Effects of soluble molecules and cellular interactions in the selection of thymocytes: a mouse model

PhD Thesis

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INTRODUCTION

T lymphocytes derive their name from their site of maturation in the thymus. Like B lymphocytes, these cells have membrane receptors for antigen and belong to the adaptive immune system. T cells are important regulators of the immune response, and also take part in the recognition and elimination of virally infected and tumour cells, and are also implicated in transplant rejection. During its maturation T cell starts to express a unique antigen-binding molecule, called the T-cell receptor (TCR), on its membrane. TCR can recognize protein antigens that is bound to cell-membrane protein called major histocompatibility complex (MHC). TCR is associated on the membrane with a signal-transducing complex called CD3. Two different T cell subsets have been discovered according to their TCR: T lymphocytes expressing αβ and cells carrying γδ chains. The majority of T cells have a TCR composed of α- and β- TCR chains, while γδ T cells represent only a small subset of T cells that possess a distinct TCR on their surface.

Mature αβ T lymphocytes can be divided into subsets according to the markers they express: the CD4+ helper T cells recognise extracellular antigens presented by MHCII molecules and CD8+ cytotoxic T cells that recognise intracellular antigens presented by MHCI molecules.

As thymocytes mature in the thymus, the diversity of their TCRs is generated by a series of random gene rearrangements.

Commitment to T cell and the disjunction of αβ and γδ T cell differentiation take place in the early phase of T cell maturation in the thymus. The rearrangement of TCR genes is followed by the selection of αβ T cells and the commitment to CD4+ T helper or CD8+ T cytotoxic cells in the thymus. The maturation of thymocytes, which is a complex process controlled by numerous cellular and humoral factors is not completely understood yet.

After expressing antigen-binding receptors, thymocytes are subjected to a two-step selection process. Any developing double positive (CD4+ CD8+, DP) thymocyte that is unable to recognize self-MHC molecules (positive selection) or that do have a high affinity for self-antigen plus self MHC (negative selection) are eliminated by programmed cell death (apoptosis). According to the mutual antagonism model interactions between glucocorticoid

hormone (GC) and TCR induced signalising will determine the fate of developing thymocytes.

Apoptosis is the process of programmed cell death which involves a series of biochemical events leading to a characteristic cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, loss of mitochondrial membrane potential, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. During positive selection both TCR activation or GC effect alone result in apoptosis. While according to the mutual antagonism model locally produced glucocorticoids and TCR signal together induce the survive of DP thymocytes. The same mutual antagonism model describes the interaction of different cytokines and glucocorticoids.

OBJECTIVES

1. Comparison the ratio of the four different thymocyte subpopulations in BALB/C and TCR transgenic AND mouse strain after *in vivo* GC hormone, specific antigen or anti-CD3 treatment (TCR activation). Long term effect of a single dose GC treatment on the cellular compositon of thymus.

2. Detection of glucocorticoid receptor (GCR) expression level in the different thymocyte subpopulations. Changes of GCR expression after T cell receptor activation, GC or GC antagonist treatment.

3. Effects of *in vivo* GC antagonist pre-treatment on GCR induced signal transductions and cellular composition of thymus.

4. Measurement of early and late apoptotic cell ratio, mitochondrial membrane potential changes and pro-apoptotic caspase-3 activation and anti-apoptotic Bcl-2 expression in thymocytes after *in vivo* TCR activation and GC treatment.

5. Effect of *in vitro* GC treatment in thymic organ culture and thymocyte culture in the present of GCR antagonists or GC hormone synthesis inhibitor.

6. Detection of CD69+ DP thymocyte ratio in BALB/C and TCR transgenic AND mouse strain after TCR activation, GC and GC antagonist treatment.

MATERIALS AND METHODS

MICE. We used 3–4-week-old (10 g body weight) BALB/C and B10.Cg-TgN (TCR AND) 53Hed (AND) pigeon cytochrome C specific I-Ek (MHC-II) restricted Vb3, Va11 TCR transgenic mice. The animal experiments were carried out in accordance with the regulations set by the University's Committee on Animal Experimentations.

TREATMENT OF ANIMALS AND THYMOCYTE PREPARATION. Mice were injected i.p. with high dose (20,0 or 10,0 mg/kg), middle dose (2,0 mg/kg) or low dose (1,0 or 2,0mg/kg) dexamethasone (DX) alone or with other treatments. We used i.p. 1mg/kg GCR competitive antagonist RU486 and RU43044 dissolved in sesame oil. AND mice were injected i.p. with 10 mg/kg PCC dissolved in PBS once per day for 2 days. Activating anti-CD3 (145.2C11) mAb (5 or 50 μg/animal) was injected i.v. in 100μl PBS. Thymocytes were prepared as dexcribed by Compton and Cidlowski. In brief, mice were killed by rapid decapitation, and the thymus glands were removed and placed on ice-cold PBS. Thymus tissue was homogenized in a glass/glass homogenizer; the suspension was filtered through a nylon mesh filter. The thymocytes were washed in PBS, and the cell number and viability determined by counting on a hemocytometer using the Trypan blue dye-exclusion test.

IN VITRO CULTURE OF THYMUS LOBES AND THYMOCYTES. One lobe of a removed thymus gland of 1 or 2 day old BALB/C mice were incubated in DMEM medium containing 10^{-7} mol/l DX, GCR antagonist RU43044 or steroid synthesis inhibitor Methyraponnal, for 24 hours.

CMX-ROS STAINING. CMX-Ros is a lipophilic cationic fluorescent dye that is sequestered in active mitochondria because of their negative mitochondrial membrane potential, therefore living cells show high CMX-Ros fluorescence. Since apoptotic cells lose their mitochondrial membrane potential CMX-Ros staining of mitochondria decreases. The lower CMX-Ros positive cell ratio indicates the loss of mitochondrial membrane potential, i.e. the presence of mitochondrial type of apoptosis. 10 μl CMX-ROS stock solution (1 mg/ml in DMSO) was

added to 10^6 cells in 1ml of RPMI medium then cells were incubated for 30–45 min in 37 °C and after cell surface labelling (a-CD4-FITC, a-CD8-CyC) analysed with flow cytometry.

FLUORESCENT CELL SURFACE AND INTRACELLULAR LABELLING OF THYMOCYTES. Thymocyte samples (10^6) were incubated with monoclonal antibody cocktails (anti-CD4-PE, anti-CD8-CyChr, anti-CD69-FITC) for 30 min in 100 μl binding buffer on ice, then washed twice in PBS, and finally resuspended in 500 μl 0.1% buffered PFA in PBS. We used triple labelling technique for the simultaneous detection of cell surface CD4, CD8, and intracellular GR or Bcl-2 protein. Briefly, after cell surface CD4, CD8 labelling thymocyte samples (10^6) were fixed in 4% PFA buffer for 20 min at room temperature then cells were further washed and intracellularly labelled in the permeabilisation buffer. Thymocytes were incubated with FITC-conjugated anti-GR or FITC-conjugated anti-Bcl-2 or anti-activated Caspase-3 monoclonal antibodies for 30 min at room temperature. Afterwards cells were washed twice in permeabilisation buffer then once in PBS and finally resuspended in 500 μl 0.1% PFA in PBS.

DETECTION OF INTRACELLULAR FREE CALCIUM LEVEL. Intracellular free calcium was measured using Fluo-3 AM according to the protocol described by Minta et al. Briefly, 10^6 cells in 100 μl RPMI containing 10 μM Fluo-3 AM were incubated for 30 min at room temperature. Afterwards, the cell suspension was diluted with 10 ml RPMI+10% FCS and incubated for a further 30 min. Finally, samples were washed three times in RPMI+10% FCS, then cell surface anti-CD4-PE, anti-CD8-CyChr labelling were carried out and immediately measured in a Becton Dickinson FacsCalibur flow cytometer using the CellQuest program. The mean fluorescence intensity of Fluo-3 AM dye was determined at 526 nm (FL1 channel) in the different samples, which is proportional to the cytosolic calcium level.

ANNEXIN V AND PI LABELLING. For the detection of early apoptotic thymocytes Annexin V – FITC staining was performed. Briefly, samples (10^5 thymocytes) were incubated with Annexin V – FITC for 20 min in 100 μl Annexin binding buffer at room temperature, then diluted with 400 μl Annexin binding buffer, immediately followed by flow cytometric measurement.

FLOW CYTOMETRIC ACQUISITION AND ANALYSIS. Samples were measured and analysed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), using the CellQuest software.

STATISTICAL ANALYSIS. The effect of various treatments between groups was tested for statistical significance using Student's t-test. $P<0.05$ denoted statistical significance.

RESULTS AND CONCLUSIONS

The molecular events leading to positive and negative selection steps during thymocyte development are still unclear. The investigation of the role and signaling pathways of known thymic cytokines and growth factors did not solve this problem. Recently it has been suggested that thymic GC synthesis by epithelial cells and local GC action might influence the selection steps by inhibiting TCR-mediated apoptotic signals, but only few *in vivo* results are available. For the further clarification of the maturation process of thymocytes we used a TCR transgenic AND mouse strain beside the well known BALB/C mouse model. Almost all T cells of the transgenic AND mouse express the same TCR specific for antigen 88-104 fragment of the Pigeon Cytochrome C (PCC: KAERADLIAYLKQATAK). Thus we could explore the effects of not only the indirect activation (anti-CD3 mAb) but also the direct, antigen specific activation of TCR *in vivo*.

CELLULAR COMPOSITION OF BALB/C AND TRANSGENIC AND MOUSE THYMUS. We showed that the cellular composition of the two mouse strains differ: in BALB/C the DP population dominated (75-80%) while in the thymus of the transgenic AND mouse strain the CD4 single positive (CD4 SP) cells were more frequently found and the CD8 single positive cells (CD8 SP) were almost totally absent. The reason of this is the transgen in the transgenic mouse strain: in the T cell precursors only one kind of rearranged TCR ($V\beta3/V\alpha11$) can be generated from the germ line gene configuration, which through MHCII molecules could ensure only the CD4 SP cell survival during positive selection.

GCR EXPRESSION IN THE DIFFERENT THYMOCYTE SUBPOPULATIONS. It is well known that the glucocorticoid analogue Dexamethasone (DX) treatment diminishes thymus size and cellularity. Glucocorticoids cause apoptosis in both mature and immature

thymocytes, but the different thymocyte subpopulations have different glucocorticoid sensitivity. The cause of this phenomenon is not jet known.

The variant expression levels of GCR in the different thymocyte subpopulations could be a plausible explanation. Our laboratory produced a monoclonal antibody against GCR which recognises both free and ligand bound form of the receptor. We showed that the GCR level differ in the different thymocyte populations: DP cells had the lowest and double negative (DN) cells the highest level of the receptor. In the transgenic AND mice the low GCR level decreased after both DX treatment and TCR activation in the DP cells. For the explanation of GCR expression fall after TCR activation further research should be done, but it is known that during TCR signal transduction AP-1, NFAT, NFκB and Elk activation occurs that could have some effects on GCR expression level. GCR level alone could not determine glucocorticoid sensitivity, because DP cells, that had the lowest GCR expression, showed the most intensive apoptosis after glucocorticoid treatment. Other signalling mechanisms also influence the glucocorticoid sensitivity of thymocytes.

The explanation of low GCR expression in DP thymocytes could be the homologues down regulation of GCR expression caused by the high concentration of the locally produced glucocorticoid hormone in the thymus cortex around DP cells. After high dose DX treatment we could detect an initial increase of GCR level before the decrease of its expression but only in DP population. In the SP populations a week after the treatment the GCR levels was still lower. This could be the result of homologues down regulation of GCR expression in the surviving and further maturing DP cells. Within 30 minutes the GCR-ligand complex translocates to the nucleus where acts as a transcription factor of different genes (glucocorticoid responsible elements, GRE). The gene of GCR is also a GRE which involves the glucocorticoids in the regulation of GCR expression. In the literature the role of glucocorticoid hormone in the determination of GCR levels in other cell lines is described.

The low GCR level in DP cells and their high sensitivity to glucocorticoid induced apoptosis raise the possibility of non-genomic GCR signalisation. One mechanism of this non-genomic signal transduction could be the interaction of GCR and other signal transduction factors in the cytoplasm. Hsp-90 may be one of this factors because it is knonwn that through Lck, Raf and ERK binding it affects TCR signaling and has a role in thymocyte selection. It has been

shown that ligand free GCR is bound to Hsp-90 in the cytoplasm, thus this molecule could be an important connection between TCR and GCR signalisation providing an explanation for mutual antagonism theory. Results of other researches in our institute lead to ZAP-70 molecule and another possible connection between TCR and GCR signal transduction. Interactions of GCR-glucocorticoid complex with other transcription factors such as NFκB, AP-1, CREB and STAT-5 could be responsible for some of our results. Using glucocorticoid antagonists we could not inhibit the DX induced thymocyte loss and the appearance of early apoptotic markers on DP cells after 4 hours of DX administration.

One of our goals was to detect the glucocorticoid induced apoptotic markers (Annexin V / PI), the kinetics of apoptosis and GCR level changes in mouse thymocytes. We found an interesting GCR expression kinetics in DP cells: after high dose DX administration the receptor expression increased for 16 hours and then decreased during the examined 24 hours. Similar results were reported in rat hepatic cells.

It seems that in DP cells the homologeus downregulation of GCR is not typical like in other thymocyte subpopulations which is important in the development of glucocorticoid resistance. The impaired GCR downregulation of DP cells could explain the high glucocorticoid sensitivity of DP cells beside the unchanged GCR level.

TCR INDUCED ACTIVATION OF THYMOCYTES. Elevation of the free Ca^{++} level in the cytoplasm is one result of TCR activation. We determined the free cytoplasmic Ca^{++} level in the four thymocyte subpopulations to clarify its possible role in the different TCR activation. The four thymocyte subpopulations can be characterised by different free intracellular Ca^{++} level and with different kinetics after TCR activation with anti-CD3 mAb. The highest and quickest increase of Ca^{++} level was found in the CD4 SP population. It is supported by previous data from literature that free Ca^{++} level plays a role in the CD4 SP or CD8 SP commitment of DP cells. The higher intracellular Ca^{++} level induce the further maturation into the CD4 SP direction.

EFFECTS OF GLUCOCORTICOID HORMONE AND TCR ACTIVATION ON THYMOCYTE APOPTOSIS. Apoptosis of thymocytes is a complex process regulated by a number of different factors. To examine this process we analyzed Bcl-2 expression and

Caspase-3 activation in parallel with two sensitive detection methods of the early phase of apoptosis.
Four hours after DX treatment we observed an increase in the proportion of early apoptotic cells which was followed by an increase in the ratio of late apoptotic cells 16-20 hours after DX treatment. Very similar kinetics of apoptosis were found in C57BL/6 mice. Changes in GCR expression, increase of apoptotic cell ratio and depletion of DP cells were observed simultaneously in the thymus.

The activation of GCR or TCR alone or their combination resulted in increased externalization of membrane phosphatidylserine molecules (Anenxin V positivity) and decreased mitochondrial potential in DP cells. The ratio of early apoptotic DP cells was lower when the two activation signals were combined similarly to the in vitro stimulation results. The co-activation of GCR and TCR caused higher DP survival (compared to single activations) due to the interaction of the two different signaling pathways supporting the mutual antagonism model.

Since in TCR transgenic AND mice more than 90% of thymocytes are DP or CD4 SP, we focused on these two populations in our further work. Both GC treatment and TCR activation caused drastic loss of immature DP cells while the loss of mature CD4 SP cells was not so dramatic. Significantly more DP thymocyte escaped from apoptosis in both BALB/C and TCR transgenic AND mice when GCR and TCR activation were used together which combined effect was not detectable in CD4 SP population.

In the AND transgenic mice the presence of the specifi antigen caused significant decrease of DP cells, while glucocorticoid and combined treatment resulted in almost total loss of this thymocyte population. At the same time the increase of CD4 SP cells was detectable suggesting that a portion of the lost DP cells presumably differentiated further into mature CD4+ cells.

Bcl-2 is an anti-apoptotic member of the Bcl-2 family, which includes both pro- and anti-apoptotic molecules. Similarly to other research groups we found different Bcl-2 expression levels in the different thymocyte populations. An in vitro examination with a T cell hibridoma showed that Bcl-2 and Bcl-xL selectively antagonised glucocorticoid induced apoptosis. We proved that TCR and GCR activation increased the number of Bcl-2 positive

DP thymocytes giving them advantage during selection steps. Other papers claimed that after successful positive selection the increased anti-apoptotic Bcl-2 expression resulted in DP thymocyte survival.

Activated Caspase-3 is a protein having a role in the effector phase of apoptosis. We did not find Caspase-3 activation after anti-CD3 mAb treatment, but it could be shown in DX induced DP apoptosis. When the two treatments were combined the Caspase-3 activation was partial. Data from literature showed that the ratio of Caspase-3 activation is proportional to DNA fragmentation, FAS and Bax expression during apoptosis. These results reflect a different apoptotic pathway due to GCR or TCR activation.

GCR could have a role in the regulation of early apoptosis since changes of GCR expression and Annexin V positivity could both be detected 4 hours after DX administration. Non-conventional GCR effects can be responsible for that since glucocorticoid antagonist treatment did not affect early apoptosis. Glucocorticoid antagonist pre-treatment inhibited the late (12-24 hours) apoptosis induced by a single high dose DX treatment which is an already known phenomenon.

EFFECTS OF GCR ANTAGONISTS. To inhibit the conventional GC effects we treated our BALB/C mice twice a day with glucocorticoid receptor antagonist RU43044 or RU486 alone or together with 20 mg/kg DX for 2 days. Neither receptor antagonist alone had any effect on thymic cell count or the cellular composition of thymus. After combined treatment with a receptor antagonist and DX the early apoptotic signs of thymocytes (Annexin V in the outer cell membrane) were detectable in thymocytes, while the late signs of apoptosis were absent suggesting that GCR ligation is not needed for the early steps of apoptosis but it is necessary in the late phase (PI positivity).

IN VITRO EFFECTS OF GLUCOCORTICOID HORMONE. In parallel with our *in vivo* experiments we also explored the *in vitro* effects of glucocorticoid hormone on thymocyte apoptosis and survival. In thymic organ culture, where the original microenvironment was conserved we were able to minimize the local glucocorticoid production with the help of Methyrapon. With keeping the thymus lobes in steroid free medium we could provide a glucocorticoid free environment. DP cells were sensitive both to the lack of GC and to the high level of the hormone. According to the mutual antagonism model the lack of GC caused

DP cells only to be activated through TCR and this signalisation alone resulted in the apoptosis of thymocytes. With the use of GCR antagonist major changes in the thymus could not be induced and the effects of DX treatment could not be totally inhibited. These results indicate possible GCR independent effects of DX.

In the thymic organ and cell cultures of 1-2 day old BALB/C mice high degree of spontaneous apoptosis was found, which could be Caspase dependent since the aminisitration of Caspase inhibitor decreased its rate. We are planning to use the reaggregate thymus organ culture method for further in vitro research of the maturation and selection processes of thymocytes.

MODELING POSITVE SELECTION OF THYMOCYTES. After successful positive selection of thymocytes the CD69 expression can be detected on the surface of DP and SP cells. We used this phenomenon to detect the positive selection after TCR and GCR co-activation in DP and CD4 SP cells. In both mouse strains antigen or anti-CD3 mAb induced TCR activation or DX treatment alone increased the ratio of CD69 positive DP cells, but co-activation of TCR and GCR had a stronger effect. In the CD4 SP population antigen itself, low dose DX and co-activation of TCR and GCR increased the CD69+ cell ratio, but after high dose DX treatment there was no increase in the number of CD69+ cells. After high dose DX treatment CD69 positivity of DP cells could be transient since CD69 and CD25 positivity has been showed during spontaneous and steroid induced apoptosis of different cell types. On the other hand low dose DX and simultaneous antigen treatment caused positive selection of thymocytes which can be indicated by the increased ratio of CD69 positive, surviving CD4 SP cells.

According to our results after GCR and TCR co-activation, maturing DP thymocytes show the early signs of apoptosis (membrane phospholipid asymmetry, loss of mitochondrial functions), while the effector phase of apoptosis is inhibited probably through Ras activation.

In summary we can say that the combined effect of stimuli which alone cause apoptosis of thymocytes results in the survival of DP cells. A key element of this phenomenon could be the Bcl-2 molecule since TCR and GCR co-stimulation resulted in the increased expression of this molecule in positively selected thymocytes.

SUMMARY OF NEW RESULTS

1. We proved that in BALB/C and TCR transgenic AND mouse strains the four different thymocyte subpopulations express GCR in different level: DP cells that are the most sensitive to GC hormone expresses the lowest GCR level.
2. We demonstrated that free cytoplasmic Ca^{2+} level and the kinetics of Ca^{2+} level after TCR activation are different in the thymocyte subpopulations.
3. We confirmed that the spontaneous and DX induced apoptosis of thymocytes are caspase dependent, while in GC hormone and TCR activation induced apoptosis caspase independent factors are also important.
4. With the help of GCR antagonists we proved that ligand binding of GCR is not essential for the early steps of GC induced apoptosis, but without it apoptosis declines.
5. GCR and TCR co-activation results in higher ratio of surviving DP thymocytes caused by the inhibition of early steps of apoptosis which suggests a link between the two signalling pathways.
6. We showed that in TCR transgenic AND mouse strain low dose glucocorticoid treatment with simultaneous antigen stimulus induced further maturation of surviving DP thymocytes into CD4 positive direction.
7. Our experiments successfully confirmed the presence of the mutual antagonism model in thymocyte selections.

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