $B220^+$ B cells is increased. Under SPF conditions they can live up to 10 months, however because of the higher susceptibility to infections resulting from the immunodeficiency under conventional housing conditions they survive only for 7-10 weeks (own observations). Weiss and colleagues have produced another ZAP-70 deficient mouse strain with the deletion of a segment of the protein kinase coding region, the phenotype of these mice is identical to the one described above.

1.5 TcR signalling

T cells recognize peptide antigens presented on the appropriate MHCs by the antigen presenting cells using their T cell receptors. The T cell receptor signalling uses the T cell receptor complex, wich is comprised of TCRα and β chains and the CD3 complex. After antigen recognition the CD45

phosphatase cleaves the inhibitory phosphate group on the Lck tyrosine kinase, and the thus activated Lck phosphorylates ITAM regions of the ζ chain of the TcR/CD3 complex. ZAP-70 gets activated after docking to the phosphorylated ITAM regions and phosphorylates the membrane-bound LAT and SLP-76 molecules. Various adapted and signalling molecules can attach to these complex, e.g. PLCγ1, the p85 regulatory subunit of PI3K, Grb2, Gads, Vav1, Itk, Nck, Shc and ADAP. Vav1 activates the MAPK/JNK pathway, while Itk activates the MAPK/ERK pathway through PLCγ1. ZAP-70 might contribute tot he MAPK activation through the p38α, independently of LAT and MAPK kinaeses As the result of the activation of the above mentioned pathways complex gene expression changes are initiated in the activated T cells, leading to cell proliferation and cytokine production. From the costimulatory molecules the CD28 contributes most significantly to the T cell receptor signalling after antigen recognition after binding to CD80/86 found on the surface of antigen presenting cells.

1.6 Rheumatoid arthritis

Rheumatoid arthritis is a systemic inflammatory autoimmune disease which primarily affects the synovial joints. Chronic inflammation leads to erosive joint damage and bone destruction, causing functional impairment and disability for patients. RA has an incidence of 0.5-1% in the Caucasian population and its prevalence is higher in females. Extraarticular symptoms affecting the lungs, skin, gastrointestinal tract and eye might accompany the synovial inflammation, with increased susceptibility to infections, atherosclerosis and vasculitis. The presence of autoantibodies is typical for RA, increased levels of rheumatoid factor (RF) and anti-citrullinated antibodies (anti-CCP, ACPA) are common serological alterations. In the developing synovitis significant leukocyte infiltration is visible, mostly comprised of T and B cells, granulocytes and dendritic cells. The proinflammatory cytokines and chemokines produced locally by these cells lead to the destruction of joint and bones. Abnormal activation of the complement system has been described in both RA and its animals models, a role of C5a anaphylatoxin and C3b opsonin in tissue damage has been hypothesized. Traditionally, RA has been considered a Th1 disease, but after the discovery of IL-17 an important role of Th17 and Th1/Th17 cells was proven.

1.7 Animal models of rheumatoid arthritis

Animal models play an important role in the study of the pathogenesis of RA. Most models are murine models, where arthritis might develop spontaneously or in an induced manner.

Among the spontaneous models K/BxN-, TNF-transgenic and SKG mouse models are the most characterized. In K/BxN mice uniform, severe inflammatory arthritis spontaneously develops at the age of 4 weeks, accompanied with the production of antibodies agains glucose-6-phosphate isomerase (GPI). With the transfer of serum from arthritic mice the disease can be transferred, the recipients develop arhritis (serum transfer model). TNF-transgenic mice overexpress the modified version of the gene coding for the human TNF-α. Mice develop symmetrical, erosive polyarthritis, in which the TNF-α expressed by cells of the synovium is the main contributory factor. In SKG mice the point mutation

of the ZAP-70 coding gene results in inadequate T cell receptor signalling, which disturbs the selection processes of thymocytes. As a result, autoreactive T cells reach the periphery, contributing to the development of autoimmune arthritis.

The most frequently used inducible arthritis model is the collagen induced arhritis (CIA), in which arthritis is induced with the repeated immunization of DBA/1 mice with type II collagen dissolved in complete Freud adjuvant (CFA). During our experiments, we used the recombinant human G1 (rhG1)-induced arthritis model (GIA), as it resembles human RA in both its clinical and immunological properties. To induce the GIA 4-5-month-old female mice with BALB/c background have to be immunized with the rhG1 antigen, which is a fusion protein consisting of the G1 domain of the human cartilage proteoglycan aggrecan and the Fc region of mouse IgG. The G1 domain contains three arthritogenic T cell epitopes, clearly showing the importance of T cells in the pathogenesis of the disease. The rhG1-induced arthritis is a T- and B cell-dependent process with autoantibody production (rheumatoid factor, anti-cyclic citrullinated protein). In GIA significant IL-17 and IFNγ production can be observed, suggesting that the induced arthritis shows significant Th1/Th17 polarization. Based on these clinical, histological and immunologival characteristics and the progressive and irreversible nature of the observed arthritis it can be concluded that GIA is suitable for the complex modelling of the human RA.

2 Objectives

ZAP-70 is a molecule indispensable for both T cell development and activation. Our group has previously investigated the role of ZAP-70 in the signalling through the T cell receptor, and in the non-genomic signalling pathways of glucocorticoids. A few years ago we have obtained the ZAP-70 knockout mouse strain, which enables us to conduct *in vivo* investigations regarding the ZAP-70 molecule. In our department we carry out studies using the GIA model of rheumatoid arthritis, in which, according to our knowledge the role of ZAP-70 has not been investigated yet.

During my work our aim was to conduct *in vivo* studies in ZAP-70 deficient mice.

In the first part of our investigations we studied T cell reconstitution in ZAP-$70^{-/-}$ mice in order to gain further insight into the role of ZAP-70 in the development of T cells and to see whether the severe combined immunodeficiency can be ameliorated using adoptive transfer of wild-type T cells.

1. Characterisation of the immunophenotype of ZAP-$70^{-/-}$ and ZAP-$70^{+/-}$ mice using histology and flow cytometry
2. Monitoring early and long-term changes in peripheral lymphoid organs and in the thymus after T cell reconstitution
3. Functional studies of transferred T cels
4. Investigation of the homing patterns of donor thymocytes to the thymus

In the second part of our work we studied the effect of the partial deficiency of ZAP-70 on the development of autoimmune arthritis in ZAP-$70^{+/-}$ mice.

1. Investigation of development and severity of GIA in ZAP-$70^{+/-}$ mice
2. Immunological characterisation of the induced autoimmune arthritis in ZAP-$70^{+/-}$ mice (cytokine patterns, autoantibodies, T cell responses)
3. Investigation of the effects of autoimmune arthritis and the partial absence of ZAP-70 on T cell activation and apoptosis

3 Materials and methods

3.1 Experimental animals

During our experiments we used BALB/c, ZAP-70 deficient and EF1α-GFP transgenic mice. All experimental animals were kept and bred in the transgenic mouse facility of the Department of Immunology and Biotechnology under conventional, non-SPF conditions Mice deficient for the ZAP-70 kinase (B6.129X1-Zap70tm1Weis/J) were purchased from Jackson Laboratories and backcrossed to BALB/c background. In the transfer experiments EF1α-GFP transgenic mice were used as donors. For the GIA experiments 4-5-month-old BALB/c and ZAP-$70^{+/-}$ female mice were used. All animal experiments were performed in accordance with the regulations set out by the Animal Welfare Committee of the University of Pécs (BA02/2000-3/2012, BA02/2000-48/2017).

3.2 Thymocyte transfer

For adoptive transfer experiments, homozygous ZAP-$70^{-/-}$ recipient mice were collected. As donors for thymocytes,we used either ZAP-$70^{+/+}$ littermates or GFP-transgenic mice. Briefly, thymi were isolated from donor mice and cells were released by mechanical dissociation then washed once in PBS. Recipients received 5–10x10^6 thymocytes with a single ip.injection at the age of 3–4 weeks. We took blood from the transferred animals regularly to check the appearance of $CD3^+$ T cells, which was the ultimate sign of the successful adoptive transfer. At the end of the experiments mice were sacrificed and we isolated the thymus, spleen, lymph nodes and Peyer's patches for histological and flow cytometric analysis. In some experiments, before the i.p. injection, we labelled the donor thymocytes with carboxyfluorescein succinimidyl ester (CFSE) in vitro, according to the manufacturer's instructions.

3.3 Flow cytometry

Cell surface markers of peripheral blood, lymph nodes, spleen and Peyer's patches were analysed by multi-colour flow cytometry, as described. Briefly, anti-coagulated blood was haemolyzed while the solid lymphoid organs were homogenized in PBS containing 0.1% sodium azide first, and then filtered. After washing with PBS containing 0.1% sodium azide and 01% BSA 106 cells were labelled with fluorochromeconjugated antibodies for 30 min in the dark; cells were then resuspended in PBS containing 1% paraformaldehyde. Data acquisition and analysis were performed on a FACS Canto II flow cytometer using FACS Diva Software. For the detection of regulatory T cells the intracellular staining was carried out after the cell surface staining using the FoxP3 Transcription Factor Staining Buffer Set, according to manufacturer's instructions. For the experiments we used the following antibodies: anti-CD3-APC-Cy7, anti-CD3-FITC, anti-TCRαβ -Alexa Fluor 700, anti-TCRγδ -BV 421, anti-CD4-PE.Cy5, anti-CD25-APC, anti-FoxP3-PE, anti-B220-PE-Cy7, anti-CD4-PE, anti-CD8-PE-Cy5.5.

3.4 Histology

Multiple immunofluorescent staining of frozen sections from thymi, spleens and peripheral lymph nodes was performed as described earlier. Acetone fixed, frozen sections were blocked for 20 min with PBS containing 5% BSA, followed by a 45-min incubation with the appropriate antibody cocktails (anti-CD3-FITC, anti-B220-Alexa Fluor 647, anti-CD4-PE, anti-CD8-FITC, anti-Ly51-PE, anti-EpCAM1-FITC). After washing with PBS, samples were mounted and analysed. For immunohistochemistry, frozen thymus sections were blocked with 5% BSA in PBS. Then, samples were treated with 1 mg/ml phenylhydrazine in PBS for 10 min. After 45-min incubation with unlabelled anti-EpCAM1, rat anti-mouse mAb samples were washed with PBS and anti-rat Ig peroxidase was used as secondary antibody for 1 h at room temperature. The sections were developed by DAB colour reaction. Immunofluorescent and immunohistochemical samples were analysed using an Olympus BX61 fluorescent microscope. The acquisition of digital images was performed with a CCD camera using analySIS software. Omenta were processed for whole-mount immunohistochemistry as described. Briefly, harvested omenta were fixed in 4% paraformaldehyde and, after washing with 01% saponin in PBS, were blocked with PBS containing 5% BSA and 0.1% saponin for 2 h. After washing three times for 20 min, samples were incubated with rabbit anti-fibronectin at 4°C overnight, then washed. Anti-rabbit-Cy3 was used as secondary antibody for 3 hours at 4°C. Samples were viewed using an Olympus Fluo-View FV-1000 laser scanning confocal imaging system.

3.5 T cell isolation and *in vitro* activation

T cells were isolated from the spleens of wild-type (WT) or transferred mice using the EasySepTM mouse T cell isolation kit, according to the manufacturer's instructions. Purified T cells were then stimulated in vitro using MACSiBeadTM particles loaded with CD3 and CD28 antibodies for 48 h (bead/cell ratio 2:1). Stimulated T cells were then divided into to: proliferation capacity of the stimulated T cells (20 000 cells/well in a 96-well plate) was assessed using Promega CellTiter 96® Non-Radioactive Cell Proliferation Assay, according to the manufacturer's instructions and the other half of the samples were processed for western blotting.

3.6 Western blot

Cells were lysed in Triton lysis buffer (50 mM HEPES, 10 mM Na-pyrophosphate, 10 mM EDTA, 100 mM Na-fluoride, 10 % glycerol, 1 % Triton X) complemented freshly with protease inhibitor and Na-orthovanadate. After centrifugation (3000 rpm for 10 min), supernatants were boiled immediately in sodium dodecyl sulfate (SDS) sample buffer. Samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), where we used the following gel concentrations: 7.5 % (for Cbl), 10% (for phosphotyrosine, Caspase-9) and 15% (for Bim, Caspase-3 and 8, Bcl-2, Cytochrome C). The gels were blotted for two hours to nitrocellulose membranes using Mini Trans-Blot Cell blotting equipment. After blotting, nitrocellulose membranes were incubated with the recommended blocking buffer (2 or 5 % BSA, 5% NFDM) and then incubated with the primary

antibodies. Blots were developed with the appropriate peroxidase-conjugated secondary antibody. Anti-β-actin was used as loading control. Western blots were visualized using enhanced chemiluminescent reagent (SuperSignal West Femto Chemiluminescent substrate) as described in the manufacturer's instructions. Luminescent light signals were detected with Fujifilm LAS 4000 blot imaging system.

3.7 Arthritis induction

For induction of arthritis 4-5-month-old BALB/c or ZAP-70$^{+/-}$ female mice were immunized three times every three weeks intraperitoneally (ip.) with the mixture of 40 μg rhG1 antigen and dimethyl-dioctadecyl-ammonium (DDA) adjuvant dissolved in PBS. After the second immunization the development and severity of arthritis was monitored using a clinical scoring system. Mice were sacrificed 3 weeks after the third immunization, sera and spleen were collected. Autoantibody and cytokine levels were determined from the serum, spleen cells were used to start *in vitro* cell cultures, to perform flow cytometric measurement and in some experiments for T cell activation studies.

3.8 *In vivo* bioluminesce imaging

For *in vivo* imaging anaesthetized mice were injected intraperitoneally with 20 mg/ml luminol in PBS. The myeloperoxidase enzyme originating from neutrophils oxidates luminol, resulting in the emission of blue light (λmax = 425 nm). Pictures were acquired 10 minutes after injection of luminol using an IVIS Lumina II machine.

3.9 Intracellular cytokine measurement

For the intracellular cytokine measurements 10^6 cells were stimulated with PMA/ionomycin for 12 hours with the addition of Brefeldin. Intracellular amounts of IL-17 and IFN-γ cytokines were determined using flow cytometry, using the protocoll described in section 3.3.

3.10 *In vitro* spleen cell culture

In vitro cell cultures were established from the isolated spleens of experimental animals on a 48-well plate ($1.8x10^6$ cells in DMEM + 10% FCS cell culture medium) and cells were cultured with/without the addition of 1,5 μg rhG1 antigen for 5 days. Supernatants were collected and frozen for later ELISA experiments.

3.11 Proliferation

Another part of cells isolated from the spleen of immunized animals was cultured on a 96-well plate with/without the addition of rhG1 antigen for 5 days ($3x10^5$ cells/well in triplicates in DMEM + 10% FCS cell culture medium). The rate of proliferation was measured using the Promega CellTiter 96® Non-Radioactive Cell Proliferation Assay.

3.12 ELISA measurements

Concentration of cytokines (IL-4, IL-6, IL-17, IFNγ, TNFα) in sera and supernatants of in vitro spleen cell cultures were determined using sandwich ELISA, according to the manufacturer's instructions. Serum-concentration of autoantibodies specific for rhG1 antigen was determined using ELISA, as well. We coated 96-well ELISA plates with rhG1 antigen (0,1 μg/well in 100 μl carbonate coating buffer) at room temperature overnight. Plates were blocked using 200 μl/well 1,5% NFDM in PBS for 1 hour and washed five times with 300μl PBS-Tween (0,5% Tween in PBS) solution. Sera were incubated for 2 h (100 μl/well) on the plates, followed by washing with 5x300μl PBS-Tween. As secondary antibody we used anti-IgG1-peroxidase antibody (2 h, room temperature) then the plates were developed using ortho-phenylenediamine chromophore and hydrogen-peroxide substrate.

3.13 Statistical methods

Statistical analysis of the data was performed using the GraphPad software. Statistical significance was determined using the unpaired, two-sample Student's t-test, where $P<0.05$ was considered significant. Data is presented as mean ± SEM (standard error of mean).

4 Results

4.1 Characterisation of the ZAP-70 knockout mouse model

4.1.1 ZAP-70 expression in ZAP-70 deficient mice

We investigated the expression of ZAP-70 fehérje using Western blot and flow cytometry. In the lymph nodes and spleen of ZAP-$70^{+/+}$ and ZAP-$70^{+/-}$ mice ZAP-70 is detectable, while in the samples of ZAP-$70^{-/-}$ mice it is not expressed according to our expectations. After quantification of our results, we proved that the expression of ZAP-70 in ZAP-$70^{+/-}$ mice is approximately the half of that seen in ZAP-$70^{+/+}$ mice.

4.1.2 Cellular composition of peripheral lymphoid organs

The cellular composition of blood, peripheral lymphoid organs and thymi of ZAP-70 knockout mice was investiated using flow cytometry. As expected,there are virtually no T cells in the organs of ZAP-$70^{-/-}$ mice in comparison to ZAP-$70^{+/+}$ controls, while the ratio of B cells increased significantly. In ZAP-$70^{+/-}$ animals T cells can be detected in peripheral lymphoid organs, but their ratio is significantly decreased in comparison to ZAP-$70^{+/+}$ controls, with the ratio of B cells significantly increased, however not as remarkably as in ZAP-$70^{-/-}$ mice. In the thyimi of ZAP-$70^{+/-}$ and ZAP-$70^{+/+}$ mice, cellular composition was similar, less, than 1% of thymocytes was DN, the majority was DP, the ratio of CD4 SP cells was 7.4±0.4%, that of CD8 SP was 2.3±0.2% in the ZAP-$70^{+/+}$ micel while 9.8±0.9% and 2.4±0.3%. in the ZAP-$70^{+/-}$, respectively. In thymi of ZAP-$70^{-/-}$ mice nearly all thymocytes are DP (99.9±0.1%), the ratio if DN is similar that of wild-type, however the SP cells are missing entirely. We also studied the ratio of T_{reg} cells in the lymphoid organs and blood of ZAP-$70^{+/-}$ and ZAP-$70^{+/+}$ mice.

There was no significant difference in the T_{reg} ratios in the thymus and blood of ZAP-$70^{+/-}$ and ZAP-$70^{+/+}$ mice, however in the lymph nodes and spleen of ZAP-$70^{+/+}$ mice the ratio of T_{reg} cells was found to be higher, than in ZAP-$70^{+/-}$mice.

4.1.3 Histology

In the spleen of ZAP-$70^{+/+}$ mice follicules with B cells and PALS regions formed by T cells are well visible. Despite the reduction in T cell ratios in the spleen of ZAP-$70^{+/-}$ mice PALS regions are present, the morphology of similar to that of ZAP-$70^{+/+}$. In contrast, in the spleen of ZAP-$70^{-/-}$ mice the PALS regions are missing, there are no T cell zones next to the central arterioles, however the B cells form follicles here, too. In the inguinal lymph nodes of ZAP-$70^{+/-}$ mice the paracortical T cell zone is present, but it contains less cells and is less structured, as ZAP-$70^{+/+}$ lymph nodes. In ZAP-$70^{-/-}$ lymph nodes the few T cells that are present does not form an ordered structure in the paracortex. In contrast the changes in the mesenteric lymph nodes are less remarkable, in ZAP-$70^{+/-}$ samples significant amount of T cells is present, the morphology is similar to wold-type. In ZAP-$70^{-/-}$ mesenteric lymph nodes a quite a few T cells are present, however these form no structured T cell zone. In the thymi of ZAP-$70^{+/+}$ and ZAP-$70^{+/-}$ mice the cortex, containing the DP cells and the medulla, where the SP cells are is well separated. In the thymus of ZAP-$70^{+/-}$ mice the size of the medulla is reduced, in ZAP-$70^{-/-}$ samples the medulla is entirely missing, which is in line with the flow cytometry findings. In parallel to the changes observed in the ratio of thymocytes, we detected changes in the thymic epithelial cell distribution, as well. In the thymi of ZAP-$70^{-/-}$ mice medullary epithelial cells are present only in small numbers,the tissue is mainly filled with cortical epithelial cells. In ZAP-$70^{+/-}$ mice the medulla and the cortex is well devided, however the area filled with medullary epithelial cells is smaller, than that seen in ZAP-$70^{+/+}$ thymi.

4.1.4 T cell activation and apoptosis

T cells originating from ZAP-$70^{+/-}$ mice show a decreased, altered tyrosine phosphorylation pattern following anti-CD3/CD28 stimulation for both 24 and 48 hours in comparison to ZAP-$70^{+/+}$ controls. The presence of cleaved caspase-3 shows the activation of apoptotic pathways. In the stimulated T cells of ZAP-$70^{+/-}$ mice less cleaved caspase-3 can be detected after both 24 and 48 hours, than in that of BALB/c mice. The cleaved caspase-8 is the initiator caspase of the extrinsic pathway. After 24 hours of stimulation, in BALB/c T cells the caspase-8 activation decreased slightly, however in T cells of ZAP-$70^{+/-}$ mice this difference was more remarkable. After 48h of stimulation this difference disappears, the amount of cleaved capsase-8 decreases in non-stimulated BALB/c T cells. We detected the activation of intrinsic apoptotic pathway using the cleaved caspase-9. In the T cells of ZAP-$70^{+/-}$ mice the level of caspase-9 is significantly higher after both 24 and 48 hours, than in the T cells of BALB/c mice. In the T cells of BALB/c mice after 48 hours the level of cleaved caspase-9 increases, however in the T cells of ZAP-$70^{+/-}$ mice the level of caspase-9 is unchanged after stimulation.

4.1.5 T cell reconstitution in ZAP-70$^{-/-}$ mice

First, for follow-up, we monitored the ratio of T cells in the peripheral blood: 10 days after the i.p. injection of thymocytes T cells had already appeared in the blood (4%), and their ratio remained steady at between 4 and 6% during the 120 days of the monitoring. Four months after the i.p. thymocyte transfer, we sacrificed the recipient mice and performed a histological analysis of the peripheral lymph nodes and spleen which showed that, indeed, T cells appeared in the periphery, as the organs of mice receiving i.p. thymocyte transfer showed restored T cell areas in contrast to ZAP-70$^{-/-}$ mice, where no PALS was seen in the spleen and lymph nodes also lacked defined T cell zones.

Thymocyte subpopulations were assessed 4–8 months after the transfer. We observed that, compared to the ZAP-70$^{-/-}$ mice, where virtually all thymocytes are DP, after transfer the thymocyte composition shifted: the ratio of DP thymocytes decreased from 99.0 ± 0.7% to 90.5 ± 3.7%, accompanied by a significant increase in CD4$^+$ and CD8$^+$ SP thymocytes.

To study the long-term effects of the transfer in several cases we monitored mice up to 12 months after the transfer and found that the T cell reconstitution remained stable even for these extended periods of time, which suggested the successful reconstitution of T cell development. The normalized lifespan of ZAP-70$^{-/-}$ mice, which are otherwise seriously immunocompromised and susceptible to early death due most probably to infections, was further important evidence for the success of T cell repopulation.

4.1.6 Early changes in the thymus after thymocyte transfer

Upon i.p. thymocyte transfer, we observed changes already in the thymocyte composition as early as 10–17 days. The ratio of DN thymocytes elevated from aránya 0.8±0.1% to 4.3±1.1% by day 17 while, simultaneously, the ratio of DP cells dropped from 99±0.7% to 94.7±1.1%. In parallel, the ratio of mature SP cells started to increase after day 4, and reached a peak at day 17 in both the CD4$^+$ (0.6±0.1%), and CD8$^+$ (0.5±0.1%) SP subpopulations, indicating the restoration of thymic T cell development. Interestingly, the ratios reverted to the starting point in all thymocyte subpopulations by day 24. These results have raised the question of whether the observed changes were also mirrored by changes in the morphology of the thymus.. Seventeen days after the i.p. thymocyte transfer, patches of reorganized medullary regions appeared. Quantitative measurements revealed that the area of the medullary regions changed parallel to that detected in the thymocyte subpopulations: i.e. an initial expansion of the medullary area until day 17 (from 1.3 ± 0.17 to 2.05 ±0.27) was followed by a reduction in the size of the medulla (1.17 ± 0.12).

4.1.7 Transfer of thymocytes expressing the GFP transgene

To confirm that the above-described T cell repopulation after i.p. thymocyte transfer in ZAP-70$^{-/-}$ mice was indeed due to the development of stable chimerism, in the next experiments we used thymocytes derived from GFP-transgenic mice. The ratio of T cells in peripheral blood was 31.6± 6.4%- (from which 83.5 ±3.9% GFP$^+$), and 27.1 ± 4.4% (from which 78.5 ± 6% GFP$^+$) at weeks 7 and 12

weeks after the transfer, respectively. We investigated the cellular composition of the peripheral lymphoid organs of reconstituted mice and found that the percentage of T cells ranged between 16 and 24% in peripheral lymph nodes, mesenteric lymph nodes, spleen and Peyer's patches. Approximately 70% of the T cells were present in lymph nodes, while 87 and 90% of the T cells in the Peyer's patches and spleen were GFP^+ donor cells, respectively. Fluorescent microscopic images supported the flow cytometric findings, as GFP^+ cells could be visualized only in the T cell-rich PALS areas of the spleen, but not in the B cell follicles. We confirmed strong GFP expression in thymocytes isolated from the i.p. transferredrecipients using reverse transcription–polymerase chain reaction (RT–PCR), but only very low expression in the bone marrow of the same mice.

4.1.8 Peritoneal homing pattern of thymocytes

The next interesting question regarded which pathways the injected thymocytes use to exit from the peritoneum. To address this, we injected CFSE-labelled thymocytes intraperitoneally. After analysing various organs in the peritoneal cavity and the draining lymph nodes (mediastinal, inguinal, axillary) 12, 24, 48 and 72 h after the transfer, we identified the omentum as the main collection site for thymocytes, as 45% of the lymphocytes isolated from the omentum were of donor origin ($CFSE^+$). Interestingly, the majority of these $CFSE^+$ lymphocytes were of the DN (86%) phenotype. Whole mount images have shown that the $CFSE^+$ donor cells were not scattered randomly in the omentum, but they formed aggregates at specific sites. To identify these specific entry zones, we performed immunofluorescent staining with a fibronectin-specific antibody, which revealed that the $CFSE^+$ cell aggregates were localized in the lymphoid compartments of the omentum (known as 'milky spots').

4.1.9 Functional tests

To test the functional properties of transferred tests, we performed *in vitro* T cell activation tests on isolated T cells from transferred mice. We assessed cell proliferation and activation of donor T cells upon anti-CD3/CD28 stimulation. We could detect similar tyrosine phosphorylation patterns and proliferative capacity in the T cells from transferred mice as in T cells deriving from WT mice. These data showed clearly that the activation and proliferation of the donorderived T cells was normal.

4.2 Investigation of recombinant human G1-induced arthritis (GIA) in partially ZAP-70 deficient mice

4.2.1 Clinical picture of autoimmune arthritis

We immunized normal control ($ZAP\text{-}70^{+/+}$) and partially ZAP-70 deficient ($ZAP\text{-}70^{+/-}$) mice to induce GIA. The two groups of mice developed GIA with similar time kinetics: significant elevation in the severity score was observed a week after the third immunization. Importantly, partially ZAP-70 deficient mice showed similar clinical scores to the controls in the early stages of the experiment, however, after day 52 we observed significantly milder arthritis in the $ZAP\text{-}70^{+/-}$ group. We did not see any differences in the incidence of arthritis when we compared the $ZAP\text{-}70^{+/-}$ and $ZAP\text{-}70^{+/+}$ groups,

apart from some insignificant variations during the immunization period, both groups reached 100% incidence one week after the third immunization. To objectively quantify the severity of paw inflammation, we performed *in vivo* bioluminescent imaging. In accordance with the clinical scores, in the hindlegs of the arthritic ZAP-70$^{+/-}$ mice myeloperoxidase activity was significantly reduced in comparison to arthritic ZAP-70$^{+/+}$ mice . However, arthritic mice in both groups showed clearly higher luminescence than the healthy controls.

4.2.2 Antigen-specific proliferation, cytokine- and autoantibody production

Based on the clinical differences, next we compared the immune responses of the ZAP-70$^{+/-}$ and ZAP-70$^{+/+}$ mice to the G1 antigen. Spleen cells isolated from arthritic ZAP-70$^{+/-}$ mice proliferated at a significantly decreased level after rhG1 stimulation, than the cells of arthritic BALB/c. ZAP-70$^{+/-}$ spleen cell cultures stimulated with rhG1 antigen produced less IL-4, IL-6, IL-17 and IFNγ than the controls, while TNF-α levels were approximately the same in arthritic BALB/c and ZAP-70$^{+/-}$ supernatants. The level of G1-specific antibodies was decreased in the serum of ZAP-70$^{+/-}$ mice, however the difference was not statistically significant. The serum levels of IL-17 and IL-4 were similar in the two groups, however IL-6 was present in increased amounts in the serum of arthritic ZAP-70$^{+/-}$ mice.

4.2.3 Identification of helper T cell subgroups using intracellular cytokine measurements

We compared the intracellular cytokine production of healthy and arthritic T cells from ZAP-70$^{+/-}$ and control mice after PMA/ionomycin stimulation using flow cytometry. In arthritic mice we detected elevated percentage of IFNγ-producing T cells than in healthy mice (in arthritic BALB/c 17.88±0.25%, in arthritic ZAP-70$^{+/-}$ 13.43±3.64% vs. in healthy BALB/c 5.15±0.72% and in healthy ZAP-70$^{+/-}$ 6.42±0.25%, respectively). When comparing the arthritic mice, a significantly higher percentage of CD4^{+} T cells from BALB/c mice produced IFNγ (17.88±0.25%) than those from ZAP-70$^{+/-}$ mice (13.43±3.64%). Similarly, from arthritic mice, more IL-17-producing CD4+ T cells could be detected than from healthy animals, however, in case of the IL-17, no prominent difference was observed between BALB/c and ZAP-70$^{+/-}$ mice either arthritic or healthy.

4.2.4 Alterations in apoptotic and activation patterns of partially ZAP-70 deficient mice

Since ZAP-70 plays an important role in the activation of T cells, finally, we wanted to investigate whether the differences in the clinical picture observed in the partial absence of ZAP-70 could arise from the altered activation/apoptosis of T cells. According to the Western-blot analysis of tyrosine-phosphorylation patterns, T cells from healthy BALB/c or ZAP-70$^{+/-}$ mice activated similarly as a result of *in vitro* anti-CD3/anti-CD28 stimulation. However, in the T cells of arthritic BALB/c and ZAP-70$^{+/-}$ mice we observed a more pronounced tyrosine phosphorylation after stimulation than in the healthy T cells. Importantly, the T cells deriving from the arthritic ZAP-70$^{+/-}$ mice showed decreased tyrosine-phosphorylation compared to the arthritic BALB/c.

Since activation-induced cell death is critical to down-regulate the immune responses, and its dysregulation is thought to be in the background of some autoimmune pathologies, next, we checked the molecular components of the apoptotic cascade. We used cleaved Caspase-3 as a general apoptotic marker, cleaved Caspase-8 as a marker of activation of the extrinsic pathway and cleaved Caspase-9 as a marker of the intrinsic pathway. In the stimulated T cells of arthritic BALB/c mice cleaved Caspase-3 was present in lower amounts as in healthy BALB/c mice, however in T cells of healthy and arthritic ZAP-$70^{+/-}$ mice we observed similar levels of activated caspase-3. Importantly, when we compared the arthritic mice groups, the amount of cleaved Caspase-3 was lower in stimulated BALB/c T cells than in the cells from ZAP-$70^{+/-}$ mice. Cleaved Caspase-8 was present in all samples, however while in arthritic T cells its amount decreased after stimulation, in T cells of healthy BALB/c mice it remained unchanged and in healthy ZAP-$70^{+/-}$ T cells its expression increased after stimulation. In T cells of both healthy and arthritic BALB/c mice the amount of cleaved Caspase-9 increased after stimulation with the highest expression seen in stimulated arthritic T cells. In healthy ZAP-$70^{+/-}$ T cells the levels of cleaved Caspase-9 did not change as a result of stimulation, the signal intensity was similar to that of arthritic, non-stimulated ZAP-$70^{+/-}$ T cells. In arthritic, ZAP-$70^{+/-}$ T cells the activation of Caspase-9 increased slightly after anti-CD3/anti-CD28 stimulation, but did not reach the level observed in arthritic stimulated BALB/c T cells.

Cytochrome C is released to the cytoplasm from the mitochondria as a result of the activation of the intrinsic pathway of apoptosis. In T cells of healthy BALB/c mice independently of stimulation the levels of Cytochrome C are similar in the cytoplasm, while in healthy ZAP-$70^{+/-}$ T cells, interestingly, we could observe higher amounts in the non-stimulated samples. On the other hand, in arthritic samples, stimulation triggered the release of Cytochrome C, it should be noted though, that non-stimulated samples of arthritic T cells contained less Cytochrome C than healthy non-stimulated T cells.

Bcl-2 is an anti-apoptotic protein, exerting its effects in the intrinsic pathway by neutralizing pro-apoptotic proteins. In T cells isolated from BALB/c mice Bcl-2 could only be detected after stimulation, which was even more prominent in arthritic samples. In contrast, in T cells of healthy ZAP-$70^{+/-}$ mice, Bcl-2 was only present in non-stimulated samples in small amount, but in arthritic ZAP-$70^{+/-}$ T cells stimulation increases the expression of Bcl-2, which, however did not reach the level detected in T cells of arthritic BALB/c mice.

Bim is a pro-apoptotic member of the Bcl-2 family, a key player in the intrinsic apoptotic pathway. During our experiments we investigated the BimEL and BimL (23 and 15 kDa, respectively) isoforms of Bim. Both isoforms were present at varying levels in all samples after 72h of anti-CD3/anti-CD28 stimulation, however we could detect BimEL in higher amounts. As a result of stimulation, the amount of BimEL elevated in all sample pairs, although the change was more pronounced in arthritic samples. The increase resulting from stimulation was more robust in arthritic BALB/c T cells in comparison to the healthy cells. Contrary to this, in ZAP-$70^{+/-}$ T cells, there was no significant difference in healthy and arthritic T cells regarding the BimEL induction. BimL was detected in all samples, as

well, although the changes in its level resulting from stimulation were not as pronounced as in the case of BimEL, with the exception of arthritic BALB/c T cells, where the increase observed in BimL levels after stimulation were the most robust observed in both isoforms.

Finally, Cbl-b is a negative regulator of ZAP-70, it plays a role in blocking T cells activation and stopping the signaling through the T cell receptor. The amount of Cbl-b increased upon activation in T cells isolated from healthy and arthritic BALB/c mice, to a similar extent. While in healthy ZAP-$70^{+/-}$ T cells we could not observe any Cbl-b expression even after stimulation, in arthritic ZAP-$70^{+/-}$ T cells Cbl-b appeared in the non-stimulated samples and expression increased upon stimulation, however, it did not reach the level of that seen in stimulated arthritic BALB/c T cells.

5 Discussion

5.1 Correction of T cell immunodeficiency with adoptive transfer of thymocytes

In our present work, we demonstrated that, with the ip. adoptive transfer of ZAP-$70^{+/+}$ (WT) thymocytes, T cells can be stably reconstituted, thus correcting the immunodeficiency in ZAP-$70^{-/-}$ mice. Here, we have shown that with a single, simple ip. injection of 5-10 million thymocytes from WT (ZAP-$70^{+/+}$) donor mice corrected the T cell deficiency effectively on the long run. The correction of the immunodeficiency was clearly shown by the significantly longer survival of transferred ZAP-$70^{-/-}$ animals: ZAP-$70^{-/-}$ mice usually have a short life span (see above), however, some of the transferred animals were alive even 8-10 months after the ip. injection. The efficacy of long-term T cell reconstitution was confirmed by the presence of mature αβ T cells up to several months after the transfer in blood and peripheral lymphoid organs, with the majority expressing TCRαβ. Histological analysis revealed that the lymph node and spleen microstructure in transferred animals was similar to WT, implicating that transfer-originated T cells were able to restore the disrupted morphology of ZAP-$70^{-/-}$ peripheral lymphoid organs to normal. Furthermore, using GFP-transgenic thymocytes we could clearly demonstrate that the T cell repopulation was donor-derived.

The long-term stability of our chimeras suggested a continuous T cell production; thus, the possibility of thymic repopulation was also investigated. We observed SP thymocytes appearing in the thymus of ZAP-$70^{-/-}$ recipient mice already 17 days after the ip. injection of WT thymocytes, accompanied by an increase in the area of the medullary region indicating that the thymic T cell development was reset by the treatment. Cross-talk between thymic epithelial cells and thymocytes during T cell development have been studied extensively. Here, the re-organization of the medullary region took place after ip. thymocyte transfer which was in line with a previous study. In another study, SP cells appeared in the thymus with similar kinetics, i.e. 3 weeks after intrathymic electroporation of ZAP-70 coding plasmids. As ZAP-70 deficient host thymocytes suffer a developmental block at the DP stage, the appearance of SP cells suggests that they originated from the donor ZAP-$70^{+/+}$ thymocyte population. The continuous increase in the ratio of T cells in peripheral blood, following the appearance

of SP cells in the thymus with a lag (peak at day 21) suggested that these SP cells populated the peripheral lymphoid organs successfully. By day 21, αβ/γδ T cell ratios in peripheral lymphoid organs resembled WT, providing a further proof of donor thymocytes being able to repopulate host lymphoid organs in the form of mature, αβ T cells. Interestingly, the changes observed in the thymus (appearance of SP cells and medullary islets) became more pronounced 4-8 months after ip. transfer than in the first month, although the composition of thymocytes was still far from WT (data not shown). However, similar percentages of SP thymocytes were reported by others.

Overall, the emergence of SP thymocytes and medullary epithelial cells suggested that in the first month following the i.p. thymocyte transfer, a wave of T cell development took place, which was followed by long term thymic recovery which generated T cells for several months. As these findings implied that the injected thymocytes were capable of entering the thymus we also analyzed which route the thymocytes could have used to leave the peritoneum. Although it was described earlier that the mediastinal and ipsilateral inguinal lymph nodes drain the peritoneal cavity after ip. transfer of cells, in our experiments we have observed no CFSE-labeled donor cells in these localizations (data not shown). Contrary to this, significant $CFSE^+$ cell aggregates were present in the omental milky spots, which were already described as gateways for trafficking of B2-B cells, dendritic cells, as well as preferential sites for tumour cell adhesion. Milky spots are covered by a discontinuous layer of mesothelial cells. These intracellular gaps, too might serve as entry sites for ip. injected cells homing via lymphatic vessels and blood capillaries. We have observed that the majority of $CFSE^+$ cells in the omentum were the most immature $CD4^-CD8^-$ DN thymocytes, which might suggest that this population was the main source of donor thymocytes that entered the host thymus and supported the development of mature SP thymocytes.

Based on these results, we propose the following mechanism of the T cell recovery in ZAP-$70^{-/-}$ mice after WT thymocyte transfer. When we injected thymocytes into the peritoneal cavity of recipient mice, we transferred a mixed cell population containing approximately 3-5% DN, 75-80% DP, 10-15% CD4 SP and 5-10% CD8 SP cells. This means that both immature (DN and DP) and mature SP cells entered the recipient. We hypothesize that this mixed cell population repopulated the recipient's lymphoid organs in a complex manner. First, mature SP cells have most likely the capacity to enter peripheral lymphoid organs/tissues where they could augment the immune response. This peripheral homeostatic expansion could be beneficial especially in the early stages of the transfer to recapitulate the immune response (at least partially) and promote survival. On the other hand, immature cells could have the potential to repopulate primary lymphoid organs and provide a long term, stable T cell production for several months after the transfer. Since DP cells are extremely sensitive to apoptosis, in our view, they might not play a key role in restoring the T cell development. Instead, it is more likely, and also supported by the aggregation in the omentum, that the DN, most immature T cells reached the thymus and/or the bone marrow and they colonized these organs. Since the recipient's own T cell progenitors do not have the capacity to mature into SP cells, there is a selective survival advantage of the ZAP-$70^{+/+}$ donor cells.

In summary, we demonstrated that ip. injected ZAP-$70^{+/+}$ thymocytes were able to enter the thymus of ZAP-$70^{-/-}$ mice, reorganize its morphology and restore T cell development. Mature, donor-originated αβ T cells were present in the peripheral blood and lymphoid organs, where they were organized into structured T cell zones. The established chimerism was stable on the long-run, as the immunodeficiency of ZAP-$70^{-/-}$ mice was resolved, as shown by the long term survival of mice after thymocyte transfer.

5.2 Alterations of the T cell activation/apoptosis signalling pathways in the autoimmune murine model of arthritis

Here, we successfully induced arthritis in ZAP-$70^{+/-}$ mice, by the end of the experiment the incidence was similar to that of BALB/c mice, although the severity of articular inflammation was reduced based on clinical scores, supported by in vivo imaging, as well. In line with the less severe clinical picture, the rhG1-specific immune responses in arthritic ZAP-$70^{+/-}$ mice showed significant alterations in comparison to control mice: in the in vitro spleen cell cultures cellular proliferation rates were significantly reduced and the production of IL-4, IL-6, IL-17 and IFNγ decreased. When we analyzed the Th1/Th17 polarization, which are prominent in GIA, we found that the ratio of IL-17 producing CD4+ T cells was similar in arthritic BALB/c and ZAP-$70^{+/-}$ mice, but the we found a reduction in the ratio of $IFN\gamma^+$ $CD4^+$ T cells in ZAP-$70^{+/-}$ animals. Based on these results, the partial deficiency of ZAP-70 influenced the Th1/Th17 polarization as well, in contrast to the Th1/Th17 intermediate form typical for the GIA model, in ZAP-$70^{+/-}$ mice production of IL-17 seems to be more characteristic with a reduced ratio of $IFN\gamma^+$ cells.

Surprisingly, in the sera, the levels of inflammatory cytokines (IL-6, IL-17) were similar in arthritic and ZAP-$70^{+/-}$ mice. However, serum cytokine data is difficult to interpret in many cases since we measure the sum of cytokine amounts produced by multiple types of immune cells taking part in the systemic inflammatory response in the whole body. However, the amount rhG1-specific antibodies was clearly lower (although not statistically significant) in the partial lack of ZAP-70. This suggests that the cooperation of T cells and B cells was still sufficient in ZAP-$70^{+/-}$ animals and the role of B cells in autoimmune arthritis remains unaltered in the partial absence of ZAP-70.

Based our current and previous results we hypothesize that the alterations seen in the clinical picture and the laboratory parameters might be consequences of the molecular alterations of apoptotic and activator pathways in T cells resulting from the partial deficiency of ZAP-70. Therefore, finally, we focused on various molecules of apoptotic pathways in T cells isolated from healthy and arthritic mice in the presence/absence of anti-CD3/anti-CD28 stimulation.

First, based on tyrosine phosphorylation levels the T cells from arthritic BALB/c mice showed higher levels of activation after anti-CD3/anti-CD28 restimulation than T cells from healthy animals. This might be the result of the increased ratio of activated effector T cells in arthritic mice, that are characterized by robust tyrosine phosphorylation after stimulation through the T cell receptor. In

arthritic ZAP-70$^{+/-}$ mice tyrosine phosphorylation also increased, although slightly weaker than that seen in BALB/c controls, upon anti-CD3/anti-CD28 stimulation, which might suggest that those cells which are capable of becoming efficiently activated even with a reduced ZAP-70 expression might have been "selected" for survival during the induction of GIA. It has to be noted though, that using the anti-phospho-tyrosine antibody we can only detect changes in the overall pattern of tyrosine phosphorylation, which not only shows activation processes, but also alterations in the phosphorylation of negative regulators of activation or even molecules involved in cell death signaling might be detectable.

The resolution of T cell activation is part of the physiological immune response, and one of the regulators of this process is the Cbl-b molecule, which is part of the Cbl family of proteins, expressed mostly by peripheral T cells. Cbl-b down-regulates signaling through the T cell receptor: it blocks the activation of ZAP-70, Vav1, PLCγ 1 and PKC-θ, thus contributing to the development of T cell anergy. As expected, in the T cells of healthy BALB/c mice the amount of Cbl-b increased after anti-CD3/anti-CD28 restimulation. Surprisingly, in ZAP-70$^{+/-}$ T cells from healthy animals, Cbl-b was undetectable even after stimulation. This might be explained by the fact that ZAP-70$^{+/-}$ T cells are less activated after anti-CD3/anti-CD28 stimulation than BALB/c T cells, thus the negative inhibition by Cbl-b is not needed to suppress activation. The phosphorylation of Cbl-b changes in parallel with that of SLP-76 and as SLP-76 is phosphorylated by ZAP-70, a reduced expression of ZAP-70 might lead to lower phosphorylation levels in both molecules. Nevertheless, as ZAP-70 has a direct contact with Cbl-b as well, thus alterations in its expression levels might alter the expression of Cbl-b, too. It was described that in Cbl-b deficient mice the T cell tolerance is disturbed, resulting in more severe collagen induced autoimmune arthritis in comparison to wild-type controls even in the absence of adjuvant. In our GIA model the amount of Cbl-b in arthritic T cells from BALB/c mice was similar to that of healthy animals, similar to what was described in a human study with T cells of RA patients. Interestingly, Cbl-b was induced in the T cells of arthritic ZAP-70+/- mice. We hypothesize, that this could be due to the in vivo selection of activated effector cells.

Apoptosis resistance of T cells was described both in RA patients and animal models of arthritis, which in addition to the continuous T cell activation might contribute to the development of autoimmunity. Similar to this, we have also found, that in T cells isolated from arthritic BALB/c mice, apoptosis was reduced based on cleaved Caspase-3 expression. In contrast, in arthritic ZAP-70$^{+/-}$ T cells apoptosis levels were similar to healthy controls, higher amounts of cleaved Caspase-3 were observed than in arthritic BALB/c T cells. These results might help to explain the differences in the severity of arthritis of BALB/c and ZAP-70$^{+/-}$ mice: when apoptosis decreased, activated T cells persisted in BALB/c mice, which kept the inflammation active and contributed to the more pronounced tissue injury, whereas in the partial absence of ZAP-70 the apoptotic processes remained close to normal. The extrinsic apoptotic pathway is an important mechanism of the activation induced cell death. Interestingly, in the T cells of arthritic mice we could not detect cleaved Caspase-8 after *in vitro* anti-CD3/CD28 stimulation. This is in line with the previously found reduced level of apoptosis in BALB/c

mice (see the previous paragraph). We hypothesize, that, in arthritic mice, activated T cells are supposedly resistant of activation induced cell death. In a similar mouse model, using human proteoglycan aggrecan to induce arthritis it was described that in the T cells of immunized BALB/c mice reduced activation induced cell death was observable after in vitro anti-CD3 stimulation, as an altered expression of FLIP inhibited the relocation of Caspase-8 to the DISC. On the other hand, in arthritic, but non-stimulated T cells significant amounts of cleaved Caspase-8 was detectable. This might be explained by the method of arthritis induction – the three immunizations with the antigen might be seen as repeated antigen-stimuli resulting in activation induced cell death through the extrinsic pathway. The processes inhibiting activation induced cell death seen in arthritic, anti-CD3/anti-CD28 stimulated T cells are probably not initiated in the non-stimulated samples because expression of FLIP increases only after signaling through the T cell receptor and the strength of signaling, the level of co-stimulation and the activation levels of signaling molecules are different after in vitro (anti-CD3/anti-CD28 beads) and in vivo (immunization during arthritis induction) stimulation. ZAP-70 is also important for activation induced cell death, as in its absence up-regulation of FasL does not take place, and the extrinsic pathway cannot be activated. This might explain why the amount of cleaved Caspase-8 is lower in arthritic ZAP-$70^{+/-}$ T cells than in BALB/c T cells.

Activation induced cell death has also a death-receptor-independent version, triggering the intrinsic apoptotic pathway. The key molecules of this process are Bim and Bcl-2. Based on our results, in arthritic ZAP-$70^{+/-}$ T cells the intrinsic pathway is active independently of stimulation with anti-CD3/anti-CD28, shown by the presence of cleaved Caspase-9, however in arthritic BALB/c T cells only the anti-CD3/anti-CD28 stimulation triggers the activation of the intrinsic pathway. Although the amount of pro-apoptotic Bim protein increased in all samples as a result of anti-CD3/CD28 stimulation, we observed higher expression in arthritic samples in comparison to the healthy T cells. Changes in the anti-apoptotic Bcl-2 were similar; in vitro stimulated, arthritic samples showed significant elevation of Bcl-2. The fate of the cell is decided by the ratio of Bcl-2 and Bim, if Bcl-2 is expressed in higher amounts it can inhibit the pro-apoptotic effects of Bim and the cell survives, but if the amount of Bim outweighs that of Bcl-2, the cell goes through apoptosis via the intrinsic pathway. As a result of this process Cytochrome C is released from the mitochondria into the cytoplasm, which is indeed what we observed in our samples: after anti-CD3/CD28 stimulation Cytochrome C was present in increased amounts in cell lysates from arthritic T cells. According to our results, in arthritic T cells upon stimulation, Bcl-2 cannot block the pro-apoptotic effects of elevated Bim levels, thus Cytochrome C is released from the mitochondria, leading to Caspase-9 activation through the apoptosome. Although similar amounts of cleaved Caspase-9 were observed in arthritic, anti-CD3/anti-CD28 stimulated T cells of BALB/c and ZAP-$70^{+/-}$ the amount of Caspase-3 was lower in BALB/c T cells. On one side it is possible that the cleavage of Caspase-3 by Caspase-9 was inhibited in BALB/c mice, but, on the other side, increased activation of Caspase-3 in ZAP-$70^{+/-}$ T cells might be caused by other activation pathways, not investigated in this study. It is interesting to note, that activation induced cell death

(Caspase-8) was increased in non-stimulated arthritic BALB/c T cells, however the effector cleaved Caspase-3 was undetectable. Furthermore, as a consequence of anti-CD3/CD28 stimulation the level of apoptosis was diminished in arthritic BALB/c T cells in comparison to healthy T cells. Based on this, we propose that activation induced cell death via the extrinsic pathway (Caspase-8) might not play an important role in these T cells, however as Bim is expressed in higher levels as Bcl-2, Bim can exert its pro-apoptotic effects, leading to Cytochrome C release from the mitochondria, activating Caspase-9. Furthermore, it is also possible that the activation of Caspase-3 does not take place as a consequence of anti-CD3/CD28 stimulation in T cells from arthritic mice, because of the activation of various inhibitors or anti-apoptotic molecules.

The partial deficiency of ZAP-70 changed the above mentioned situation: in non-stimulated arthritic ZAP-$70^{+/-}$ T cells activation induced cell death (Caspase-8) was less pronounced than in the BALB/c, and, regarding the intrinsic pathway, the Bcl-2 expression was stronger than in BALB/c, blocking the pro-apoptotic effects of Bim, and, as a sum of these processes Caspase-3 was not cleaved. In arthritic, anti-CD3/anti-CD28-stimulated ZAP-$70^{+/-}$ T cells the intrinsic pathway seems to dominate, while in healthy, anti-CD3/anti-CD28-stimulated ZAP-$70^{+/-}$ T cells the extrinsic pathway might be triggered more efficiently.

As a conclusion, we propose that partial ZAP-70 deficiency changes the balance between the activation and apoptotic processes of T cells. In arthritic BALB/c mice, the T cell activation/apoptosis balance shifted to activation leading to severe arthritis and the accumulation of pathogenic T cells. In partial ZAP-70 deficient mice, on the other hand, the T cell activation is impaired and the apoptotic processes are more pronounced leading to milder inflammation in the joints. Our work clearly demonstrates the importance of ZAP-70 in the regulation of TcR-dependent activation and apoptosis signaling pathways in autoimmune arthritis.

6 New results

We have shown, that with the intraperitoneal adoptive transfer of ZAP-$70^{+/+}$ thymocytes:

1. T cell reconstitution can be restored in ZAP-$70^{-/-}$ mice (improved survival, long-term T cell reconstitution)
2. after the transfer T cell development normalized in the thymus
3. the newly produced, donor-originated T cells are functional
4. the injected thymocytes leave the peritoneum through the milky spots of the omentum

Using the recombinant human G1 arthritis (GIA) model we have found that:

1. autoimmune arthritis can be induced in ZAP-$70^{+/-}$ mice
2. in ZAP-$70^{+/-}$ mice the clinical picture of the disease was milder
3. in arthritic ZAP-$70^{+/-}$ mice antigen-induced T cell proliferation and IFNγ production was decreased, however production of IL-17 was significant

In the T cells of arthritic mice:

1. without anti-CD3/CD28 stimulation in ZAP-$70^{+/-}$ the intrinsic, in the wild-type the extrinsic apoptotic pathway was more pronounced
2. after anti-CD3/CD28 stimulation this differences disappear, but in the wild-type T cells the intensity of apoptotic pathways is reduced in comparison to ZAP-$70^{+/-}$ T cells, in line with the *in vitro* results (higher proliferation late, more pronounced activation) and the observed more severe clinical picture

7 Publications

Publications related to the thesis:

Kugyelka R, Kohl Z, Olasz K, Prenek L, Berki T, Balogh P, Boldizsar F: Correction of T cell deficiency in ZAP-70 knockout mice by simple intraperitoneal adoptive transfer of thymocytes. CLINICAL AND EXPERIMENTAL IMMUNOLOGY 2018 Jun;192(3):302-314. doi: 10.1111/cei.13114. IF: 3,410

Kugyelka R, Kohl Z, Olasz K, Mikecz K, Rauch TA, Glant TT, Boldizsar F. Enigma of IL-17 and Th17 Cells in Rheumatoid Arthritis and in Autoimmune Animal Models of Arthritis. MEDIATORS OF INFLAMMATION 2016;2016:6145810. doi: 10.1155/2016/6145810. IF: 3.232 (shared first author with K.Z.)

Kugyelka R, Prenek L, Olasz K, Kohl Z, Botz B, Glant T. T, Berki T, Boldizsár F: ZAP-70 regulates autoimmune arthritis via alterations in T cell activation and apoptosis. Submitted (under review) CELLS (Special issue: „The Molecular and Cellular Basis of Rheumatoid Arthritis")

Other publications:

Prenek L, Boldizsar F, Kugyelka R, Ugor E, Berta G, Nemeth P, Berki T The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells. APOPTOSIS 22:(2) pp. 239-253. (2017) IF: 3,833

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